TITLE:

Evolution of resistance and disease tolerance mechanisms to oral bacterial infection in *D. melanogaster*

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ABSTRACT

Pathogens exert strong selection on hosts, that evolve and deploy different defensive strategies, namely minimizing pathogen exposure (avoidance), directly promoting pathogen elimination (resistance), and/or managing the deleterious effects of illness (disease tolerance). However, how the response to pathogens partitions across these processes has never been directly assessed in a single system, let alone in the context of known adaptive trajectories under controlled selection regimes. Here, an experimental evolution system composed of *D. melanogaster* and its natural pathogen *P. entomophila* is used to independently assess the role of behavioural traits, and of resistance and disease tolerance mechanisms on host evolution. We compare one replicate of a population adapted to oral infection with *P. entomophila* (BactOral) to a replicate of its control population to find no evidence for behavioural change but measurable differences in both resistance and disease tolerance. In BactOral, we identify a relative decrease in bacterial loads correlated with an increase in gut production of specific AMPs, but no differences in bacterial intake, in gut cell renewal rate, or in the rate of bacterial defecation, pointing to a strengthening in resistance. Additionally, we posit that disease tolerance also contributes to the adaptive response of the BactOral population through a tighter control of its immune response and of the deleterious effects of exposure. This study reveals a genetically complex and mechanistically multi-layered response, possibly reflecting the structure of adaptation to infection in natural populations.

BACKGROUND

Host-parasite interactions are main drivers of evolution[1,2]. In natural environments, parasites are constantly attempting to penetrate host defences, which consist of multiple mechanisms to fight infections[3]. Such mechanisms include behavioural strategies (e.g. avoidance)[4], the active reduction or elimination of pathogens (i.e. resistance) and/or the maintenance of homeostasis and fitness without interfering with pathogen burden (i.e. disease tolerance)[5–8]. While many studies in the past decades have explored the mechanistic and genetic bases of these different host defence strategies in diverse organisms [9–11], few have focused on how these processes can be shaped by evolution[12–15]. Experimental evolution is a powerful method by which it is possible to infer the evolutionary response of a host species to a specific pathogenic pressure under controlled conditions[16]. Previous studies of experimental evolution have addressed the impact of different hosts on parasite fitness[17], of different pathogens on the host's evolutionary trajectory[13,18,19], of immune priming[20,21], and of different routes of infection on host adaptation[19,22].

It is postulated that oral exposure to pathogens constitutes a common process of infection in *D. melanogaster*[23,24], as it lives on decaying substrates. When feeding on pathogenic microorganisms, *Drosophila* deploys different responses that include avoidance behaviours[25,26], the barrier action of the peritrophic matrix[27], localized high acidity of the midgut[28], intensification of gut transit[29], local production of Anti-Microbial Peptides (AMPs)[30] and Reactive Oxygen Species (ROS) by gut epithelial cells[31,32] and increase of enterocyte delamination and renewal rates[33–36]. In addition, *Drosophila* can activate a systemic humoral response, when the pathogen manages to cross the gut epithelium into the haemolymph[37,38] or otherwise provokes a systemic reaction[23,39–41]. In the specific case of oral infection with the natural entomopathogen *Pseudomonas entomophila*, its high pathogenicity to *D. melanogaster* is linked to the production of toxins and virulence factors[42,43] causing translational arrest in host tissues and the inability to renew damaged gut epithelium[44,45]. The

72 above-presented plethora of possible host responses provides a clear picture of the multi-
73 layered and mechanistically complex nature of the immune response, understood in its broad
74 sense[3].

75 In this work, we used a previously experimentally evolved outbred population of *D.*
76 *melanogaster* (hereafter called "BactOral") showing a fast evolutionary response to a strong
77 selection against oral *P. entomophila* infection[19,46], to investigate the mechanisms targeted by
78 adaptation. We systematically tested for behaviour, resistance, and disease tolerance
79 contributions, to provide a comprehensive account of the partitioning of these defense
80 strategies in adaptation.

81

82 RESULTS

83 Evolved population shows higher survival upon infection

84 In previous work[19,46], experimental evolution was conducted on an outbred population
85 of *D. melanogaster* against oral infection either with the bacterium *P. entomophila* (hereafter,
86 BactOral) or with a control solution (hereafter, Control). Since then, these populations[46] have
87 been kept under relaxed selection for over 80 generations. To understand to which extent this
88 relaxation affected the response to the original selection regime, we measured survival of
89 Control and BactOral populations upon *P. entomophila* oral infection. We found significantly
90 higher survival of BactOral for both sexes (Fig. 1, Infected Control females *versus* Infected
91 BactOral females: *z.ratio*= 9.123, $p < 0.0001$; Infected Control males *versus* Infected BactOral
92 males: *z.ratio*= 9.909, $p < 0.0001$)(Suppl. Table 1). Moreover, these differences are not due to
93 an inherent frailty of the Control as compared to BactOral. Indeed, survival under uninfected
94 conditions for both long term longevity experiments (Suppl. Fig. 1 and Suppl. Table 2) and
95 over the equivalent time period shows similar trends in both populations (Fig. 1, Uninfected

96 Control females *versus* Uninfected BactOral females: *z.ratio*= 0.004, *p*= 1; Uninfected Control
97 males *versus* Uninfected BactOral males: *z.ratio*= -1.080, *p*= 0.9611)(Suppl. Table 1).

98 Taking these results into account, we undertook a systematic description of multiple
99 traits previously associated to immune response to oral infections aiming to identify different
100 processes targeted by selection and estimate their relative contribution.

101 Evolved population has not changed bacterial uptake or defecation 102 rates

103 Our systematic approach started by screening behavioural traits that impact bacterial
104 numbers in the gut: feeding and defecation. To test for differences in feeding behaviour, we
105 performed a Capillary Feeding assay (CAFE)[47] on females of both Control and BactOral
106 populations at different timepoints over 24 hours of exposure to bacterial suspension. We
107 allowed individual flies to feed on a *P. entomophila* suspension mixed with food coloring in
108 graduated glass capillaries and measured the amounts eaten by each female at 2, 8 and 24
109 hours of exposure. We found no significant differences between the amount ingested by
110 individuals of the two populations (power analysis for a t-test: *Cohen's d*= 1,06). Pairwise
111 comparisons of their cumulative bacterial intake after 2 hours, 8 hours and 24 hours exhibited
112 a comparable amount of total bacteria ingested (Suppl. Table 3 and Fig. 2A, Control *versus*
113 BactOral at 2 hours, *t*= 0.097, *p*= 1; at 8 hours, *t*= 0.643, *p*= 0.9873; at 24 hours, *t*= 0.574, *p*=
114 0.9925).

115 Additionally, we estimated the average slopes from linear regression where microliters
116 of bacteria eaten were modelled between different time points, concluding that the bacterial
117 quantities ingested by both populations remain undistinguishable as the infection progresses.
118 Thus, exposure affects feeding ability of both populations to a comparable degree and,
119 consequently, adaptation to oral infection with *P. entomophila* in the BactOral regime did not
120 involve an alteration of feeding behaviour.

121 Considering the absence of significant differences in bacterial intake, an alternative
122 would be that the BactOral population had evolved a more effective bacterial defecation, which
123 would reduce the time of pathogen permanence and proliferation in the gut. We established a
124 protocol to measure live bacteria loads in the faeces, hence considering the number of
125 bacteria defecated as a proxy for defecation rate itself, assuming that no interaction between
126 the two parameters exists. To this purpose, we took individual flies upon oral infection and
127 quantified the CFUs defecated over a six-hour and the subsequent twelve hour-periods, post-
128 exposure (Fig. 2B).

129 We observed a few instances where flies did not expel alive bacteria during that period
130 or did so in a quantity that fell below our detection threshold, resulting in a few counts of zero.
131 Therefore, a zero-inflated model with a negative binomial distribution was fitted on the counts
132 data (accounting for overdispersion) jointly with a binomial distribution on the zeros. For the
133 counts portion of the model, none of the three factors significantly predicted the measured
134 bacterial loads (Suppl. Table 4, model parameters - Population: *z.value*= 1.324, *p*= 0.185;
135 Sex: *z.value*= 0.073, *p*= 0.942; Timepoint: *z.value*= -0.359, *p*= 0.720). However, looking at the
136 zero portion of the model, we find a significant effect of Population (Population: *z.value*= -
137 2.407, *p*= 0.016; Timepoint: *z.value*= -0.006, *p*= 0.996).

138 We then performed post-hoc pairwise comparisons on these regressions and identified
139 a significant decrease in bacterial loads defecated by males of BactOral, between the two
140 timepoints (males: *z.ratio*= -2.719, *p*= 0.033), but no other significant effects among bacterial
141 counts between populations and/or timepoints, in a sex-dependent manner. However, when
142 considering the zeros, we found a significant difference in the proportion of BactOral flies with
143 undetectable amounts of bacteria in the faeces between the first and second timepoints (B6h–
144 B18h: *z.ratio*= 4.092, *p*= 0.0002), as well as compared to Control at both timepoints (B6h–
145 C6h: *z.ratio*= 3.612, *p*= 0.002; B6h– C18h: *z.ratio*= 3.530, *p*= 0.002)(Suppl. Table 4).

146 These results demonstrate that both Control and BactOral populations defecate alive
147 bacteria in comparable amounts upon infection. Nonetheless, we detected that males of

148 BactOral decrease the amount of live bacteria they defecate as time progresses, hinting at a
149 possible resistance mechanism more evident in males of the adapted lines, that was not
150 significant in females. This observation is corroborated by the difference in the number of
151 "zeros" found between both populations, with BactOral exhibiting more instances of expelling
152 undetectable bacterial loads.

153 Evolved population shows a faster decrease and clearance of 154 bacteria

155 Next, we studied the physiological responses to estimate the roles of resistance and
156 disease tolerance. First, we tested for structural changes in the gut itself that could influence
157 the "carrying capacity" of the structure and create difficulties in interpreting the quantification
158 experiments. We measured the capacity of BactOral to regenerate its gut epithelium upon
159 bacterial infection relative to Control, using initial length and extent of the shortening induced
160 by infection[36], as a proxy. For this, we measured the total length of the guts of females, before
161 and after 24 hours of oral infection with *P. entomophila* and performed multiple pairwise
162 comparisons on a linear model with mixed effects to pinpoint how population and infection
163 status affected this trait. We found a shortening of approximately 2 mm in the total length of
164 infected guts by comparing the post-infection timepoint of 24 hours to the initial timepoint, prior
165 to exposure, in both populations (Suppl. Table 5 and Suppl. Fig. 2, Control before infection
166 *versus* Control 24 hours post-infection: t= 6.274, p<0.0001; BactOral before infection *versus*
167 BactOral 24 hours post-infection: t = 5.453, p <0.0001). Nonetheless, no significant differences
168 between populations in the total length of the guts could be detected both before and after
169 infection (Without infection: BactOral *versus* Control – p= 0.6719; after 24 hours of infection –
170 p= 0.9925), (power analysis for a multiple linear regression: *Cohen's d* for Population= 0,71;
171 *d* for Treatment= 0,71) (Suppl. Fig. 2).

172 To explore the dynamics of bacterial proliferation inside the host, we performed a time-
173 course characterization of bacterial loads during and after oral infection by counting the

174 number of colony-forming units (CFUs) obtained from individual flies. In Figure 3, we show the
175 progression of bacterial loads from 1 hour until 52 hours post-infection (Fig. 3A) as well as the
176 cumulative number of individuals without detectable live bacteria (Fig. 3B). The infection
177 dynamics of both populations start with very high numbers of *P. entomophila* at early stages,
178 confirming the absence of differences in initial inoculum reported above (Table 1, parameter
179 *a*: 3.072± 0.016). Bacterial loads continuously decrease and, at approximately 20 hours of
180 infection, Control and BactOral population begin to differ significantly in a sex-dependent
181 manner. From this point onwards we observe a steady decrease of bacterial quantities in the
182 two populations, always more severe in males than in females, and in BactOral than in Control
183 flies (Fig. 3A). In addition, the number of flies that clear the infection also increases with time
184 in both populations, but at a faster rate and reaching higher levels in BactOral (Fig. 3B). By 52
185 hours post-infection, in both males and females, the number of "cleared" flies in the BactOral
186 population was twice as high as that of its Control counterpart (Fig. 3B). Together, these
187 analyses indicate that the evolved population eliminates bacteria faster and more efficiently
188 than Control.

189 In order to pinpoint more accurately the parameters contributing to this overall
190 difference, we modelled the bacterial load and zero dynamics, and assessed differences in
191 parameter estimates considering the initial load or inoculum (*a*), the load in the long-term or
192 set-point bacterial load (*b*), the timepoint at which the load is reduced in half (*k*) and the highest
193 rate of decrease or slope at timepoint *k* (*h*) (see Materials and Methods for details). In Table
194 1, we show the mean estimates, standard deviation, and high-density intervals (3% and 97%)
195 for all four parameters of the fitted sigmoidal model, for Control and BactOral populations.
196 Overall, these results show that sex has a significant effect on all curve parameters (k= -0.053
197 ± 0.068; h= -0.330 ± 0.227; a= -0.130 ± 0.038; b = -0.147 ± 0.066), reinforcing the notion of a
198 strong sexual dimorphism in response to infections[48–50]. Additionally, populations differ on
199 "time to half height" (k= -0.108 ± 0.046) and final load (b= -0.285 ± 0.053), but not on initial
200 load (a= -0.002 ± 0.024) nor on highest rate of decrease (h= 0.042 ± 0.182).

201 Because this time-course experiment yielded a significant amount of zero counts,
202 corresponding to individual flies for which no CFUs could be counted, we treated this as an
203 independent dataset for which we developed a separate model focused on infection clearance
204 (Table 2). With that, we found that a_1 (final fraction of zeros) was significantly affected by both
205 sex and population (with no observable overlap between the mean estimates for each
206 condition) (a_1 Females= -0.781 ± 0.07; a_1 Males= -0.061 ± 0.069; a_1 Control= -0.700 ± 0.070;
207 a_1 BactOral= -0.144 ± 0.069), with more BactOral individuals having cleared the infection by
208 the end of the experiment, and males more so than females. There were significant overlaps
209 between the estimates of the remaining parameters, evidencing a difference in clearance rate
210 only in late stages of bacterial infection. In sum, these data align with those of bacterial load
211 dynamics, whereby flies from BactOral population clear *P. entomophila* faster and better from
212 20h of exposure onwards.

213 These results support the hypothesis that resistance mechanisms participate in the
214 adapted response of BactOral. Indeed, bacterial numbers are affected differently between
215 BactOral and Control populations despite the equivalent initial bacterial inocula (Fig. 2A, Table
216 1) and rates of live bacteria defecation (Fig. 2B). The differences in number of detected live
217 bacteria inside flies suggests a direct bacterial control mechanism is operating more efficiently
218 in BactOral individuals.

219 Differences in AMP expression are consistent with distinct bacterial
220 loads dynamics

221 In an attempt to pinpoint which resistance mechanisms contribute to the differences in
222 bacterial clearance rates observed between populations, we infected female flies from Control
223 and BactOral and dissected their guts prior to exposure, and after 8, 24, and 32 hours of
224 infection with *P. entomophila*. We quantified expression levels for a panel of 10 AMPs covering
225 different molecular families, dedicated pathways and pathogen specificities, including *Attacin-*

226 *A*, *Bomanins Short 2* and *3*, *Cecropin-A1*, *Defensin*, *Diptericin-A*, *Drosomycin* and
227 *Drosomycin*-like *2*, *3* and *5* (Fig. 4, Suppl. Tables 6-7, and Supp. Fig. 3).

228 We first verified if the baseline levels of AMPs (i.e., expressed under homeostatic
229 conditions) differed between populations, by relativizing expression levels to the Control
230 population at the unchallenged timepoint. With this approach, we established that BactOral
231 and Control populations express comparable amounts of 8 AMPs when unchallenged, the two
232 exceptions being *Cecropin-A1* (*CecA*) with lower expression levels (t = 2.892, p= 0.0445) and
233 *Drosomycin-like 5* (*Drsl5*) with higher levels (t = -4.686 , p= 0.0094) (Supp. Fig. 2) in the
234 Control compared to the BactOral population.

235 In addition, and since our main goal was to characterize the temporal dynamics of AMP
236 expression in both populations, we performed the analysis for the entirety of the time-course
237 using the unchallenged timepoint as reference (power analysis for a t-test: *Cohen's d*= 0,62)
238 (Fig. 4 and Suppl. Tables 6-7). Overall, we detect consistent dynamics with the reduction of
239 expression happening sooner (by 32 hours) in BactOral than Control across 7 of the 10 AMPs
240 tested, the exceptions being *Attacin A*, *Defensin* and *Drosomycin-like 2* (*Drsl2*) (Fig. 4). This
241 earlier down-regulation in BactOral relative to Control flies (at 32-hours) of *BomaninS2*
242 (*BomS2*) (t = 4.347, p= 0.0047), *BomaninS3* (*BomS3*) (t = 4.035, p= 0.0089), *CecA* (t = 4.529,
243 p = 0.0010), *Diptericin* (t = 2.613, p= 0.0439), *Drosomycin* (t = 4.795, p= 0.0056), *Drosomycin-*
244 *like 3* (*Drsl3*) (t = 3.194, p= 0.0105) and *Drsl5* (t = 4.266, p= 0.0021). Furthermore, we detected
245 a significantly higher up-regulation of *AttacinA* and *CecA* in BactOral at 8 hours of infection
246 (*AttacinA*: Control 8h *versus* BactOral 8h – t = -2.563, p= 0.0389; *CecA*: Control 8h *versus*
247 BactOral 8h – t = - 3.034, p= 0.0123), consistent with the role of these peptides as main players
248 against infections with Gram-negative bacteria[51,52]. A similar trend was observed for other
249 tested AMPs such as *Defensin* and *Drsl2*, although these changes were not statistically
250 significant (*Defensin*: Control 8h *versus* BactOral 8h – t = -2.101, p= 0.231; *Drsl2*: Control 8h
251 *versus* BactOral 8 h.p.i. – t = -0.899, p= 0.465).

252 Principal Component Analysis (PCA) supports these trends (Suppl. Fig. 4). The two
253 main Principal Components (PC1 and PC2) evidence the time-dependent differences
254 observed between populations by showing an initial indistinguishable grouping of both,
255 followed by a dissimilar change in expression patterns at 8 hours of infection which culminates
256 with a maximum divergence between Control and BactOral at the later timepoint (32 hours).

257 Increase of AMP levels in the Control population phenocopies
258 BactOral response to infection

259 Having correlated the improved bacterial clearance of BactOral flies (Fig. 3) with
260 significant differences in expression levels of AMPs between populations (Fig. 4), we sought
261 to functionally associate these two observations. For that, we conducted a priming experiment
262 to induce higher basal levels of AMPs by feeding flies a solution of heat-killed bacteria. These
263 primed flies were, subsequently, infected orally with live *P. entomophila*, and followed daily for
264 mortality. This exposure to dead bacteria has been shown to "artificially" increase AMP
265 levels[53–55]. We expect the priming with heat-killed bacteria to increase the basal expression of
266 AMPs and thus provoke, particularly in Control flies, an up-regulation of the immune system,
267 allowing test of the influence of this heightened condition in survival to oral infection.

268 Flies previously fed with heat-killed bacteria increased survival to infection with live *P.*
269 *entomophila* in BactOral and Control populations, confirming the stronger immune response
270 expected upon priming (Suppl. Table 8 and Fig. 5, BactOral females fed with live bacteria
271 (control) *versus* BactOral females primed with heat-killed bacteria (primed): $z.ratio = 7.193$, $p <$
272 0.0001; BactOral males treated with control *versus* BactOral males primed with Heat-killed
273 bacteria: $z.ratio = 4.368$, $p = 0.0001$; Control females fed with control *versus* Control females :
274 $z.ratio = 10.011$, $p < 0.0001$; Control males treated with control *versus* Control males primed :
275 $z.ratio = 7.299$, $p < 0.0001$). Females from the Control population primed with heat-killed
276 bacteria increased their survival to oral infection, virtually phenocopying BactOral infected with
277 live bacteria, whereas in males, the same tendency is observed but to a lesser degree (Suppl.

278 Table 8 and Fig. 5, BactOral females fed with control *versus* Control females primed: *z.ratio*=
279 1.629, *p*= 0.3620 and BactOral males fed with control *versus* Control males primed: *z.ratio*= -
280 4.547, *p*<0001). This effect is supported by a time-course of relative expression levels of IMD
281 pathway key players, *Relish* (its devoted transcription factor) and its associated AMP,
282 *Diptericin*, as well as the Toll-activated AMP, *Drosomycin* (Suppl. Fig. 5). For both populations,
283 we observed an increase in levels of *Diptericin* to a comparable degree upon exposure to
284 heat-killed *P. entomophila*, but not of *Drosomycin*.

285 With this experiment we strengthened the case for a role of improved resistance in the
286 adapted phenotype of the BactOral population.

287 BactOral maintains higher survival upon immune response
288 activation

289 To ascertain whether disease tolerance may also contribute to BactOral increased
290 survival upon oral infection, we continuously fed flies with a mixture of heat-killed *P.*
291 *entomophila* and followed survival (Fig. 6).

292 With this setup we verified that both females and males of BactOral survive significantly
293 more than Control after the specific damages induced by constant exposure to heat-killed
294 bacteria. Importantly, the survival differences detected between populations occur up until the
295 time at which flies were selected to reproduce during the experimental evolution protocol (10
296 days upon infection), evidencing the adaptive nature of this response. (Suppl. Table 9 and Fig
297 6, Control - HK *versus* BactOral - HK females: *z.ratio*= 6.579, *p*< 0.0001; males: *z.ratio*=
298 9.612, *p*< 0.0001). No significant differences exist under control conditions (Control - PBS
299 *versus* BactOral - PBS females: *z.ratio*= 1.392, *p*= 0.504; males: *z.ratio*= 0.587, *p*= 0.936).

300

301

302 # DISCUSSION

303 In this work, we enquired systematically for the bases of adaptation of one evolved line
304 of *D. melanogaster*, against an oral bacterial infection considering the three general layers
305 that constitute the immune response: behaviour, resistance and disease tolerance[3,8,56]. Upon
306 experimental evolution of an outbred population of *D. melanogaster* against oral infection with
307 *P. entomophila*, and after approximately 6 generations, the selected population (BactOral)
308 increased survival upon infection[19]. This phenotype was maintained throughout the selection
309 experiment but, since then, the population has been kept for more than 80 generations under
310 relaxed selection. Despite the fact that no physiological costs associated to this response
311 could be detected in any of the original four replicates[46], we tested the immunocompetence of
312 one selected representative replicate per evolved population. The average survival rate after
313 oral infection of flies from BactOral was approximately 63% (50% for females and 75% for
314 males) and approximately 10% in the Control population (5% for females and 15% for males).
315 Considering that at the end of selection, BactOral survival reached 90 %[19], this partial
316 decrease in overall survival after infection with bacteria can potentially underestimate the
317 contributions of each of the different mechanisms behind adaptation. Importantly, these losses
318 could be attributed to genetic drift acting on the populations during the long relaxed-selection
319 period they sustained. Notwithstanding, we established that it is methodologically possible to
320 ascertain the relative importance of behavioural, resistance, and disease tolerance
321 mechanisms.

322 ## No role for behavioural or structural differences

323 It is well described that the route of infection is of paramount importance to the outcome
324 of host-parasite interactions. In *D. melanogaster*, the mode of entry of a bacterial pathogen
325 into the host's body elicits different physiological responses[19,57–61], which can start by the
326 avoidance of the pathogenic substrate, in the case of infection through oral route[26,62,63], both
327 by inhibiting feeding or choosing oviposition sites according to bacterial composition[26,53,62–64].

328 *P. entomophila* is a natural pathogen of Drosophila that causes high mortality when ingested
329 across diverse genetic backgrounds and conditions[23,43,44,65,66]. Thus, it is plausible to consider
330 that fasting could constitute a general response against oral infection. The simplest behaviour
331 that could explain the observed differences in survival between populations would be that
332 BactOral flies evolved a reduced intake during exposure to bacteria, thereby reducing their
333 "inoculum" size. Indeed, fasting is a prevalent response to infection and behavioural avoidance
334 is a common strategy to limit exposure to infected substrates in different systems[4,62,63].
335 However, we showed similar amounts of bacteria were ingested by females of both
336 populations (Figure 2A)[23,67]. Moreover, we consubstantiate the findings from the CAFE assay
337 with the bacterial loads time-course, that show equivalent amounts of *P. entomophila* colonies
338 inside infected flies at early timepoints. Our results indicate that behavioural inhibition of
339 bacterial intake was not one of the mechanisms that evolved in BactOral flies, eliminating
340 fasting as a component of the adaptation to *P. entomophila* exposure.

341 Previous reports have shown that pathogen intake can affect gut function and
342 structure[34–36,45] as well as influence the dynamics of exposure by altering defecation rates[29].
343 However, we could not find evidence for any differences in these features between control
344 and evolved populations. Firstly, there are no changes in gut length in steady-state conditions
345 that would somehow enable the BactOral population to *a priori* face bacterial infection more
346 favourably. Increased enteric cell renewal, which often leads to a temporary shortening of gut
347 length[36], has been described as one of the main mechanisms of *D. melanogaster* defense
348 against oral bacterial infection[35,36,45]. In our experiments, we detected a significant decrease
349 in total gut length after 24 hours of oral infection, but this change was equivalent in both
350 populations, not supporting a role for altered renewal rate in BactOral adaptation.

351 These experiments, however, do not completely discard the hypothesis of differential
352 gut structure underlying the adapted phenotype of BactOral. For example, proportions of gut
353 cell types can change upon infection[68] and such qualitative shift could contribute to higher

354 fitness upon exposure to *P. entomophila* in the adapted population. Also, it could be that
355 relevant changes in gut length occur at later timepoints. Notwithstanding, it has been
356 previously shown that infection-induced gut cell loss starts as early as 2 hours after exposure,
357 with a return to 75% of the total original gut length after 24 hours[36]. This time window is well
358 within the boundaries defined in our setup. Another important aspect to note is that renewal
359 capacity is sexually dimorphic under diverse physiological contexts[69,70]. For instance, females
360 have higher gut cell renewal rates than males upon oral infection with *P. carotovorum* 15 [71], a
361 bacterium that provokes higher mortality in males than females, contrary to oral infection with
362 *P. entomophila* (see Results). The disparity between those and our observations may be
363 explained by the different mechanisms of virulence employed by these two pathogens to infect
364 *D. melanogaster*[45]. Be as it may, we envision further experiments testing for potential
365 differences in gut delamination rates between evolved populations at distinct timepoints.

366 BactOral flies also did not expel pathogenic bacteria from their guts differently from
367 Control. However, males of BactOral show a decrease in the number of alive bacteria
368 defecated between 6- and 18-hours post-exposure, evidencing, to some extent, improved
369 bacterial clearance and suggesting a role for resistance. Since our protocol only detects live
370 bacteria, we could potentially be missing an important component of the response and future
371 work should quantify defecation of alive and dead bacteria, for example through fluorescent
372 microscopy, to compare populations with regards to the contribution of overall peristalsis to
373 the elimination of total gut content[29]. In addition, considering the slightly higher number of of
374 BactOral flies that defecated alive bacteria below our detection limit (zeros), we hypothesize
375 that this population either retains *P. entomophila* for longer inside guts before they begin to
376 expel it (which could point to higher disease tolerance), or reduce bacterial loads more
377 effectively at earlier stages, even though, with time, bacterial proliferation recovers (again
378 suggesting stronger resistance).

379

380 ## A role for resistance

381 To further test the suggested role for resistance, we characterized the bacterial load
382 dynamics in evolved populations over 52 hours and determined that BactOral flies eliminate
383 pathogens in a more efficient way than Control. This significant change in bacterial loads
384 indicates that BactOral has evolved higher resistance, that control earlier and clear better gut
385 bacterial loads. As this model was applied to the logarithm of mean bacterial counts at each
386 timepoint, it is possible that information regarding intra-populational variation is not considered
387 in the analysis. Nevertheless, the conclusions from the comparison of the bacterial counts are
388 further supported by the separate analysis on the number of "zeros" as a function of time,
389 which is a proxy for bacterial clearance.

390 Even though there is no discernible difference in the amount of CFUs infecting both
391 males and females between both populations until approximately 20h of infection, by this time
392 individuals from BactOral begin to exhibit gradually decreasing levels of bacteria. This trend
393 aligns with the higher clearance rate observed in the adapted population (and even higher in
394 males than females) as measured by the increase in the number of flies from which we cannot
395 grow colonies of *P. entomophila* ("zeros"). Flies from the Control population control their
396 bacterial loads (at around 20h of infection) but do it at a slower pace and in a less effective
397 way (i.e. many individuals exhibited high bacterial counts by the end of the experiment). It is
398 possible that our proxy for clearance (i.e., flies classified as "zeros"), could be overestimated
399 due to a low detection threshold. Indeed, it has been shown that *D. melanogaster* can endure
400 chronic bacterial infections[72–74], albeit at rather low levels, which can be the case in our
401 experimental setup. Nonetheless, this holds true for both populations, therefore not
402 invalidating our interpretation of a different bacterial clearance rate between genotypes.

403 Having shown that BactOral clears *P. entomophila* infection more rapidly, we
404 hypothesized that this response could rely on an increased production of immune effectors,
405 such as AMPs. Analysis of AMP gut gene expression showed that in unchallenged condition,
406 only two AMPs (*CecA1* and *Drsl5*) differed in expression between populations (down- and up-

407 regulation respectively), indicating that the base levels of AMP expression are unlikely to have
408 been a major target of selection. The detected expression difference of *Drsl5*, a gut-specific
409 AMP peptide, could represent a pre-emptive resistance mechanism acquired by BactOral, by
410 establishing a more antagonistic environment for *P. entomophila* proliferation in the guts of
411 these flies after ingestion.

412 At 8 hours of exposure, comparing BactOral to Control, two AMPs show a significantly
413 faster up-regulation while seven of the panel members display a pattern of faster
414 downregulation, later on (32h). These results suggest that a two-step process might be taking
415 place, whereby the response is initially (8h) more aggressive, permitting a more effective
416 bacterial control, and is turned off sooner (32h), limiting the secondary effects of prolonged
417 immune responses (immunopathology). Interestingly, *Drosomycin-like* genes (*Drsl2*, *Drsl3*,
418 and *Drsl5*), whose expression is specific to the gut epithelium and are regulated by JAK-
419 STAT[35], show up-regulation after 8-hours of infection in both populations. For all three genes,
420 there is a slight tendency for a faster response that could represent a more effective bacterial
421 control in the BactOral population and a faster down-regulation at the 32-hour timepoint that
422 could limit self-inflicted damage. Likewise, we observed a similar process of down-regulation
423 of other AMPs specifically deployed to fight off infections with Gram-negative pathogens (e.g.
424 *CecA1* and *Diptericin*) at the 32-hour timepoint. In contrast, some AMPs typically associated
425 to response to Gram-positive bacteria, (e.g. *BomS2*, *BomS3* and *Drosomycin*[51,75,76)], do not
426 exhibit significant up-regulation after exposure to *P. entomophila* until 32-hours post-infection
427 and only in the Control population.

428 Strikingly, and even though *Diptericin* has been shown to be deployed by *D.*
429 *melanogaster* to resist *P. entomophila* infection[67], we did not detect a difference in the relative
430 expression of this gene between Control and BactOral until after 24 hours of infection. At this
431 timepoint, adapted flies are already returning to basal levels of AMP production whereas
432 Control flies keep actively fighting off the infection.

433 The same pattern is observed also in the priming experiment (Fig. 5 and Suppl. Fig.
434 5). After priming with heat-killed bacteria and subsequent exposure to live bacteria, both
435 populations maintain an equivalent expression increase until 8 hours but from 24 hours
436 onwards BactOral shows, again, an earlier downregulation of *Diptericin* and a quicker return
437 to homeostatic levels of expression (Suppl. Fig. 5). In brief, the dynamics of *Diptericin*
438 expression mimic the expected effect of priming by establishing higher basal levels of this
439 AMP with which to counteract an incoming infection. This is not seen for the AMP *Drosomycin*,
440 which again is consistent with our previous result that this AMP is not deployed against
441 infection with *P. entomophila* (Fig. 4 and Suppl. Fig. 3).

442 With this we re-enforce the notion that the process of earlier AMP down-regulation
443 could be an active response rather than a consequence of lower bacterial loads. This points
444 to a potential role for disease tolerance mechanisms in this population through the prevention
445 of AMP-induced immunopathology.

446 A role for Disease Tolerance

447 Disease tolerance encompasses many distinct mechanisms of action, and describes
448 multiple different physiological processes, which have in common the wide umbrella of
449 maintaining host fitness without directly impacting pathogen load[6,77]. Maintenance of fitness in
450 the face of stress-inducing or homeostatic conditions is assured by tissue damage control
451 mechanisms[6,77]. Furthermore, these damage control responses can be activated in the event
452 of an infection, either due to direct action of pathogens (i.e., injury-inducing toxins) or indirectly
453 from immune-derived damages (i.e., immunopathology).

454 With this in mind, a putative role for disease tolerance was tested by measuring survival
455 of the evolved populations to prolonged exposure to heat-killed bacteria. This protocol
456 provided a means by which to test the response to damage, whether provoked by
457 pathogenicity of the bacteria or by the host's own response, independently of active pathogen
458 elimination. Although we hypothesize that the heat-killing treatment was insufficient to

459 denature all the virulence factors present in the suspension, we are yet to disentangle the
460 relative contribution of virulence factors versus immunopathology to the longevity
461 measurements. A similar longevity was observed for females and males of Control and
462 BactOral after feeding on heat-killed bacteria, but an interesting trend appeared when
463 comparing survival profiles (Fig. 6). We found that BactOral survived better to the stress of
464 prolonged exposure to heat-killed *P. entomophila*, specifically under an evolutionarily relevant
465 timeframe, that is, the period when flies reproduced during the selection experiment. Although
466 both Control and BactOral individuals fully succumbed to the chronic exposure to heat-killed
467 bacteria, significant differences in survival distinguish the two populations. Physiological
468 responses measured approximately at 10 days post-infection (the age at which populations
469 were selected to reproduce), can potentially represent evolutionary costs[78–81], which are not
470 the focus of this study.

471 Additionally, together with the gene expression data presented in Fig. 4, showing that
472 BactOral represses the activation of immune effectors earlier upon infection, it is tempting to
473 hypothesize that a similar process happened during the course of this experiment, and a role
474 may be attributed to tighter control and negative regulation of immune over-activation.

475 The improved response of adapted flies to exposure to heat-killed bacterial substrate
476 can be explained by a higher capacity to withstand damage, whether 1) provoked by their own
477 immune response, or 2) caused by virulence factors present in inactivated bacterial
478 supernatant. Additionally, process 1) could be correlated with the previously identified tighter
479 immune repression. Disentangling the relative contribution of these mechanisms to the overall
480 adapted phenotype would need further empirical characterization.

481

482

483

484 # CONCLUSION

485 In sum, we were able to identify resistance and disease tolerance mechanisms as
486 targets of experimental evolution for increased survival to *P. entomophila* oral infection. Our
487 observations align with previous work that identifies a positive genetic correlation between
488 these two distinct immune defense strategies[82]. The increased survival of an experimentally
489 adapted population to oral infection directly relates to its capacity to decrease bacterial loads
490 after infection, independently of bacterial feeding rate, gut renewal capacity and bacterial
491 defecation. Previous work found resistance to be targeted by selection upon immune
492 challenge in *D. melanogaster*[79,83]. Our work adds to this approach by approaching the other
493 mechanisms at play in immunity, namely disease tolerance and behaviour, in the framework
494 of a single evolutionarily coherent system. We report one additional case of resistance
495 evolution that exhaustively approaches the different processes by which immunity may evolve
496 in populations fighting off persistent infections. In addition to resistance, we find a quantifiable
497 role for disease tolerance in the evolved response of BactOral, revealing the complex nature
498 of this adaptative process[82], which is likely to rely also on improved tissue repair coupled with
499 a more controlled deployment of damage-inducing immune effectors, such as AMPs[84,85]. This
500 interplay between disease tolerance and resistance, underscores the complex mechanistic
501 nature of immune response evolution with, undoubtedly, parallels in other host-pathogen
502 systems. Future work will focus on identifying the specific mechanisms of disease tolerance
503 and their physiological mode of action and interactions with resistance and their potential
504 evolutionary repeatability.

505

506 # METHODS

507 ## Maintenance of Drosophila populations under relaxed selection

508 Experimentally evolved Control and BactOral populations were initially derived from a
509 highly outbred population of *D. melanogaster*, as described in previous studies [19,46]. During
510 experimental evolution, four replicates of each selection regime were maintained by orally
511 infecting 310 females and 310 males per replicate, with a suspension of *P. entomophila*
512 previously determined to cause approximately 66% of mortality in the starting population. After
513 selection was terminated, populations were kept at high census (1500-2000 individuals per
514 cage), allowing for maintenance of high genetic variability and optimal larval development. For
515 all generations, flies were kept under constant temperature (25°C), humidity (55–65%) and
516 light-darkness cycle (12:12), and fed with a standard cornmeal-agar medium, consisting of
517 4.5% molasses, 7.5% sugar, 7% corn-flower, 2% granulated yeast extract, 1% agar and 0.25%
518 nipagin, mixed in distilled water. Control and BactOral were under selection for 24 generations
519 and under relaxed selection for approximately 80 generations, after which one replicate of
520 each regime was singled out to perform mechanistic experiments.

521 Each generation cycle lasted approximately 3 weeks, during which flies eclosed and
522 remained undisturbed until they were 7-8 days old. At this time, fresh food was placed in the
523 cages for 2 days, which allowed for oviposition of retained eggs. After this period (when flies
524 were 9-11 days old), similarly to the protocol applied for selection, new fresh food was placed
525 in the cages for controlled egg-lays (1-3 hours long) and establishment of the next generation.

526 ## Pathogen culture and infections

527 Oral infection protocol with *P. entomophila* (Rifampicin resistant strain kindly provided
528 by Bruno Lemaitre) was adapted from the one used in Martins and Faria et al[19]. Briefly, single
529 bacterial colonies were grown in kick-start cultures (5mL of LB) for approximately 8 hours after
530 which they were transferred into larger volumes (1 L of LB) for overnight growth, both periods
531 at 29ºC. After centrifugation and resuspension of bacterial pellets, concentration was adjusted

532 to OD_{600}= 100 and finally diluted 1:1 with a 5% sucrose solution. For infection, 3-5 days-old
533 flies were separated by sex into groups of twenty with the use of CO_2 (at least 24 hours before
534 the start of the experiments) and allowed to feed on a filter disk embedded with the bacterial
535 solution described above for 24 hours (for survival assays) or for the amount of time specific
536 to each experiment (see Results). After this period, infected flies were flipped onto clean food
537 and survival was scored daily. Controls were fed a 1:1 LB/PBS:5% sucrose solution.

538 For the priming experiment, a solution of *P. entomophila* at OD_{600}= 100 was previously
539 heat-killed for 1 hour at 55ºC, mixed 1:1 with a 5% sucrose solution and fed to flies for 2 hours,
540 in a similar way as described above for regular oral infection. Efficiency was confirmed by
541 plating the heat-killed culture and confirm absence of bacterial growth. After this time, flies
542 were transferred into tubes containing a live solution of *P. entomophila* for 24 hours, after
543 which they were changed back to clean food and their survival scored.

544 For the experiment with prolonged exposure to heat-killed *P. entomophila* a similar
545 bacterial preparation and heat-killing protocol were performed as described above, but the
546 final dead bacteria suspension was mixed 1:1 with standard fly food and dispensed into fly
547 vials. To the final solution, extra agar powder was added to account for viscosity and to
548 optimize texture and left to cool down and solidify for at least 2 hours, before being fed to flies.
549 The same protocol was performed for the control treatment, in which fly food was mixed with
550 sterile 1X PBS. Exposure to these treatments was constant throughout the experiment, during
551 which replicates of 15 females and 15 males were co-housed in vials, exclusively feeding on
552 the dead-bacteria or PBS mixtures. Survival scoring began at the start of exposure and lasted
553 14 days. During the treatment, flies were frequently flipped onto fresh food mixture and survival
554 scoring continued daily. Overall efficiency of the bacterial heat-killing protocol was tested by
555 culturing the heat-killed suspension and confirm the absence of bacterial growth, prior to
556 storage at -20 ºC, which anteceded the mixing with the food or sucrose solution.

557 Survival analysis was done for the multiple experiments by fitting data with Cox
558 Proportional Hazards models (with *coxme* or *coxph* functions from the *survival* package[86])
559 using population, treatment, and sex as fixed factors and replicate (or replicate nested within
560 block) as a random factor. To assess the effect sizes of each factor, type 3 ANOVAs (*Anova*
561 from the *car* package[87]) were ran on the survival models. Additionally, multiple comparisons
562 with *tukey* adjustments were done on all the models to disentangle which specific pairwise
563 comparisons were significantly different (*emmeans* function[88]). Finally, the *cld* function of the
564 *multcomp* package[89] (with *sidak* confidence-level adjustments) was used to summarize the
565 results of the multiple comparisons across conditions. Survival models used were:

566 - immunocompetence (Fig. 1)- *coxme*(~Population*Treatment*Sex+(1|Replicate));
567 - priming (Fig. 5)- *coxme*(~Population*Treatment*Sex+(1|block/Replicate)) and
568 - immune over-activation (Fig. 6)- *coxme*(~Population*Treatment*Sex+(1|Replicate),
569 data = survival_HKexposure).

570 **Bacterial defecation assay**

571 To have a measure of the rate of gut purge/defecation a change was introduced to the
572 bacterial infection protocol, whereby the feeding period was reduced to a 3-hour period. After
573 this time, flies were surface sterilized and separated individually into plastic spectrophotometry
574 cuvettes, previously filled with fly food. After 6 hours (6 hours.), the same flies were changed
575 into fresh cuvettes where they remained for an additional 12 hours (18 hours.), after which
576 they were discarded. Bacterial quantification was performed for both timepoints by washing
577 the interior surface of the cuvettes with PBS and plating, as described below.

578 Statistical analysis on number of defecated bacteria was done by fitting a zero-inflated
579 on the CFU counts and zeros (using the *zeroinfl* function[90] from the *pscl* package[91]). This
580 model allowed for the fitting of a negative-binomial distribution on the counts data and of a
581 binomial distribution on the zeros (*zeroinfl* (formula= Counts ~ Population * Sex * Timepoint |
582 Population * Timepoint, dist= "negbin")). To assess differences between specific pairwise

583 comparisons we used *emmeans* with *tukey* adjustments, on both distributions of the zero-
584 inflated model. In the instances where flies died during either timepoint, their count at that time
585 was removed from further analysis and the linear regression was not included in Figure 2B.

586 Quantification of food intake by CApillary FEeder Assay - CAFE

587 To measure the amount of bacterial solution ingested by single flies, an adapted
588 CAFE[47] assay was performed. We substituted the food of small fly vials with agarose, to
589 ensure humidity, and placed a glass capillary in each lid, filled with 5% sucrose solution. We
590 allowed individual flies to habituate to this experimental setup for 24 hours before the infection.
591 After this period, capillaries filled with *P. entomophila* suspension (prepared as described
592 before) were given to individual females to feed on and consumption estimated, at different
593 timepoints. To facilitate scoring in capillaries, the bacterial solution was mixed with blue food
594 colouring.

595 Modelling of cumulative amounts of bacteria eaten over time was done with a
596 Generalized Linear Model with a Gamma distribution, (*glm*(Quantity ~ Population*Timepoint))
597 using the *glm* function from the base *stat* R package[93]. For better visualization of feeding over
598 time, linear regressions were drawn between mean values of microliters eaten cumulatively
599 by each population at each timepoint, using the *stat_poly_eq* function of the *ggpmisc*
600 package[94]. Finally, multiple comparisons were done with *emmeans*[88], using *tukey*
601 adjustments, to disentangle differences between populations across timepoints.

602 Measurement of gut length

603 Females were individually dissected in PBS 1X, and their guts removed for imaging by
604 adapting a protocol described in[96]. Dissections started by fixing flies in the head with a Minutien
605 pin and gradually separating the gut from the remaining tissues, in a posterior-to-anterior
606 direction. After the gut was exposed, we added a few drops of a solution of 5% Acetic Acid
607 and 50%EtOH, which slightly whitens the tissues, allowing for better visualization and a slight

608 tissue fixation. After having isolated the guts, they were individually transferred to a coverslip
609 and photographed using a uEye camera installed on an Olympus SZX7 stereoscope.

610 Images were processed in FIJI v2.0.0 and measurements were collected by drawing
611 consecutive transects along the total length of guts, as illustrated in Supplementary figure 2A.
612 Each individual gut measurement was repeated 3 times, to account for errors in the manual
613 drawing of the transects and averaged to obtain a final gut length in millimeters (mm).

614 Differences in lengths of guts between populations were analysed with a Linear Mixed-
615 Effects Model (*lmer*(Length ~ Population * Treatment + (1|Block)) with *lmer* from the *lmerTest*
616 package[97]. The model was followed by multiple comparisons with *emmeans*[88], with a *tukey*
617 adjustment, for assessment of individual pairwise differences.

618 Quantification of bacterial loads

619 To quantify bacterial loads, we adapted an established protocol[19,92]. In short, individual
620 flies were surface sterilized (washed in EtOH 70%, bleach 60% and two washes with MiliQ
621 water) and placed in 96 deep-well plates, where they were homogenized with glass beads in
622 50 µL of LB using a TissueLyser II (Qiagen). Individual samples were serially diluted 5 times
623 with 1:10 dilution steps, plated as 4 µL droplets in LB+Rifampicin, and left to grow overnight
624 at 29ºC to optimize colony size for counting. For the bacterial defecation assay, cuvettes that
625 contained flies for each of the timepoints were filled with 1mL of PBS and incubated at room
626 temperature for 15 minutes, after which each sample was diluted up to 4 times (1:5 dilutions)
627 and platted as droplets in LB+Rifampicin.

628 Analysis of the time-course of infection progression was performed by dividing the data
629 obtained from the bacterial quantification into “counts” and “zeros” and using different models
630 to examine their dynamics in our evolved populations. For the counts, the modelling of the
631 mean of the load dynamics during the time-course was done using a sigmoidal function that
632 considers four parameters: *a* the log (initial load), *k* the timepoint at which the load is half of
633 the maximum value (in this case, *a*), *h* the slope at the *k* timepoint (steepest slope of the

634 curve), and *b* the log (final load). A linear model was applied to each of the parameters which
635 was fitted using a Monte Carlo Markov Chain approach. To estimate the impact of Sex and
636 Population on the likely distribution of the parameters, a normal error structure was assumed,
637 using Control females as reference. For the "zeros", a modified logistic model with four
638 parameters was ran: a_0 initial fraction of zeros, k_0 rate of zero disappearance, k_1 rate of zero
639 re-appearance, t_0 timepoint at which zeros re-appear and a_1 final fraction of zeros. Similarly,
640 to the "counts" analysis, linear models were used to assess the impact of Sex and Population
641 on all four parameters.

642 RNA extractions and quantitative PCR

643 For RNA extractions, flies from Control and BactOral population were infected as
644 described above and, at different timepoints, pools of 10 females were collected and
645 homogenized in 500 µL of Trizol. RNA extractions were performed using a Direct-zol™ RNA
646 MiniPrep w/ Zymo-Spin™ IIC Columns. After precipitation, a DNase I (RQ1 RNASE-FREE
647 DNASE 1* from Promega®) treatment was done to all samples, followed by reverse
648 transcription using the Thermo Scientific® RevertAid H Minus cDNA kit. cDNA was finally
649 diluted 1:5 for qPCR.

650 For quantification of gene expression, qPCRs were performed using SYBRTM Green
651 Master Mix (Thermo Scientific®) and reactions ran on 384-well plates (Applied Biosystems®).
652 The PCR conditions used in all experiments were: initial denaturation/ enzyme activation, 95ºC
653 for 10'; followed by 45 cycles of denaturation, 95ºC for 10"; annealing, 60ºC for 10"; extension,
654 72ºC for 30". Sequences of primers used for qPCRs are shown in Supp. Table. 1. Either EIF2
655 (eIF-2alpha) or Rpl32 were used as reference.

656 Analysis of gene expression differences was done with the relative quantification
657 technique (DDCt)[95]. Briefly, to the average of technical replicates for each candidate gene we
658 subtracted the average of the Ct values of the respective sample's house-keeping gene (EIF2
659 or Rpl32) (DCt) and normalized this value to the DCt of the respective reference condition. For

660 the time-course analysis, the reference was the “Unchallenged” timepoint while for
661 comparisons between “Unchallenged” conditions among populations, Control was used as
662 reference. Finally, we used reverse logarithm to transform the final gene expression values
663 into fold change levels (2^{-DDCt}) and analysed the Log_2(foldchange) differences across
664 Populations and/or Timepoints.

665 Statistical analysis on gene expression was done by running Linear Models (from base
666 R stats[93]) on the Log_2(foldchange) values of each individual gene (*lm*(logfold_AMP ~
667 Population * Timepoint), followed by an ANOVA (type three) from the car package[87]. To
668 individualize significant pairwise comparisons, *emmeans* was ran on the linear models (with a
669 Benjamini-Hochberg adjustment). Finally, the *cld* function of the multcomp package[89] (with
670 sidak confidence-level adjustments) was used to summarize the results of the multiple
671 comparisons across different conditions.

672 Statistical analysis and graphical representations

673 All datasets (excluding survival data) were tested for Normality before further analysis,
674 using the Shapiro-Wilk Test (*shapiro.test* function of the *stat* R package), for determination of
675 the most suitable type of statistical test to perform in each case. All model formulas used for
676 statistical analysis are discriminated throughout the Results sections. All graphical
677 representations were performed using the *ggplot2* package[98].

678 Statistical analysis and graphics for all experiments, excluding analysis of the bacterial
679 loads time-course modelling analysis (Fig. 5), were done on R version 4.2.1, through RStudio[99]
680 version 1.3.959. For the bacterial dynamics time-course, models were implemented and fitted
681 in Python[100], using the probabilistic programming language package *pymc3*[101] version 3.11.

682 Sensitivity (power) calculations for the CAFE and qPCR datasets was calculated using
683 G*Power 3.1 (https://www.psychologie.hhu.de/arbeitsgruppen/allgemeine-psychologie-und-
684 arbeitspsychologie/gpower).

Sensitivity analysis for gut length dataset was performed by simulation, using Python packages *scipy*, *numpy*, and *statsmodels*. Briefly: a synthetic dataset of the same sample sizes and structure as the real data but with a specified effect in both factors (Treatment and Genotype) was generated. Multiple linear regression was then performed and the significance (*p-value*) of both factors was recorded. This was performed 10^5 times, and the fraction of significant tests was recorded (power, independently for both factors). A numerical search for the minimum effect sizes that achieve 80% power on both factors was then performed.

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DECLARATION

The authors declare that this work is not under review elsewhere and that none of its authors has any conflict of interest to declare.

DATA AVAILABILITY

All relevant data are within the manuscript and its Supporting Information files.

AUTHOR CONTRIBUTIONS

Conceptualization: Tânia F. Paulo and Élio Sucena.

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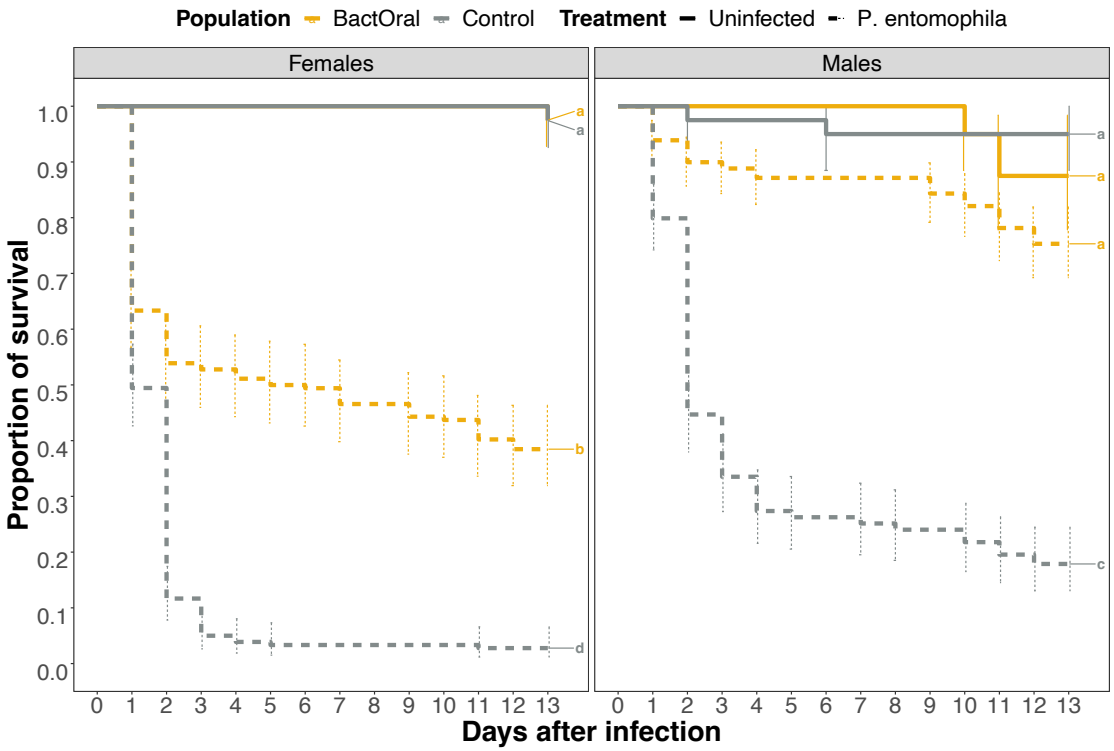
1060 # FIGURE AND TABLE LEGENDS

1061 **Figure 1.**



1062

1063 **Figure 1. Survival upon infection with *P. entomophila.***

1064 Females (left) and males (right) of Control (grey) and BactOral (yellow) populations were orally infected
1065 with a culture of *P. entomophila* OD_{600}=50 (dashed lines) or a control solution (solid lines) for 24 hours,
1066 after which they were flipped onto clean food and their survival measured daily. We identify a
1067 significantly higher survival after infection in BactOral (in both sexes) as compared with Control, in a
1068 treatment-dependent manner. Results from multiple comparisons are shown as letters in the plots.
1069 Sample sizes were 40 uninfected and 180 infected individuals, for each sex per population.

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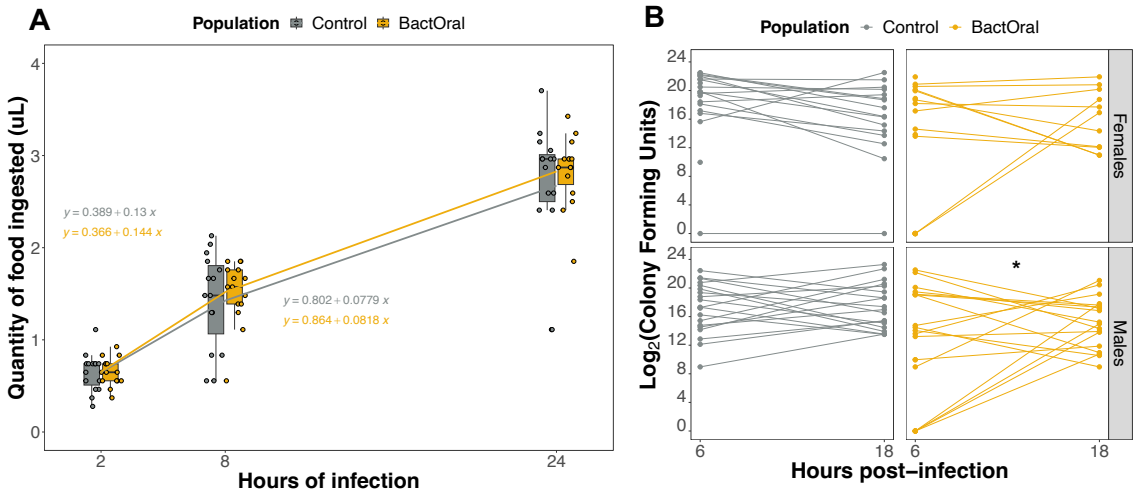
1071

1072 **Figure 2.**

1073



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1076

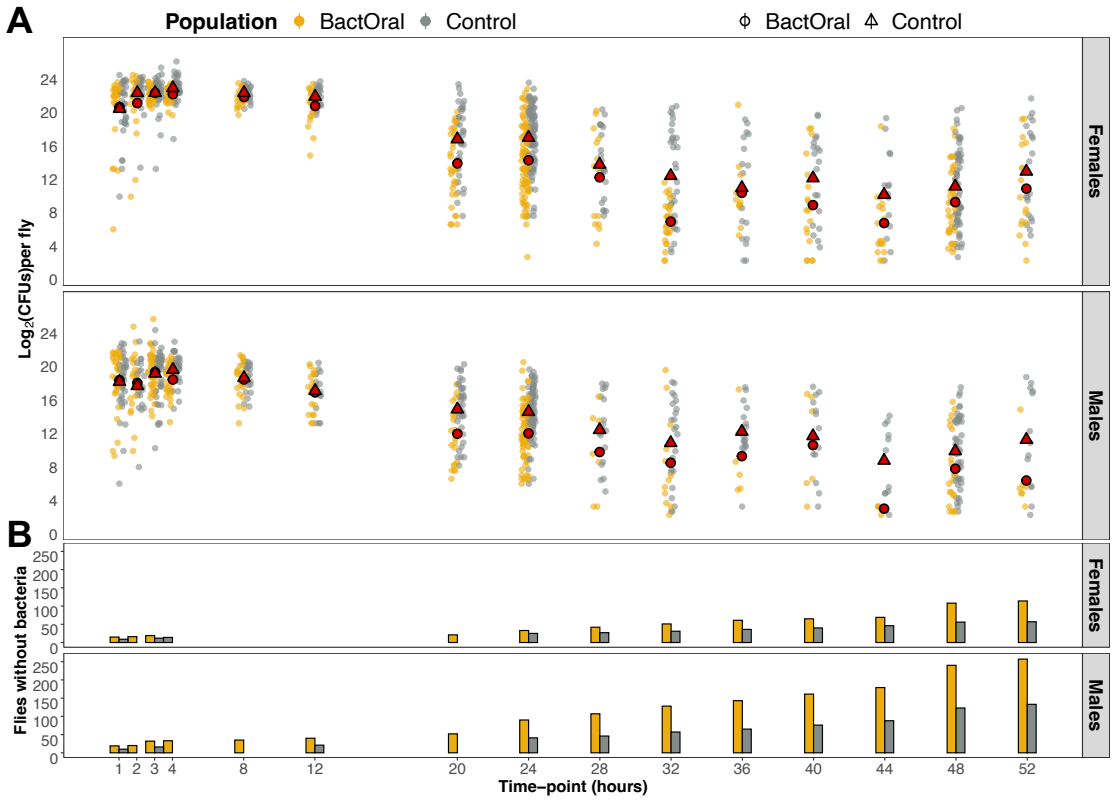
1077 **Figure 2. Evolved and control populations eat and defecate equivalent amounts of**

1078 **bacteria.**

1079 **A)** CAFE assay. Females of Control and BactOral populations were fed individually *P. entomophila*
1080 solution through a graduated glass capillary and the amount of bacterial solution ingested (in µL) per
1081 fly was registered at 2, 8 and 24 hours. Cumulative number of µL ingested per fly showed no statistically
1082 significant difference in amounts ingested by either population at the different time-points. Linear
1083 regressions between averaged values eaten by each population between time-points show a
1084 comparable decrease in feeding as infection progresses (equations shown in plot for the periods, 2–8
1085 hours (above lines) and 8–24 hours (below lines). 15 females were used from each population. **B)**
1086 Quantification of defecated bacteria. Females (top) and males (bottom) from Control (grey) and
1087 BactOral (yellow) populations were fed *P. entomophila* for 3 hours, after which the number *P.*
1088 *entomophila* defecated was estimated after 6 hours and 12 hours. Lines connecting the two points
1089 provide a sense of individual rates of live bacteria defecation within this time window. Linear regressions
1090 between the two counts of the same fly show a significant decrease in the number of bacteria defecated
1091 during the initial 6 hours and the following 12 hours by males of BactOral (shown with *). Additionally,
1092 there is a significant difference in the number of BactOral flies that defecated below our detection
1093 threshold (zero CFU counts) at 6 hours compared to 18 hours and compared to Control for both
1094 timepoints. Samples sizes are shown at the top of each panel.

1095

1096 **Figure 3.**



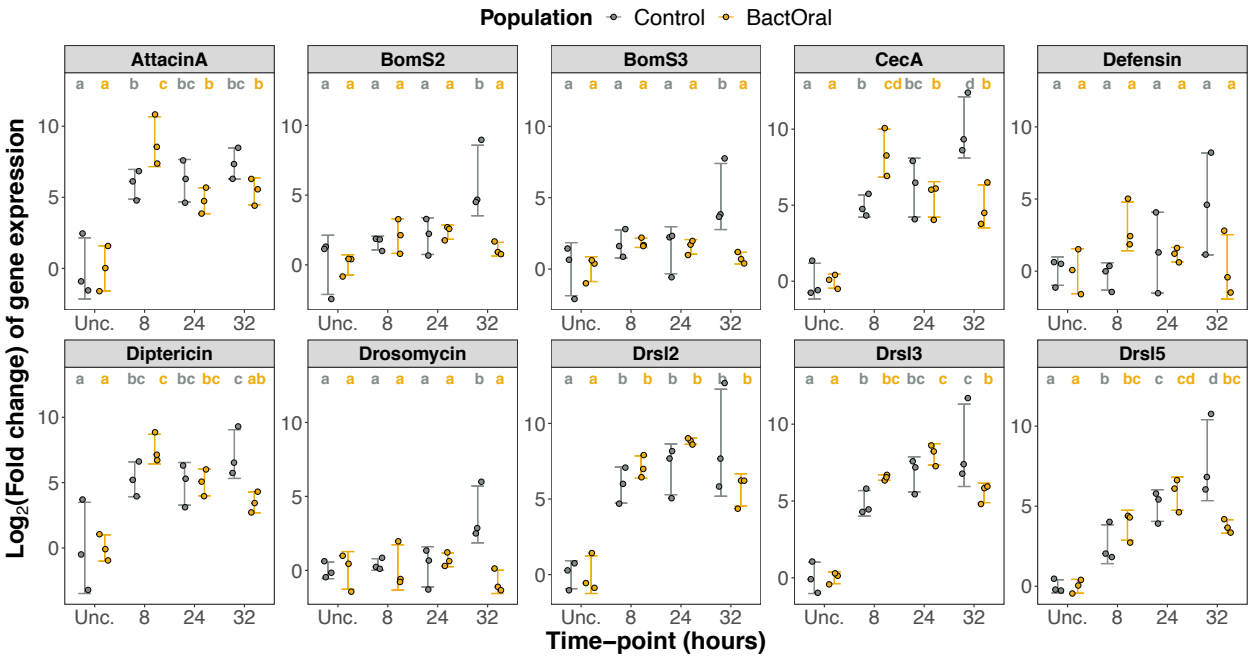
1097
1098

1099 **Figure 3. Bacterial load dynamics.**

1100 Individual flies from Control (grey) and BactOral (yellow) were collected upon exposure to *P.*
1101 *entomophila* at different timepoints for assessment of the number of Colony Forming Units (CFUs)
1102 inside their bodies. After the end of the infection period (24 hours), flies were changed onto clean food
1103 and collection continued until 52 hours post-infection. **A)** Log_2 of the number of CFUs found for females
1104 (top) and males (bottom) of either Control (grey) or BactOral (yellow) populations, with the mean value
1105 at each time-point evidenced in red (triangles for Control and circles for BactOral). There are no
1106 detectable differences in bacterial loads between both populations until 20 hours of infection, when
1107 BactOral starts to evidence a bigger decrease in bacterial counts. **B)** Cumulative number of individual
1108 flies from which no CFUs were obtained increases as time progresses, with the BactOral reaching
1109 significantly higher final values.

1110 Numbers at the top of each panel correspond to the sample size at each timepoint, with BactOral
1111 population in yellow and Control population in grey.

1112 **Figure 4.**



1113

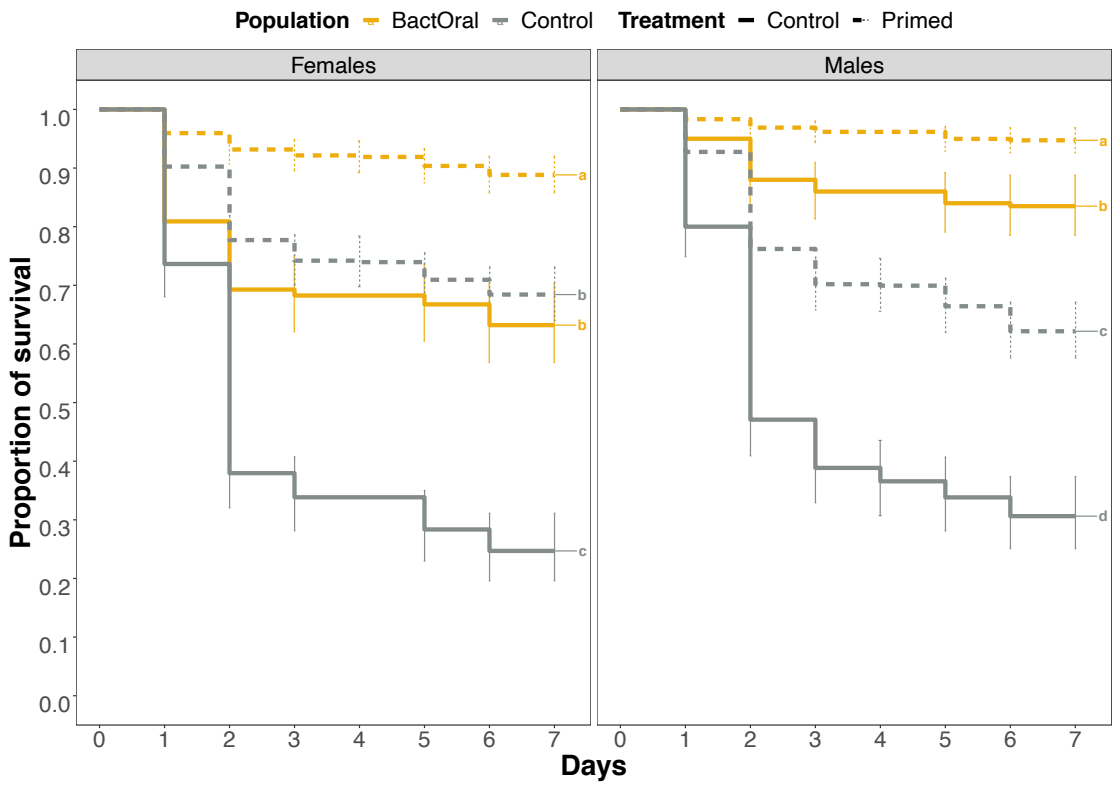
1114 **Figure 4. AMP expression differs between populations.**

1115 Guts from Control (grey) and BactOral (yellow) female flies (3 samples containing 10 guts, each) were
1116 dissected before exposure ("Unc.") and at different timepoints after oral infection (8, 24 and 32 hours)
1117 with *P. entomophila.* Relative gene expression, normalized to housekeeping gene *EIF2,* was performed
1118 for a representative panel of 10 AMPs. We detected a significantly higher up-regulation in BactOral
1119 compared to Control for *AttacinA* and *CecropinA* at 8-hours of infection. Results from these
1120 comparisons are shown in the letters above the plots.

1121

1122 **Figure 5.**

1123



1124

1125 **Figure 5. Control flies primed with heat-killed bacteria phenocopy BactOral survival to**

1126 **oral infection with *P. entomophila*.**

1127 Control (grey) and BactOral (yellow) females (left) and males (right) were fed a solution of heat-killed

1128 bacteria for 2 hours before oral infection (dashed lines) or directly infected with live *P. entomophila* (full

1129 lines) for 24 hours, after which they were changed onto clean food and their survival measured daily.

1130 Previous exposure to heat-killed bacteria before infection increased survival in both populations, leading

1131 additionally Control females to phenocopy BactOral survival levels upon infection. Results from multiple

1132 comparisons are shown as letters in the plots. Numbers at the end of each trajectory correspond to the

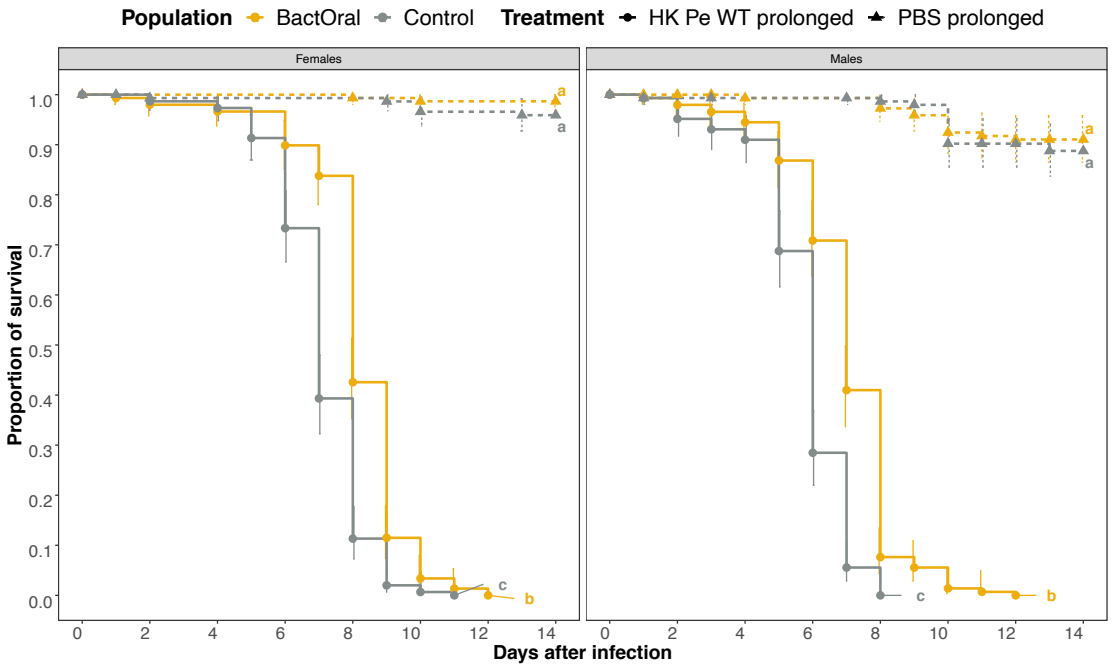
1133 sample size, with BactOral population in yellow and Control population in grey.

1134

1135

1136 **Figure 6.**

1137



1138

1139 **Figure 6. BactOral displays higher survival than Control upon immune system**

1140 **activation without bacterial proliferation.**

1141 Daily survival was measured for Control (grey) and BactOral (yellow) populations fed a mixture of food

1142 with heat-killed bacteria or PBS continuously. BactOral females and males exhibit a slower mortality

1143 compared to Control flies until around day 8. This difference fades away for both sexes after that period.

1144 Survival comparisons between populations and treatments are shown as lower-case letters. Sample

1145 sizes were 150 individuals, for each sex, per population, per treatment.

1146

1147

Table 1.

parameter	mean	sd	hdi 3%	hdi 97%
Ref. *k*	3.143	0.033	3.080	3.204
Ref. *h*	-2.309	0.144	-2.577	-2.043
Ref. *a*	3.072	0.016	3.043	3.104
Ref. *b*	2.361	0.034	2.298	2.425
Sex *k*	-0.053	0.068	-0.172	0.068
Pop. *k*	-0.108	0.046	-0.193	-0.021
Sex *h*	-0.330	0.227	-0.769	0.089
Pop. *h*	0.042	0.182	-0.301	0.376
Sex *a*	-0.130	0.038	-0.195	-0.061
Pop. *a*	-0.002	0.024	-0.047	0.043
Sex *b*	-0.147	0.066	-0.276	-0.035
Pop. *b*	-0.285	0.053	-0.384	-0.187
ss	3.671	0.062	3.552	3.785

Table 1. Parameter estimates for the model sigmoidal function fitted to the time-course of bacterial counts.

A sigmoidal function of 4 parameters (*a*, *k*, *h*, *b*) was fitted to the bacterial dynamics time-course shown in Figure 3A, for quantification of differences between Control and BactOral CFU counts. Control females were used as reference on which to estimate the contribution of tested variables (variable "Sex": males and variable "Population": BactOral) on the parameters. Effects (Sex and Population) on parameters were considered significant when the 94% highest posterior density interval (hdi) did not include zero. The "ss" parameter represents the estimate of the standard deviation of the normal error structure.

1174 **Table 2.**

1175

1176

parameter	mean	sd	hdi 3%	hdi 97%
a_0 Control	-1.214	0.570	-2.262	-0.127
a_0 BactOral	-0.731	0.570	-1.816	0.324
a_0 Female	-0.984	0.569	-2.073	0.075
a_0 Male	-0.959	0.569	-2.064	0.079
a_1 Female	-0.781	0.071	-0.914	-0.645
a_1 Male	-0.061	0.069	-0.188	0.074
a_1 Control	-0.700	0.070	-0.833	-0.570
a_1 BactOral	-0.144	0.069	-0.268	-0.010
k_0 Female	0.086	0.097	-0.096	0.269
k_0 Male	0.085	0.095	-0.088	0.268
k_0 Control	0.066	0.097	-0.114	0.250
k_0 BactOral	0.104	0.095	-0.076	0.281
k_1 Female	-0.161	0.103	-0.354	0.030
k_1 Male	-0.232	0.103	-0.426	-0.037
k_1 Control	-0.161	0.101	-0.355	0.027
k_1 BactOral	-0.231	0.105	-0.426	-0.033
t_0 Female	0.152	0.119	-0.071	0.372
t_0 Male	0.253	0.116	0.031	0.462
t_0 Control	0.168	0.119	-0.047	0.397
t_0 BactOral	0.238	0.116	0.020	0.453

1177

1178

1179 **Table 2. Parameter estimates for the model logistic function fitted to the zero counts**

1180 **of the time-course of bacterial counts.**

1181 An adapted logistic function of 4 parameters (a_0, a_1, k_0, k_1) was fitted to the progression of zero counts

1182 shown in Fig. 3B), for quantification of bacterial clearance differences between Control and BactOral.

1183 Single estimates were generated for the contribution of each variable (Sex: females and males;

1184 Population: Control and BactOral) to the model parameters. Effects were considered significant when

1185 was considered when the mean estimate ± standard deviation did not overlap for the same parameter

1186 between each variable, which is the case for a_1 when considering Population and Sex. High-density

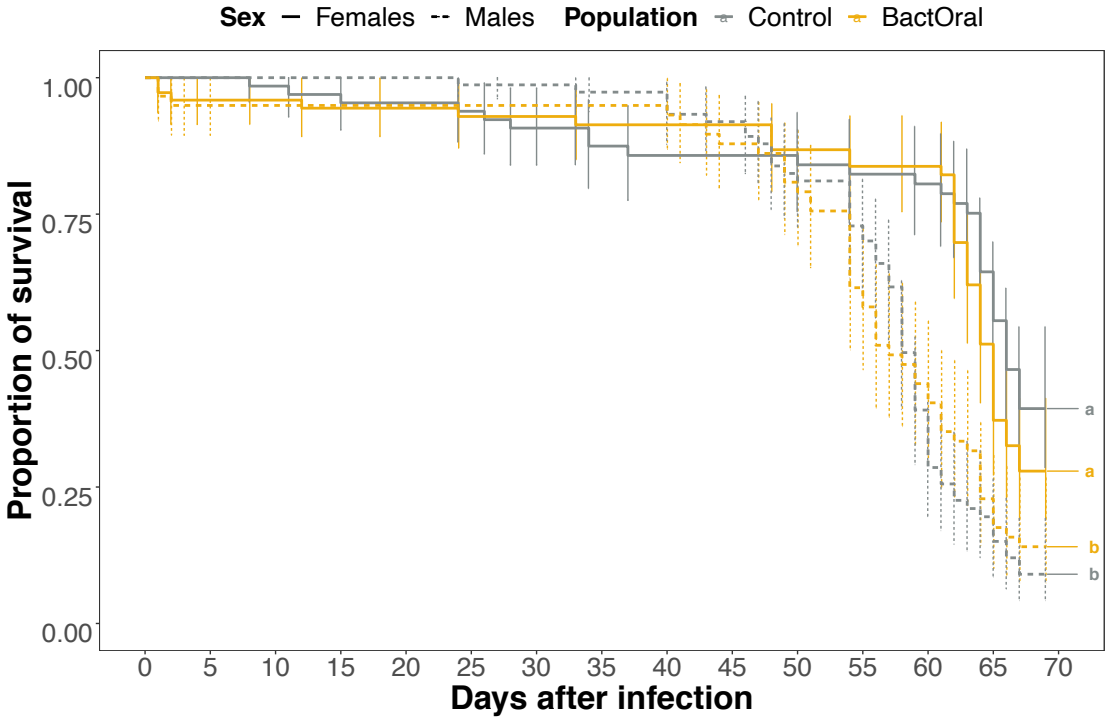
1187 intervals of 3% and 97% show that all our estimated parameters have a high-density probability (i.e.

1188 credibility) as they are all included in them.

1189

SUPPLEMENTARY FIGURE AND TABLE LEGENDS

Supplementary Figure 1.

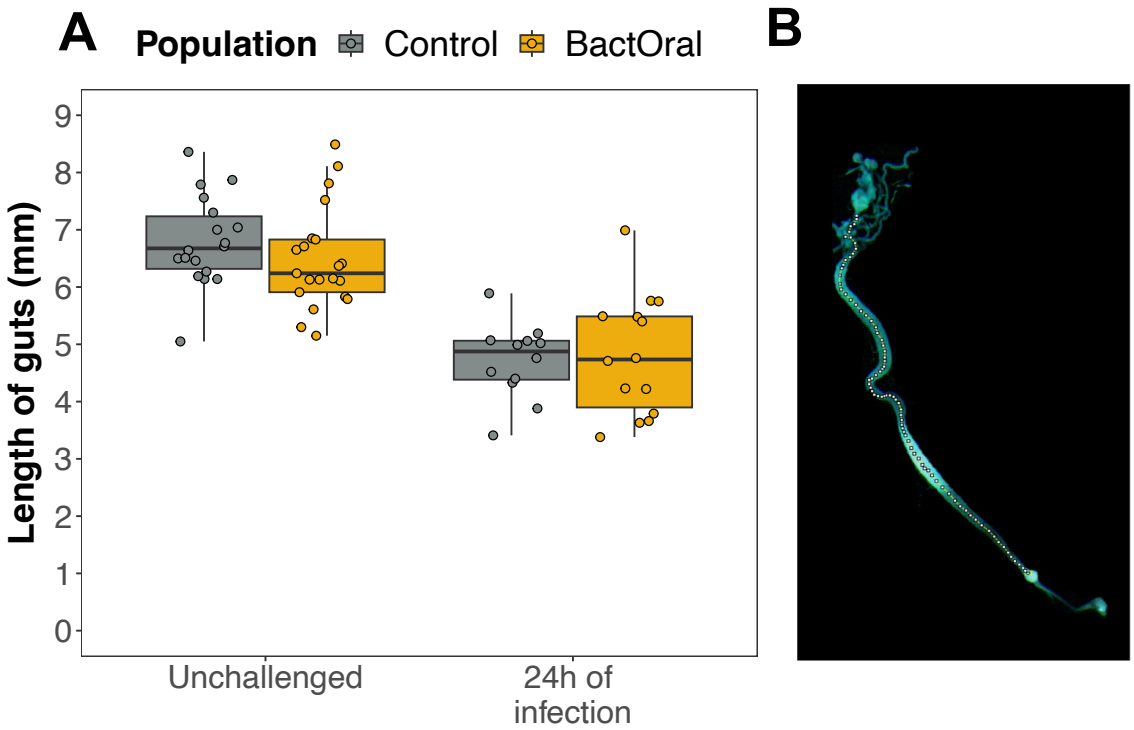


Supplementary Figure 1. Longevity of BactOral and Control populations.

Females (full lines) and males (dashed lines) of Control (grey) and BactOral (yellow) populations, are shown. No differences can be found between populations within sex (Uninfected Control females versus Uninfected BactOral females: z.ratio= 1.214, p= 0.6180; Uninfected Control males versus Uninfected BactOral males: z.ratio= 0.615 , p= 0.9272).

Results from multiple comparisons are shown as lower-case letters in the plots.

1200 **Supplementary Figure 2.**



1201

1202 **Supplementary Figure 2. No detectable differences in gut structure between Control**

1203 **and BactOral after oral infection with *P. entomophila*.**

1204 Females of Control (grey) and BactOral (yellow) were fed normal food ("Unchallenged") or orally infected with *P.*

1205 *entomophila* for 24 hours, after which their **A)** guts were dissected and photographed **B)**. Gut length (in mm) was

1206 measured by drawing consecutive longitudinal transects along the guts thrice and averaging those

1207 measurements to reach final length per sample. **A)** Exposure to bacteria shortens the guts significantly in both

1208 populations (Control before infection *versus* Control 24 hours post-infection: t= 6.274, p<0.0001; BactOral before

1209 infection *versus* BactOral 24 hours post-infection: t= 5.453, p<0.0001). No differences were detected in gut

1210 lengths between populations within each timepoint (Without infection: BactOral *versus* Control – p= 0.6719; after

1211 24 hours of infection – p= 0.9925), although a significant effect of treatment was observed in both Control

1212 (Without infection *versus* after 24 hours of infection – p< 0.0001) and BactOral (p< 0.0001), showing that gut

1213 shortening due to infection affected both regimes in a similar manner. **B)** Dissected guts of females from Control

1214 and BactOral populations were photographed and scaled for posterior length quantification. Manual transects

1215 were drawn longitudinally along the guts (as shown above) and total size of the cumulative transects was

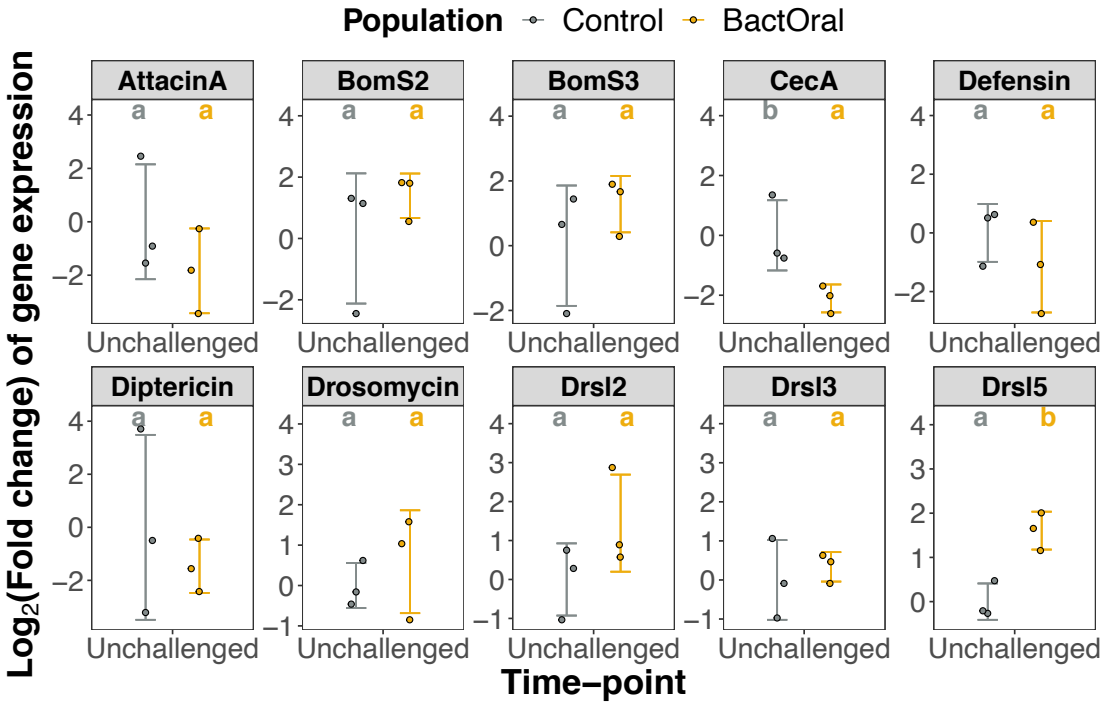
1216 calculated in FIJI. Three independent measurements were done on each gut and averaged to obtain a final

1217 value per sample (Supp. Fig. 2 A)).

1218 Samples sizes are shown at the top of the panel.

1219 **Supplementary Figure 3.**

1220



1221

1222 **Supplementary Figure 3. AMP expression differences between populations under**

1223 **homeostatic conditions.**

1224 Guts from Control (grey) and BactOral (yellow) female flies (3 samples containing 10 guts, each) were dissected

1225 at different timepoints after oral infection with *P. entomophila* and relative quantification of gene expression was

1226 performed. Expression levels of each gene in BactOral samples were normalized to expression levels of Control

1227 samples. Log_2(FoldChange) of these differences were fitted on a linear model with Population as factor and

1228 followed by multiple comparisons (*emmeans*) to uncover pairwise differences. Results from these comparisons

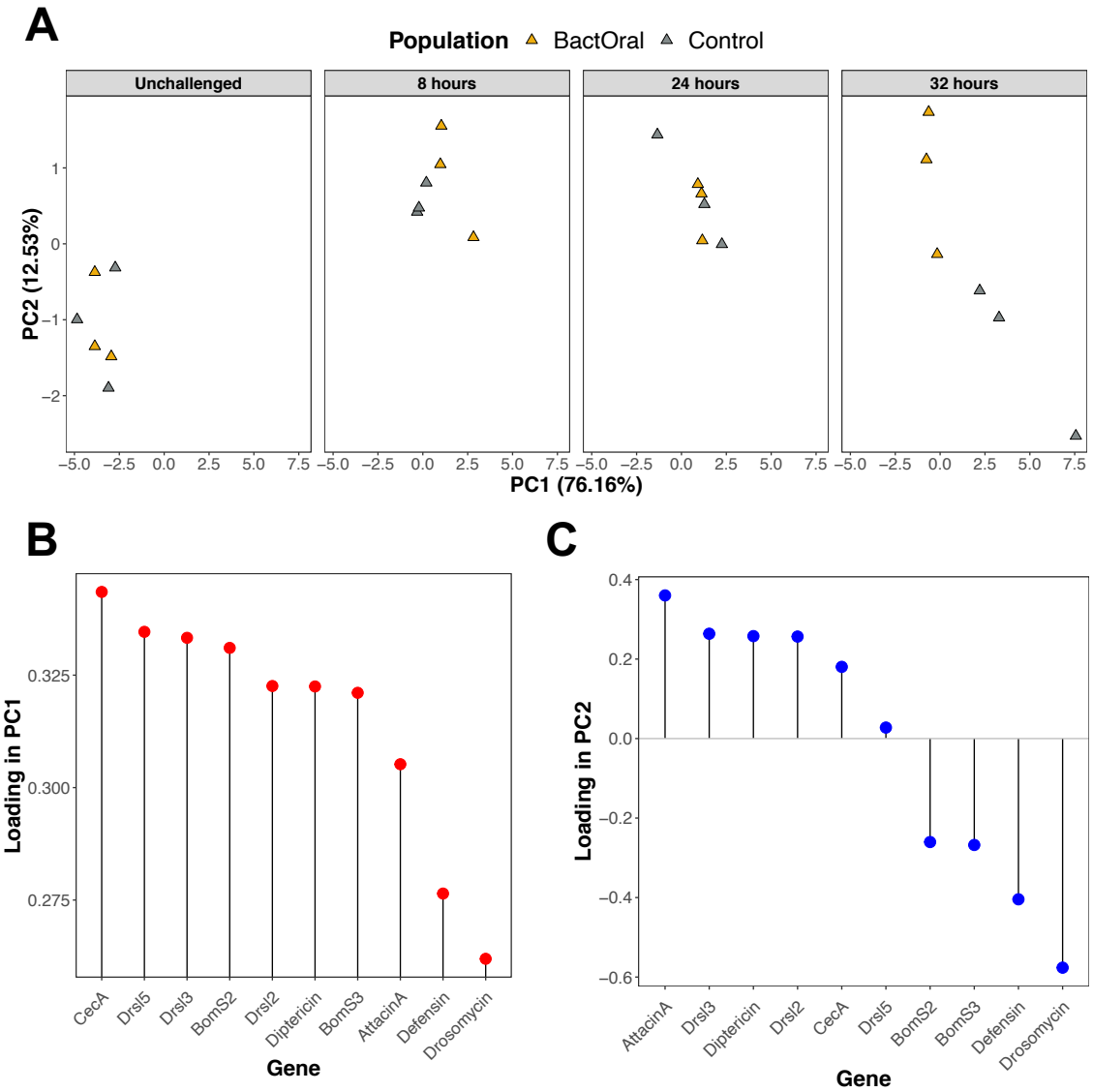
1229 are shown in the letters above the plots. There are significantly lower levels of CecropinA (CecA) (t= 2.892, p=

1230 0.0445) and significantly higher levels of *Drosomycin-like 5* (Drsl5) (t= -4.686 , p= 0.0094) being expressed in

1231 guts of BactOral flies under unchallenged conditions.

1232

1233 **Supplementary Figure 4.**



1234

1235 **Supplementary Figure 4. Principal Component Analysis on relative gene expression**

1236 **levels in evolved populations.**

1237 **(A)** Relative AMP expression levels of Control and BactOral flies upon oral infection with *P. entomophila* showed

1238 significant differences at various timepoints (see Fig. 4 and Supp. Fig. 2). **(B-C)** Relative influence of each AMP to

1239 the establishment of PC1 and PC2 (see Supp. Fig. 3 A). Importantly, Cecropin A1 represents the largest contributor

1240 to PC1 (**B**) whereas Drosomycin represents the largest contributor, although negatively correlated, to the variation

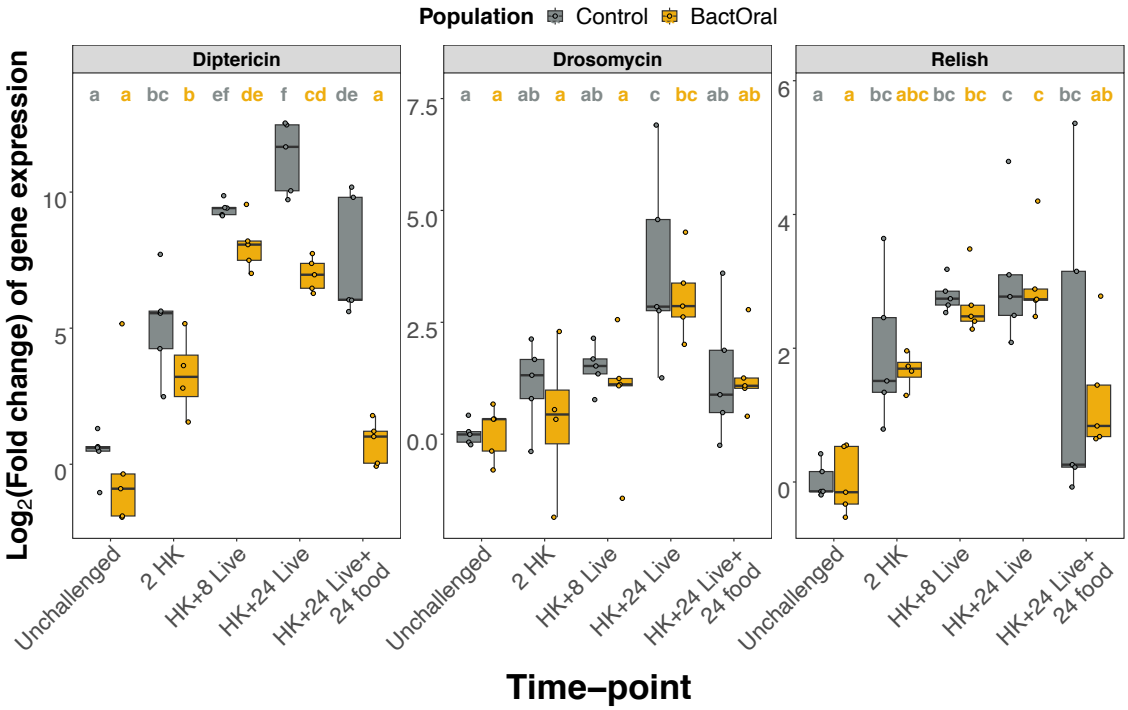
1241 in PC2 (**C**).

1242

1243

1244 **Supplementary Figure 5.**

1245



1246

1247

1248 **Supplementary Figure 5. Quantification of immunity-related gene expression upon**

1249 **artificial immune stimulation and infection**.

1250 RNA from whole-females from Control (grey) and BactOral (yellow) (4 samples containing 10 flies, each) was

1251 extracted at different timepoints. Relative gene expression, normalized to housekeeping gene *rpl32,* was performed

1252 for *Diptericin*, *Drosomycin* and *Relish*. Results from these comparisons are shown in the letters above the plots.

1253 To test for individual pairwise differences, relative Log2 (FoldChange) expression levels of each gene were fitted

1254 onto a linear model with Population and Timepoints as factors and followed by multiple comparisons (*emmeans*).

1255 No differences are observed between non-challenged individuals or between individuals after 2 hours of exposure.

1256 However, for Diptericin Unchallenged versus 2 HK show differences in both populations (Control: t= -4.778 , p=

1257 0.0001; BactOral: t= -3.122, p= 0.0052).

SUPPLEMENTARY TABLES

Supplementary Table 1:

A)
Model: coxme(Surv(time, status) ~ Population * Treatment * Sex + (1|Replicate))

fixed coefficients	**coef**	**exp(coef)**	**se(coef)**	***z***	***p***
Population-B	-0.005	0.995	1.418	0.00	1.0e+00
Treatment-Pe	5.022	151.786	1.008	4.98	**6.2e-07**
Sex-m	0.712	2.038	1.229	0.58	5.6e-01
Population-B:Treatment-Pe	-1.250	0.287	1.425	-0.88	3.8e-01
Population-B: Sex-m	0.916	2.499	1.650	0.56	5.8e-01
Treatment-Pe:Sex-m	-1.542	0.214	1.236	-1.25	2.1e-01
Population-B:Treatment-Pe:Sex-m	-1.452	0.234	1.665	-0.87	3.8e-01
Random effects					
Group	**Variable**	**Std Dev**	**Variance**		
Replicate	Intercept	0.106	0.011		

B)
Analysis of Deviance Table (Type III tests)

	Df	**Chisq**	**Pr(>Chisq)**
Population	1	0.0000	0.997
Treatment	1	24.845	**6.214e-07 *****
Sex	1	0.308	0.563
Population:Treatment	1	0.770	0.380
Population:Sex	1	0.302	0.579
Treatment:Sex	1	1.558	0.212
Population:Treatment:Sex	1	0.760	0.383

Supplementary Table 1. Statistical analysis of survival after oral infection with *P. entomophila* (immunocompetence, shown in Fig. 1).

A) Model estimates and **B)** ANOVA estimates for survival analysis testing immunocompetence of Control and BactOral populations.

Supplementary Table 2:

A)
Model: coxph(Surv(time, status) ~ Population * Sex)

	coef	**exp(coef)**	**se(coef)**	***z***	**Pr(>|z|)**
Population-B	0.271	1.312	0.224	1.214	0.225
Sex-m	1.069	2.912	0.215	4.970	**6.7e-07 *****
Population-B: Sex-m	-0.389	0.677	0.295	-1.321	0.186

B)
Analysis of Deviance Table (Type III tests)

	Chisq	**Df**	**Pr(>Chisq)**
Population	1.489	1	0.222
Sex	26.067	1	**3.298e-07 *****
Population:Sex	1.759	1	0.185

Supplementary Table 2. Statistical analysis of survival under uninfected conditions (Longevity, shown in Supp. Fig. 1).

A) Model estimates and **B)** ANOVA estimates for survival analysis testing longevity of Control and BactOral populations.

Supplementary Table 3:

A)
Model: glmer(µL.eaten ~ Population*factor(Timepoint) + (1|Replicate fly), family = "Gamma")

Fixed effects:	**Estimate**	**Std. Error**	**t-value**	**Pr(>|z)**
(Intercept)	1.56	0.104	14.940	**< 2e-16 *****
Population-BactOral	-0.015	0.140	-0.104	0.918
Timepoint-8	-0.836	0.109	-7.657	**1.91e-14*****
Timepoint-24	-1.156	0.102	-11.290	**< 2e-16 *****
Population-B:Timepoint-8	-0.028	0.153	-0.181	0.856
Population-B:Timepoint-24	-0.005	0.144	-0.035	0.972
Random effects:				
Groups	**Name**	**Variance**	**Std. Dev.**	
Replicate fly	(Intercept)	0..004	0.065	
Residual	--	0.059	0.243	

B)
Analysis of Deviance Table (Type III tests)

	Chisq	**Df**	**Pr(>Chisq)**
(Intercept)	223.2	1	**<2e-16 *****
Population	0.0107	1	0.918
Timepoint	153.151	2	**<2e-16 *****
Population:Timepoint	0.110	2	0.947

Supplementary Table 3. Analysis of the cumulative amounts of bacteria ingested by evolved populations (CAFE assay, shown in Fig. 2 A).

A) Model estimates and **B)** ANOVA estimates for the generalized linear model testing the effects of population and timepoint on ingested bacterial mixture.

Supplementary Table 4:

Model: zeroinfl(formula = avg_geom_cfus ~ Population * Sex * factor(Timepoint) | Population * factor(Timepoint), dist = "negbin")

Count model coefficients (negbin with log link)				
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	13.721	0.482	28.447	**<2e-16 *****
Population-C	0.816	0.616	1.324	0.185
Sex-m	0.047	0.647	0.073	0.942
Timepoint-18	-0.251	0.699	-0.359	0.720
Population-C:Sex-m	-0.910	0.840	-1.083	0.279
Population-C:Timepoint-18	-0.579	0.905	-0.640	0.522
Sex-m:Timepoint-18	-0.950	0.911	-1.043	0.297
Population-C:Sex-m:Timepoint-18	2.171	1.204	1.803	0.071
Log(theta)	-1.031	0.106	-9.700	**<2e-16 *****
Zero-inflation model coefficients (binomial with logit link):				
	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	-0.828	0.351	-2.357	**0.018 ***
Population-C	-3.004	1.248	-2.407	**0.016***
Timepoint-18	-18.771	3347.432	-0.006	0.996
Population-C:Timepoint-18	18.876	3347.433	0.006	0.996

Supplementary Table 4. Analysis of quantities of defecated bacteria by evolved populations (CFUs, shown in Fig. 2 B)).

A) Model estimates and for the zero-inflated model testing the effects of population, sex and timepoint on amount of defecated live bacteria and instances of non- measurable bacteria (zeros).

Supplementary Table 5:

A)
Model: lmer(Lenght ~ Population*Treatment + (1|Round), REML=TRUE)

Fixed effects	**Estimate**	**Std. Error**	**df**	**t-value**	**Pr(>|t|)**
(Intercept)	6.794	0.202	61	33.565	**< 2e-16 *****
Population-B	-0.314	0.276	61	-1.136	0.260
Treatment-Pe	-2.084	0.320	61	-6.513	**1.58e-08 *****
Population-B:Treatment-Pe	0.407	0.436	61	0.933	0.354
Random effects					
Groups	**Name**	**Variance**	**Std Dev**		
Round	(Intercept)	0	0		
Residual	---	0.7376	0.8588		

B)
Analysis of Deviance Table (Type III Wald chisquare tests)

	Chisq	Df	Pr(>Chisq)
(Intercept)	1126.6148	1	**< 2.2e-16 *****
Population	1.2914	1	0.256
Treatment	42.4139	1	**7.386e-11 *****
Population:Treatment	0.8710	1	0.351

Supplementary Table 5. Analysis of total gut lengths of females of evolved populations before and after 24 hours of infection (shown in Supp. fig. 1 A)).

A) Model estimates and **B|)** ANOVA estimates for the linear model testing the effects of population and treatment on total length of guts.

Supplementary Table 6:

A)
Model: lm(LogFold_gene ~ Population * factor(Timepoint))
AttacinA

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-3.333e-10	8.287e-01	0.00	1.00
Population-B	3.333e-10	1.172e+00	0.00	1.00
Timepoint-8	5.914e+00	1.172e+00	5.047	0.000119 ***
Timepoint-24	6.171e+00	1.172e+00	5.266	7.69e-05 ***
Timepoint-32	7.375e+00	1.172e+00	6.293	1.07e-05 ***
Population-B: Timepoint-8	3.004e+00	1.657e+00	1.813	0.088712 .
Population-B: Timepoint-24	-1.412e+00	1.657e+00	-0.852	0.406965
Population-B: Timepoint-32	-1.950e+00	1.657e+00	-1.177	0.256541

B)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.00	1.0
Population	0.0	1	0.00	1.0
Timepoint	98.317	3	15.908	**4.647e-05** ***
Population:Timepoint	22.181	3	3.589	**0.03718** *
Residuals	32.962	16		

C)
BomaninS2

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-5.881e-16	8.017e-01	0.0	1.0
Population-B	1.358e-15	1.134e+00	0.0	1.0
Timepoint-8	1.563e+00	1.134e+00	1.378	0.187
Timepoint-24	2.062e+00	1.134e+00	1.818	0.088.
Timepoint-32	**6.049e+00**	1.134e+00	5.335	**6.7e-05** ***
Population-B: Timepoint-8	5.023e-01	1.603e+00	0.313	0.758
Population-B: Timepoint-24	2.832e-01	1.603e+00	0.177	0.862
Population-B: Timepoint-32	-4.928e+00	1.603e+00	-3.074	**0.007** **

D)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	0.0	1	0.0	1.0
Timepoint	59.677	3	10.3161	**0.0005071** ***
Population:Timepoint	30.495	3	5.2715	**0.0101493** *
Residuals	30.852	16		

E)
<u>BomaninS3</u>

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-8.158e-16	7.573e-01	0.0	1.0
Population-B	1.282e+00	1.071e+00	1.197	0.249
Timepoint-8	-1.355e-17	1.071e+00	0.0	1.0
Timepoint-24	1.124e-15	1.071e+00	0.0	1.0
Timepoint-32	1.014e-15	1.071e+00	0.0	1.0
Population-B: Timepoint-8	7.635e-02	1.515e+00	0.050	0.960
Population-B: Timepoint-24	2.302e-01	1.515e+00	0.152	0.881
Population-B: Timepoint-32	-4.322e+00	1.515e+00	-2.853	**0.012***

F)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	2.467	1	1.434	0.249
Timepoint	0.0	3	0.0	1.0
Population:Timepoint	22.057	3	4.273	**0.021 ***
Residuals	27.529	16		

G)
<u>CecropinA1</u>

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-1.813e-16	8.111e-01	0.0	1.0
Population-B	-2.107e+00	1.147e+00	-1.837	0.085.
Timepoint-8	5.263e-16	1.147e+00	0.0	1.0
Timepoint-24	-3.400e-16	1.147e+00	0.0	1.0
Timepoint-32	-5.934e-16	1.147e+00	0.0	1.0
Population-B: Timepoint-8	3.480e+00	1.622e+00	2.146	**0.048 ***
Population-B: Timepoint-24	-7.721e-01	1.622e+00	-0.476	0.641
Population-B: Timepoint-32	-5.195e+00	1.622e+00	3.202	**0.006****

H)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	6.660	1	3.375	0.085.
Timepoint	0.0	3	0.0	1.0
Population:Timepoint	57.223	3	9.665	**0.0007*****
Residuals	31.576	16		

I)
<u>Defensin</u>

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-4.532e-16	1.167e+00	0.0	1.0
Population-B	-1.155e+00	1.651e+00	-0.700	0.4942
Timepoint-8	-3.333e-10	1.651e+00	0.0	1.0
Timepoint-24	-3.333e-10	1.651e+00	0.0	1.0
Timepoint-32	6.636e-16	1.651e+00	0.0	1.0
Population-B: Timepoint-8	3.468e+00	2.335e+00	1.485	0.1569
Population-B: Timepoint-24	-1.377e-01	2.335e+00	-0.059	0.9537
Population-B: Timepoint-32	-4.353e+00	2.335e+00	-1.865	0.0807 .

J)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	2.001	1	0.490	0.494
Timepoint	0.0	3	0.0	1.0
Population:Timepoint	46.098	3	3.759	**0.032 ***
Residuals	65.404	16		

K)
<u>Diptericin</u>

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	2.617e-15	1.001e+00	0.0	1.0
Population-B	3.333e-10	1.416e+00	0.0	1.0
Timepoint-8	5.259e+00	1.416e+00	3.713	**0.002 ****
Timepoint-24	4.907e+00	1.416e+00	3.465	**0.003 ****
Timepoint-32	7.187e+00	1.416e+00	5.075	**0.000113 *****
Population-B: Timepoint-8	2.307e+00	2.003e+00	1.152	0.266
Population-B: Timepoint-24	1.076e-01	2.003e+00	0.054	0.958
Population-B: Timepoint-32	-3.701e+00	2.003e+00	-1.848	0.083 .

L)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	0.0	1	0.0	1.0
Timepoint	84.314	3	9.3416	**0.0008357 *****
Population:Timepoint	27.922	3	3.0937	0.057 .
Residuals	48.137	16		

M)
Drosomycin

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	-3.333e-10	6.719e-01	0.0	1.0
Population-B	3.333e-10	9.503e-01	0.0	1.0
Timepoint-8	3.984e-01	9.503e-01	0.419	0.681
Timepoint-24	2.392e-01	9.503e-01	0.252	0.804
Timepoint-32	3.783e+00	9.503e-01	3.981	**0.001 ****
Population-B: Timepoint-8	-1.971e-01	1.344e+00	-0.147	0.885
Population-B: Timepoint-24	4.762e-01	1.344e+00	0.354	0.728
Population-B: Timepoint-32	-4.556e+00	1.344e+00	-3.390	**0.004****

N)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	0.0	1	0.0	1.0
Timepoint	28.931	3	7.1198	**0.003 ****
Population:Timepoint	24.678	3	6.0731	**0.006 ****
Residuals	21.672	16		

O)
Drosomycin-like2

Coefficients	**Estimate**	**Std. Error**	**t-value**	**Pr(>|t|)**
(Intercept)	1.807e-15	9.335e-01	0.0	1.0
Population-B	3.333e-10	1.320e+00	0.0	1.0
Timepoint-8	5.925e+00	1.320e+00	4.488	**0.0004 *****
Timepoint-24	6.968e+00	1.320e+00	5.278	**7.50e-05 *****
Timepoint-32	8.724e+00	1.320e+00	6.609	**6.01e-06 *****
Population-B: Timepoint-8	1.187e+00	1.867e+00	0.636	0.534
Population-B: Timepoint-24	1.857e+00	1.867e+00	0.995	0.335
Population-B: Timepoint-32	-3.125e+00	1.867e+00	-1.674	0.114

P)
Anova Table (Type III tests)

	Sum Sq	**Df**	**F-value**	**Pr(>F)**
(Intercept)	0.0	1	0.0	1.0
Population	0.0	1	0.0	1.0
Timepoint	128.832	3	16.428	**3.843e-05 *****
Population:Timepoint	21.934	3	2.797	0.074 .
Residuals	41.826	16		

Q)
Drosomycin-like3

Coefficients	Estimate	Std. Error	t-value	Pr(>|t|)
(Intercept)	5.143e-16	6.851e-01	0.0	1.0
Population-B	1.147e-15	9.688e-01	0.0	1.0
Timepoint-8	4.849e+00	9.688e-01	5.005	**0.0001*** **
Timepoint-24	6.738e+00	9.688e-01	6.955	**3.24e-06 *** **
Timepoint-32	8.622e+00	9.688e-01	8.900	**1.36e-07 *** **
Population-B: Timepoint-8	1.678e+00	1.370e+00	1.225	0.238
Population-B: Timepoint-24	1.297e+00	1.370e+00	0.947	0.358
Population-B: Timepoint-32	-3.095e+00	1.370e+00	-2.259	**0.038 * **

R)
Anova Table (Type III tests)

	Sum Sq	Df	F-value	Pr(>F)
(Intercept)	0.0	1	0.0	1.0
Population	0.0	1	0.0	1.0
Timepoint	123.465	3	29.230	**9.967e-07 *** **
Population:Timepoint	21.108	3	4.9972	**0.01238 * **
Residuals	22.528	16		

S)
Drosomycin-like5

Coefficients	Estimate	Std. Error	t-value	Pr(>|t|)
(Intercept)	5.815e-16	6.871e-01	0.0	1.0
Population-B	3.333e-10	9.717e-01	0.0	1.0
Timepoint-8	2.630e+00	9.717e-01	2.706	**0.016 * **
Timepoint-24	5.041e+00	9.717e-01	5.188	**8.98e-05 *** **
Timepoint-32	7.878e+00	9.717e-01	8.108	**4.66e-07 *** **
Population-B: Timepoint-8	1.179e+00	1.374e+00	0.858	0.404
Population-B: Timepoint-24	7.405e-01	1.374e+00	0.539	0.597
Population-B: Timepoint-32	-4.145e+00	1.374e+00	-3.016	**0.008 ** **

T)
Anova Table (Type III tests)

	Sum Sq	Df	F-value	Pr(>F)
(Intercept)	0.0	1	0.0	1.0
Population	0.0	1	0.0	1.0
Timepoint	101.853	3	23.972	**3.678e-06 *** **
Population:Timepoint	26.821	3	6.313	**0.00497 ** **
Residuals	22.660	16		

Supplementary Table 6. Analysis of relative gene expression levels in guts of females of evolved populations at several timepoints throughout infection (shown in Fig. 4).

Model estimates and ANOVA estimates for the linear models testing the effects of population and timepoint on expression levels of multiple AMPs during the course of oral bacterial infection.

Supplementary Table 7

Primer name	5'-Sequence-3'
rpl32 F01	CCAGTCGGATCGATATGCTAA
rpl32 R01	GTTCGATCCGTSACCGATGT
eIF(-2alpha) F01	TCGCATCAACCTGATAGCAC
eIF(-2alpha) R01	ATCGTACTCGCTGGTCTTGG
relish F01	ACAAAGGACGAGGATTCCGTGG
relish R01	TGATTCAGCAGCGAACAGAGCC
drosomycin F01	CTCTTCGCTGTCCTGATGCTGG
drosomycin R01	GACAGGTCTCGTTGTCCCAGAC
diptericin F01	TACTTTGGCTTATCCGATGCCC
diptericin R01	GTGCTGTCCATATCCTCCATTC
cecropin a1 F01	GTTTTCGTCGCTCTCATTCTGG
cecropin a1 R01	CTTGTTGAGCGATTCCCAGTC
defensin F01	TCGCTTTTGCTCTGCTTGCTTG
defensin R01	GTTGCAGTAGCCGCCTTTGAAC
drsl2 F01	CCTGGCTGTGATGACAATTGTTG
drsl2 R01	ACTGATATGTCCCTCCTCCTTGC
drsl3 F01	TCCTAATGGAGGCCAACACTG
drsl3 R01	TCCCTCCTCAATGCAGAGACG
drsl5 F01	TCCTGGCTGTGATGACCATCTTC
drsl5 R01	ACTGGATCGTCCTTCCTCCTTG
boms2 F01	AGTCGTCACCGTCTTTGTGTT
boms2 R01	GCAGTATTTGCAGTCCCCGTTG
boms3 F01	CTATCACTCGCCTTCGTTTTGG
boms3 R01	TCACATTGCAGACGCGGCAATC
attacin a1 F01	CATTACCGAGGCACTTCCCAC
attacin a1 R01	GAAAACCTGACCCACCACATTG

Supplementary Table 7. Primer sequences used in Real-Time quantitative PCRs (shown in Fig. 4).

The first four genes listed (shown in bold and italics) were used as reference (house-keeping) for relative quantifications.

Supplementary Table 8:

A)
Model: coxme(Surv(time,status)~Population*Treatment*Sex+(1|Block/Replicate))

Fixed coefficients	**coef**	**exp(coef)**	**se(coef)**	***z***	***p***
Population-B	-1.065	0.345	0.156	-6.82	**8.8e-12**
Treatment-HK	-1.324	0.266	0.132	-10.01	**0.0e+00**
Sex-m	-0.195	0.823	0.130	-1.50	1.3e-01
Population-B:Treatment-HK	-0.115	0.891	0.239	-0.48	6.3e-01
Population-B: Sex-m	-0.792	0.453	0.256	-3.09	**2.0e-03**
Treatment-HK: Sex-m	0.382	1.465	0.184	2.08	**3.8e-02**
Population-B:Treatment-HK:Sex-m	-0.172	0.842	0.391	-0.44	6.6e-01
Random effects					
Group	**Variable**	**Std Dev**	**Variance**		
Block/Replicate	(Intercept)	0.152	0.023		
Block	(Intercept)	0.173	0.030		

B)
Analysis of Deviance Table (Type III tests)

	Df	**Chisq**	**Pr(>Chisq)**
Population	1	46.577	**8.808e-12 *****
Treatment	1	100.214	**< 2.2e-16 *****
Sex	1	2.238	0.135
Population:Treatment	1	0.233	0.639
Population:Sex	1	9.553	**0.002 ****
Treatment:Sex	1	4.308	**0.038 ***
Population:Treatment:Sex	1	0.194	0.659

Supplementary Table 8. Statistical analysis of survival of evolved populations after priming with heat-killed bacteria (shown in Fig. 5).

A) Model estimates and **B)** ANOVA estimates for survival analysis testing the effect of priming on infection in Control and BactOral.

Supplementary Table 9:

A)

Model: coxme(Surv(time,status)~Population*Treatment*Sex+(1|Replicate))

Fixed coefficients	**coef**	**exp(coef)**	**se(coef)**	***z***	***p***
Population-B	-1.137	0.321	0.817	-1.39	0.160
Treatment-Pe WT	-5.354	211.507	0.430	12.45	**0.000**
Sex-m	1.032	2.807	0.479	2.16	**0.031**
Population-B: Treatment-Pe WT	0.359	1.432	0.824	0.44	0.660
Population-B: Sex-m	0.917	2.502	0.898	1.02	0.310
Treatment-Pe WT: Sex-m	0.141	1.151	0.494	0.28	0.780
Population-B: Treatment-Pe WT: Sex-m	-1.348	0.260	0.913	-1.48	0.140
Random effects					
Group	**Variable**	**Std Dev**	**Variance**		
Replicate	(Intercept)	4.149e-03	1.721e-05		

B)

Analysis of Deviance Table (Type III tests)

	Df	**Chisq**	**Pr(>Chisq)**
Population	1	1.937	0.164
Treatment	1	154.932	**< 2e-16 *****
Sex	1	4.648	**0.0312 ***
Population:Treatment	1	0.1897	0.663
Population:Sex	1	1.044	0.307
Treatment:Sex	1	0.081	0.776
Population:Treatment:Sex	1	2.178	0.140

Supplementary Table 9. Statistical analysis of survival of evolved populations after prolonged exposure to heat-killed bacteria (shown in Fig. 6).

A) Model estimates and **B)** ANOVA estimates for survival analysis testing the effects of population, treatment and sex on survival to prolonged exposure to heat-killed *P. entomophila*, for 14 days.