Title: Adaptation to oral infection in *D. melanogaster* through evolution of both resistance and disease tolerance mechanisms

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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 determine the role of behavioural traits, and of resistance and disease tolerance mechanisms on host adaption. We compare a population adapted to oral infection with *P. entomophila* (BactOral) with 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 differential gut cell renewal rate, or in the rate of defecation, pointing to a strengthening in resistance. Additionally, we posit that disease tolerance also contributes to the adaptive response of BactOral 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.

34 # Introduction

35 Host-parasite interactions are main drivers of evolution[1,2]. In natural environments,
36 parasites are constantly attempting to penetrate host defences, which consist on multiple
37 mechanisms to fight infection[3]. Such mechanisms include behavioural strategies (e.g.
38 avoidance)[4], the active reduction or elimination of pathogens (i.e. resistance) and/or the
39 maintenance of homeostasis and fitness without interfering with pathogen burden (i.e. disease
40 tolerance)[5–8]. While many studies in the past decades have explored the mechanistic and genetic
41 bases of these different host defence strategies in diverse organisms [9–11], few have focused on how
42 these processes can be shaped by evolution[12–15]. Experimental evolution is a powerful method by
43 which it is possible to infer the evolutionary response of a host species to a specific pathogenic
44 pressure under controlled conditions[16]. Previous studies of experimental evolution have addressed
45 the impact of different hosts on parasite fitness[17], of different pathogens on the host's evolutionary
46 trajectory[13,18,19], of immune priming[2021], and of different routes of infection on host adaptation[19,22].

47 It is believed that oral exposure to pathogens constitutes a common process of infection
48 in *D. melanogaster*[23,24], as it lives on decaying substrates. When feeding on pathogenic
49 microorganisms, *Drosophila* deploys different responses that include avoidance behaviours[25,26],
50 the barrier action of the peritrophic matrix[27], localized high acidity of the midgut[28], intensification
51 of gut transit[29], local production of Anti-Microbial Peptides (AMPs)[30] and Reactive Oxygen
52 Species (ROS) by gut epithelial cells[31,32], increase of enterocyte delamination and renewal rates[33–
53 36]. In addition, it can activate a systemic humoral response, when the pathogen manages to cross
54 the gut epithelium into the haemolymph[37,38] or otherwise provokes a systemic reaction[23,39–41]. In
55 the specific case of *D. melanogaster* oral infection with the natural entomopathogen
56 *Pseudomonas entomophila*, its high pathogenicity is linked to the production of toxins and
57 virulence factors[42,43] causing translational arrest in host tissues and the inability to renew damaged
58 gut epithelium[44,45]. These responses provide a clear picture of the multi-layered and
59 mechanistically complex nature of the immune response, understood in its broad sense, as
60 presented above[3].

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

67

68 # Results

69 ## Evolved population shows higher survival upon infection

70 In previous work[19,46], experimental evolution was conducted on an outbred population of
71 *D. melanogaster* against oral infection either with the bacterium *P. entomophila* (hereafter,
72 BactOral) or with a control solution (hereafter, Control). Since then, these populations[46] have been
73 kept under relaxed selection for over 80 generations. To understand to which extent this relaxation
74 affected the response to the original selection regime, we measured survival of Control and
75 BactOral populations upon *P. entomophila* oral infection. We found significantly higher survival
76 of BactOral for both sexes (Fig. 1, Infected Control females *versus* Infected BactOral females:
77 *z.ratio*= 9.123, $p< 0.0001$; Infected Control males *versus* Infected BactOral males: *z.ratio*= 9.909,
78 $p< 0.0001$)(Suppl. Table 1). Moreover, these differences are not due to an inherent frailty of the
79 Control as compared to BactOral, since survival under uninfected conditions (for the equivalent
80 time period) was similar in both populations (Fig. 1, Uninfected Control females *versus*
81 Uninfected BactOral females: *z.ratio*= 0.004, $p= 1$; Uninfected Control males *versus* Uninfected
82 BactOral males: *z.ratio*= -1.080, $p= 0.9611$)(Suppl. Table 1).

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

86

87 ## Evolved population has not changed bacterial uptake or defecation rates

88 Our systematic approach started by screening behavioural traits that impact gut bacterial
89 number: feeding and defecation. To test for differences in feeding behaviour, we performed a
90 Capillary Feeding assay (CAFE)[47] on females of both Control and BactOral populations at
91 different timepoints over 24 hours of exposure to the bacteria solution. We found no significant
92 differences between the amount ingested by individuals of the two populations. Pairwise
93 comparisons of their cumulative bacterial intake after 2 hours, 8 hours and 24 hours exhibited a
94 comparable amount of total bacteria ingested (Suppl. Table 2 and Fig. 2A, Control *versus*
95 BactOral at 2 hours, *t.ratio*= 0.097, $p= 1$; at 8 hours, *t.ratio*= 0.643, $p= 0.9873$; at 24 hours,
96 *t.ratio*= 0.574, $p= 0.9925$).

97 Additionally, we estimated the average slopes from linear regression where microliters
98 of bacteria eaten were modelled between different time points, concluding that the feeding rates
99 of both populations decreased in similar ways as infection progresses. Thus, exposure affects
100 feeding ability of both populations to a comparable degree and, consequently, adaptation to oral

101 infection with *P. entomophila* in the BactOral regime did not involve an alteration of feeding
102 behaviour.

103 Considering the absence of significant differences in bacterial intake, an alternative would
104 be that the BactOral population had evolved a more effective defecation rate, which would reduce
105 the time of bacterial permanence and proliferation in the gut. We established a protocol to measure
106 live bacteria loads in the faeces of individual flies upon oral infection through the quantification
107 of CFUs defecated over a six-hour period and the subsequent twelve hours post-exposure (Fig.
108 2B).

109 We observed a few instances where flies did not expel alive bacteria during that period
110 or did so in a quantity that fell below our detection threshold. Therefore, a zero-inflated model
111 with a negative binomial distribution was fitted on the counts data (accounting for overdispersion)
112 jointly with a binomial distribution on the zeros. For the counts portion of the model, none of the
113 three factors significantly predicted the measured bacterial loads (Suppl. Table 3, model
114 parameters - Population: *z.value*= 1.324, *p*= 0.185; Sex: *z.value*= 0.073, *p*= 0.942; Timepoint:
115 *z.value*= -0.359, *p*= 0.720). However, looking at the zero portion of the model, we find a
116 significant effect of Population (Population: *z.value*= -2.407, *p*= 0.016; Timepoint: *z.value*= -
117 0.006, *p*= 0.996).

118 We then performed post-hoc pairwise comparisons on these regressions and identified a
119 significant decrease in bacterial loads defecated by males of BactOral, between the two timepoints
120 (males: *z.ratio*= -2.719, *p*= 0.033), but no other significant effects among bacterial counts between
121 populations and/or timepoints, in a sex-dependent manner. However, when considering the zeros,
122 we found a significant difference in the proportion of BactOral flies with undetectable amounts
123 of bacteria in the faeces between the first and second timepoints (B6h– B18h: *z.ratio*= 4.092, *p*=
124 0.0002), as well as compared to Control at both timepoints (B6h– C6h: *z.ratio*= 3.612, *p*= 0.002;
125 B6h– C18h: *z.ratio*= 3.530, *p*= 0.002)(Suppl. Table 3).

126 In sum, we were not able to correlate the higher survival of the BactOral population upon
127 oral infection with a more efficient defecation. However, we observed that males of BactOral
128 decrease the amount of live bacteria they defecate as time progresses, hinting at a possible
129 resistance mechanism more evident in males of the adapted lines.

130

131 Evolved population shows a faster decrease and clearance of bacteria

132 Next, we studied the physiological responses to estimate the roles of resistance and
133 disease tolerance. First, we tested for structural changes in the gut itself that could influence the
134 "carrying capacity" of the structure and create difficulties in interpretating the quantification

135 experiments. We measured the capacity of BactOral to regenerate its gut epithelium upon
136 bacterial infection relative to Control, using initial length and extent of the shortening induced by
137 infection[36], as a proxy. For this, we measured the total length of the guts of females, before and
138 after 24 hours of oral infection with *P. entomophila* and performed multiple pairwise comparisons
139 on a linear model with mixed effects to pinpoint how population and infection status affected this
140 trait. We found a shortening of approximately 2 mm in the total length of infected guts by
141 comparing the post-infection timepoint of 24 hours to the initial timepoint, prior to exposure
142 (Suppl. Table 4 and Suppl. Fig. 1, Control before infection *versus* Control 24 hours post-infection:
143 *t.ratio*= 6.274, $p<0.0001$; BactOral before infection *versus* BactOral 24 hours post-infection:
144 *t.ratio*= 5.453, $p<0.0001$). Nonetheless, no significant differences between populations in the
145 total length of the guts could be detected both before and after infection (Without infection:
146 BactOral *versus* Control – p= 0.6719; after 24 hours of infection – p= 0.9925) (Suppl. Fig. 1).

147 To explore the dynamics of bacterial numbers inside the host, we performed a time-course
148 characterization of bacterial loads during and after oral infection by counting the number of
149 colony-forming units (CFUs) obtained from individual flies. In Figure 3, we show the progression
150 of bacterial loads from 1 hour until 52 hours post-infection (Fig. 3A) as well as the cumulative
151 number of individuals without detectable live bacteria (Fig. 3B). The infection dynamics of both
152 populations start with very high numbers of *P. entomophila* at early stages, confirming the
153 absence of differences in initial inoculum reported above (Table 1, parameter a: 3.072 $\pm$ 0.016).
154 Bacterial loads continuously decrease and, at approximately 20 hours of infection, Control and
155 BactOral population begin to differ significantly in a sex-dependent manner. From this point
156 onwards we observe a steady decrease of bacterial quantities in the two populations, always more
157 severe in males than in females, and in BactOral than in Control (Fig. 3A). In addition, the number
158 of flies that clear the infection also increases with time in both populations, but at a faster rate and
159 reaching higher levels in BactOral flies (Fig. 3B). By 52 hours post-infection, in both males and
160 females, the number of "cleared" flies in the BactOral populations was twice as high as that of its
161 Control counterpart (Fig. 3B). Together, these analyses indicate that the evolved population
162 eliminates bacteria faster and more efficiently than Control.

163 In order to pinpoint more accurately the parameters contributing to this overall difference,
164 we modelled the bacterial load and zero dynamics, and assessed differences in parameter
165 estimates considering the initial load or inoculum (a), the load in the long-term or set-point
166 bacterial load (b), the timepoint at which the load is reduced in half (k) and the highest rate of
167 decrease or slope at timepoint k (h) (see Materials and Methods for details). In Table 1, we show
168 the mean estimates, standard deviation, and high-density intervals (3% and 97%) for all four
169 parameters of the fitted sigmoidal model, for Control and BactOral populations. Overall, these
170 results show that sex has a significant effect on all curve parameters (k= -0.053 ± 0.068; h= -0.330

171 ± 0.227; a = -0.130 ± 0.038; b = -0.147 ± 0.066), reinforcing the notion of a strong sexual
172 dimorphism in response to infections[48–50]. Additionally, populations differ on "time to half height"
173 (k= -0.108 ± 0.046) and final load (b= -0.285 ± 0.053), but not on initial load (a= -0.002 ± 0.024)
174 nor on highest rate of decrease (h= 0.042 ± 0.182).

175 Because this time-course experiment yielded a significant amount of zero counts,
176 corresponding to individual flies for which no CFUs could be counted, we treated this as an
177 independent dataset for which we developed a separate model focused on infection clearance
178 (Table 2). With that, we found that a_1 (final fraction of zeros) was significantly affected by both
179 sex and population (with no observable overlap between the mean estimates for each condition)
180 (a_1 Females= -0.781 ± 0.07; a_1 Males= -0.061 ± 0.069; a_1 Control= -0.700 ± 0.070; a_1 BactOral=
181 -0.144 ± 0.069), with more BactOral individuals having cleared the infection by the end of the
182 experiment and males more so than females. There were significant overlaps between the
183 estimates of the remaining parameters, evidencing a difference in clearance rate only in late stages
184 of bacterial infection. In sum, these data align with those of bacterial load dynamics, whereby
185 flies from BactOral population clear *P. entomophila* faster and better from 20h of exposure
186 onwards.

187 These results align with the hypothesis that resistance mechanisms participate in the
188 adapted response of BactOral. Indeed, bacterial numbers are affected differently between
189 BactOral and Control populations despite the equivalent initial bacterial inocula (Fig. 2A, Table
190 1) and defecation rates (Fig. 2B). The differences in number of detected live bacteria inside flies
191 suggests a direct bacterial control mechanism is operating more efficiently in BactOral
192 individuals.

193

194 Differences in AMP expression are consistent with distinct bacterial loads 195 dynamics

196 In an attempt to pinpoint which resistance mechanisms contribute to the differences in
197 bacterial clearance rates observed between populations, we infected female flies from Control and
198 BactOral and dissected their guts prior to exposure, and after 8, 24, and 32 hours of infection with
199 *P. entomophila*. We quantified expression levels for a panel of 10 AMPs covering different
200 molecular families, dedicated pathways and pathogen specificities, including *Attacin-A*,
201 *Bomanins Short 2* and *3*, *Cecropin-A1*, *Defensin*, *Diptericin-A*, *Drosomycin* and *Drosomycin*-like
202 *2*, *3* and *5* (Fig. 4, Suppl. Table 5 and Supp. Fig. 2).

203 We first verified if the baseline levels of AMPs (i.e., expressed under homeostatic
204 conditions) differed between populations, by relativizing expression levels to the Control

population at the unchallenged timepoint. With this approach, we established that BactOral and Control express comparable amounts of 8 AMPs when unchallenged, the two exceptions being *Cecropin-A1* (*CecA*) with lower expression levels (*t.ratio*= 2.892, *p*= 0.0445) and *Drosomycin-like 5* (*Drsl5*) with higher levels (*t.ratio*= -4.686 , *p*= 0.0094) (Supp. Fig. 2).

In addition, and since our main goal was to characterize the temporal dynamics of AMP expression in both populations, we performed the analysis for the entirety of the time-course using the unchallenged timepoint as reference (Fig. 4 and Suppl. Table 5). Overall, we detect consistent dynamics with the reduction of expression happening sooner (by 32 hours) in BactOral than Control across 7 of the 10 AMPs tested, the exceptions being Attacin A, Defensin and Drosomycin-like 2 (Fig. 4). This earlier down-regulation in BactOral relative to Control (at 32-hours) of *BomaninS2* (*BomS2*) (*t.ratio*= 4.347, *p*= 0.0047), *BomaninS3* (*BomS3*) (*t.ratio*= 4.035, *p*= 0.0089), *CecropinA* (*t.ratio*= 4.529, *p* = 0.0010), *Diptericin* (*t.ratio*= 2.613, *p*= 0.0439), *Drosomycin* (*t.ratio*= 4.795, *p*= 0.0056), *Drosomycin-like 3* (*Drsl3*) (*t.ratio*= 3.194, *p*= 0.0105) and *Drosomycin-like 5* (*Drsl5*) (*t.ratio*= 4.266, *p*= 0.0021). Furthermore, we detected a significantly higher up-regulation of *AttacinA* and *CecA* in BactOral at 8 hours of infection (*AttacinA*: Control 8h *versus* BactOral 8h – *t.ratio*= -2.563, *p*= 0.0389; *CecA*: Control 8h *versus* BactOral 8h – *t.ratio*= - 3.034, *p*= 0.0123), consistent with the role of these peptides as main players against infections with Gram-negative bacteria[51,52]. A similar trend was observed for other tested AMPs such as *Defensin* and *Drsl2*, although these changes were not statistically significant (*Defensin*: Control 8h *versus* BactOral 8h – *t.ratio*= -2.101, *p*= 0.231; *Drsl2*: Control 8h *versus* BactOral 8 h.p.i. – *t.ratio*= -0.899, *p*= 0.465).

Principle Component Analysis (PCA) supports these trends (Suppl. Fig. 3). The two main Principle Components (PC1 and PC2) evidence the time-dependent differences observed between populations by showing an initial indistinguishable grouping of both, followed by a dissimilar change in expression patterns at 8 hours of infection which culminates with a maximum divergence between Control and BactOral at the later timepoint (32 hours).

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

Having correlated the improved bacterial clearance of BactOral (Fig. 3) with significant differences in expression levels of AMPs between populations (Fig. 4), we sought to functionally associate these two observations. For that, we conducted a priming experiment to induce higher basal levels of AMPs by exposing flies to a solution of heat-killed bacteria, which has been shown

238 to "artificially" increase AMP levels[53–55]. These primed flies were, subsequently, infected orally
239 with live *P. entomophila*, and followed daily for mortality.

240 Previous exposure to heat-killed bacteria increased survival to infection with live *P.*
241 *entomophila* in BactOral and Control populations, confirming the stronger immune response
242 expected upon priming (Suppl. Table 6 and Fig. 5, BactOral females fed with live bacteria
243 (control) *versus* BactOral females primed with heat-killed bacteria (primed): *z.ratio*= 7.193, *p*<
244 0.0001; BactOral males treated with control *versus* BactOral males primed with Heat-killed
245 bacteria: *z.ratio*= 4.368, *p*= 0.0001; Control females fed with control *versus* Control females :
246 *z.ratio*= 10.011 , *p*< 0.0001; Control males treated with control *versus* Control males primed :
247 *z.ratio*= 7.299 , *p*< 0.0001). Females from the Control population primed with heat-killed bacteria
248 increased their survival to oral infection, virtually phenocopying BactOral infected with live
249 bacteria, whereas in males, the same tendency is observed but to a lesser degree (Suppl. Table 6
250 and Fig. 5, BactOral females fed with control *versus* Control females primed: *z.ratio*= 1.629, *p*=
251 0.3620 and BactOral males fed with control *versus* Control males primed: *z.ratio*= - 4.547,
252 *p*<0001). This effect is supported by a time-course of relative expression levels of IMD pathway
253 key players, *Relish* (its devoted transcription factor) and its associated AMP, *Diptericin*, as well
254 as the Toll-activated AMP, *Drosomycin* (Suppl. Fig. 4). For both populations, we observed an
255 increase in levels of *Diptericin* to a comparable degree upon exposure to heat-killed *P.*
256 *entomophila*, but not of *Drosomycin*.

257 With this experiment we strengthened the case for a role of improved resistance in the
258 adapted phenotype of BactOral.

259

260 BactOral maintains higher survival upon immune response activation

261 To ascertain whether disease tolerance may also contribute to BactOral increased survival
262 upon oral infection, we continuously fed flies with a mixture of heat-killed *P. entomophila* and
263 followed survival.

264 As the experimental evolution protocol with which these populations were generated
265 consisted of selection until flies were approximately 10-day old, we analyzed survival after
266 exposure to heat-killed *P. entomophila* exclusively until day 14. With this we verified that, up to
267 this timepoint, both females and males of BactOral survive significantly more than Control after
268 this specific damage damage (Fig 6, Control - HK *versus* BactOral - HK females: *z.ratio*= 6.579,
269 *p*< 0.0001; males: *z.ratio*= 9.612, *p*< 0.0001). No significant differences exist under control
270 conditions (Control - PBS *versus* BactOral - PBS females: *z.ratio*= 1.392, *p*= 0.504; males:
271 *z.ratio*= 0.587, *p*= 0.936).

This result strongly suggests a role for disease tolerance, as exposure to heat-killed bacteria is expected to trigger host responses. Both the deleterious effects of virulence factors (at least partially) as well as of immunopathology should be at play and appear to participate differentially between control and adapted populations in their survival. Together with the gene expression data presented above (Fig. 4) showing that BactOral represses the activation of immune effectors earlier upon infection, it is tempting to hypothesize that a similar process happened during the course of this experiment, and a role may be attributed to tighter control and negative regulation of immune over-activation.

Discussion

In this work, we enquired systematically for the bases of adaptation against an oral bacterial infection in *D. melanogaster*, considering the three general layers that constitute the immune response: behaviour, resistance and disease tolerance[3,8,56]. Upon experimental evolution of an outbred population of *D. melanogaster* against oral infection with *P. entomophila*, and after approximately 6 generations, the selected population (BactOral) showed increased survival upon infection[19]. This phenotype was maintained throughout the selection experiment but, since then, the population has been kept for more than 80 generations under relaxed selection. Despite the fact that no physiological costs associated to this response could be detected[46], we tested the immunocompetence of the evolved populations. The average survival rate after oral infection of flies from BactOral was approximately 63% (50% for females and 75% for males) and approximately 10% in the Control population (5% for females and 15% for males). Considering that at the end of selection BactOral survival reached 90 %[19], this partial decrease in overall survival after infection with bacteria can potentially underestimate the contributions of each of the different mechanisms behind adaptation. Notwithstanding, we were able to ascertain the relative importance of behavioural, resistance, and disease tolerance mechanisms for BactOral adaptation to oral infection with *P. entomophila*.

No behavioural or structural differences between BactOral and Control populations

It is well described that the route of infection is of paramount importance to the outcome of host-parasite interactions In *D. melanogaster*, the mode of entry of a bacterial pathogen into the host's body elicits different physiological responses[19,57–61], which can start by the avoidance of the

305 pathogenic substrate, in the case of infection through oral route[26,62,63], both by inhibiting feeding
306 or choosing oviposition sites according to bacterial composition[26,53,62–64]. P. entomophila is a
307 natural pathogen of Drosophila that causes high mortality when ingested across diverse genetic
308 backgrounds and conditions[23,43,44,65,66]. Thus, it is plausible to consider that fasting could constitute
309 a general response against oral infection. The simplest behaviour that could explain the observed
310 differences in survival between populations would be that BactOral evolved a reduced intake
311 during exposure to bacteria, thereby reducing the "inoculum" size. Indeed, fastening is a prevalent
312 response to infection and behavioural avoidance is a common strategy to limit exposure to
313 infected substrates in different systems[4,62,63]. However, we showed similar amounts of bacteria
314 were ingested by females of both populations. With time, feeding rate decreases, which is
315 consistent with reports that *P. entomophila* ingestion can cause feeding inhibition (Figure 2A)[23,67].
316 Moreover, we consubstantiate the findings from the CAFE assay with the bacterial loads time-
317 course, that show equivalent amounts of *P. entomophila* colonies inside infected flies at early
318 timepoints. Our results indicate that behavioural inhibition of bacterial intake was not one of the
319 mechanisms that evolved in BactOral flies, eliminating fastening as a component of the adaptation
320 to *P. entomophila* exposure.

321 Previous reports have shown that pathogen intake can affect gut function and structure[34–
322 36,45] as well as influence the dynamics of exposure by altering defecation rates[29]. However, we
323 could not find evidence for any differences in these features between control and evolved
324 populations. Firstly, there are no changes in gut length in steady-state conditions that would
325 enable somehow BactOral to a priori face bacterial infection more favourably nor is there a
326 difference in their response to damage and capacity to restore lost cells more efficiently upon
327 infection. Increased enteric cell renewal, which often leads to a temporary shortening of gut
328 length[36], has been described as one of the main mechanisms of *D. melanogaster* defense against
329 oral bacterial infection[35,36,45]. In our experiments, we detected a significant decrease in total gut
330 length after 24 hours of oral infection but equivalent in both populations, not supporting a role for
331 renewal rate in BactOral adaptation.

332 These experiments, however, do not completely discard the hypothesis of differential gut
333 structure underlying the adapted phenotype of BactOral. For example, proportions of gut cell
334 types can change upon infection[68] and such qualitative shift could contribute to higher fitness
335 upon exposure to *P. entomophila* in the adapted population. Also, it could be that relevant changes
336 in gut length occur at later timepoints. Notwithstanding, it has been previously shown that
337 infection-induced gut cell loss starts as early as 2 hours after exposure, with a return to 75% of
338 the total original gut length after 24 hours[36]. This time window is well within the boundaries
339 defined in our setup. Another important aspect to note is that renewal capacity is sexually
340 dimorphic under diverse physiological contexts[69,70]. For instance, females have higher gut cell

341 renewal rates than males upon oral infection with *P. carotovorum* 15 [71], a bacterium that provokes
342 higher mortality in males than females, contrary to oral infection with *P. entomophila* (see
343 Results). The disparity between those and our observations may be explained by the different the
344 different mechanisms of virulence employed by these two pathogens to infect *D. melanogaster*[45].
345 Be as it may, we envision further experiments testing for potential differences in gut delamination
346 rates between evolved populations at distinct timepoints.

347 BactOral flies also did not expel pathogenic bacteria from their guts differently from
348 Control. However, BactOral shows a decrease in the number of alive bacteria defecated between
349 6- and 18-hours post-exposure, evidencing, to some extent, improved bacterial clearance and
350 suggesting a role for resistance. Since our protocol only detects live bacteria, we could potentially
351 be missing an important component of the response and future work should quantify defecation
352 of alive and dead bacteria, for example through fluorescent microscopy, to compare populations
353 with regards to the contribution of overall peristalsis to the elimination of total gut content[29]. In
354 addition, considering the differences in the proportion of individuals that defecated alive bacteria
355 below our detection limit (zeros), we hypothesize that BactOral either retain *P. entomophila* for
356 longer inside their guts before they begin to expel it (which could point to higher disease
357 tolerance), or reduce bacterial loads more effectively at earlier stages, even though with time
358 bacterial proliferation recovers (again suggesting stronger resistance).

359 A role for resistance

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

369 Even though there is no discernible difference in the amount of CFUs infecting both
370 females and males until approximately 20h of infection, by this time individuals from BactOral
371 begin to exhibit gradually decreasing levels of bacteria. This trend aligns with the higher clearance
372 rate observed in the adapted population (and even higher in males than females) as measured by
373 the increase in the number of flies from which we cannot grow colonies of *P. entomophila*
374 ("zeros"). Flies from the Control population control their bacterial loads (at around 20h of
375 infection) but do it at a slower pace and in a less effective way (i.e. many individuals exhibited

376 high bacterial counts by the end of the experiment). It is possible that our proxy for clearance (i.e.,
377 flies classified as "zeros"), could be overestimated due to a low detection threshold. Indeed, it has
378 been shown that *D. melanogaster* can endure chronic bacterial infections[72–74], albeit at rather low
379 levels, which can be the case in our experimental setup. Nonetheless, this holds true for both
380 populations, therefore not invalidating our interpretation of a different bacterial clearance rate
381 between populations.

382 Having shown that BactOral clears *P. entomophila* infection more rapidly, we
383 hypothesized that this response could rely on an increased production of immune effectors, such
384 as AMPs. Analysis of AMP gut gene expression showed that in unchallenged condition, only two
385 AMPs (*CecA1* and *Drsl5*) differed in expression between populations (down- and up-regulation
386 respectively), indicating that the base levels of AMP expression are unlikely to have been the
387 major target of selection. The detected expression difference of *Drsl5*, a gut-specific AMP
388 peptide, could represent a pre-emptive resistance mechanism acquired by BactOral, by
389 establishing a more antagonistic environment for *P. entomophila* proliferation in the guts of these
390 flies after ingestion. At 8 hours after exposure, two AMPs show a faster up-regulation while seven
391 of the panel members display a pattern of faster downregulation, in BactOral relative to Control.
392 These results suggest that a two-step process might be taking place, whereby the response is
393 initially (8h) more aggressive, permitting a more effective bacterial control, and is turned off
394 sooner (32h), limiting the secondary effects of prolonged immune responses (immunopathology).
395 Interestingly, Drosomycin-like genes (*Drsl2*, *Drsl3*, and *Drsl5*), whose expression is specific to
396 the gut epithelium and is regulated by JAK-STAT[35], show up-regulation after 8-hours of infection
397 in both populations. For all three genes, there is a slight tendency for a faster response that could
398 represent a more effective bacterial control in the BactOral population and a faster down-
399 regulation at the 32-hour timepoint that could limit self-inflicted damage. Likewise, we observed
400 a similar process of down-regulation of other AMPs specifically deployed to fight off infections
401 with Gram-negative pathogens (e.g. *CecA1* and *Diptericin*) at the 32-hour timepoint. In contrast,
402 some AMPs typically associated to response to Gram-positive bacteria, (e.g. *BomS2*, *BomS3* and
403 *Drosomycin*[51,75,76)], do not exhibit significant up-regulation after exposure to *P. entomophila* until
404 32-hours post-infection and only in the Control population.

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

410 The same pattern is observed also in the priming experiment (Fig. 5 and Suppl. Fig. 4).
411 After priming with heat-killed bacteria and subsequent exposure to live bacteria, both populations
412 maintain an equivalent expression increase until 8 hours but from 24 hours onwards BactOral
413 shows, again, an earlier downregulation of *Diptericin* and a quicker return to homeostatic levels
414 of expression (Suppl. Fig. 4). In brief, the dynamics of *Diptericin* expression mimic the expected
415 effect of priming by establishing higher basal levels of this AMP with which to counteract an
416 incoming infection. This is not seen for the AMP *Drosomycin*, which again is consistent with our
417 previous result that this AMP is not deployed against infection with *P. entomophila* (Fig. 4 and
418 Suppl. Fig. 2).

419 With this we re-enforce the notion that this process could be an active response rather
420 than a consequence of lower bacterial loads, thus pointing to a potential role for disease tolerance
421 mechanisms in this population.

422 A role for Disease Tolerance

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

430 With this in mind, a putative role for disease tolerance was tested by measuring survival
431 of the evolved populations to prolonged exposure to heat-killed bacteria. This protocol provided
432 a means by which to test the response to damage, whether provoked by pathogenicity of the
433 bacteria or by the host's own response, independently of resistance. A similar longevity was
434 observed for females and males of Control and BactOral after feeding on heat-killed bacteria, but
435 an interesting trend appeared when comparing survival profiles (Fig. 6). We found that BactOral
436 survived better to the stress of prolonged exposure to heat-killed *P. entomophila*, specifically
437 under an evolutionarily relevant timeframe, that is, the period when flies reproduced during the
438 selection experiment. Although both Control and BactOral individuals fully succumbed to the
439 chronic exposure to heat-killed bacteria, significant differences in survival distinguish the two
440 populations and their overall fitness. Physiological responses measured after flies are
441 approximately 10 days old, can potentially represent evolutionary costs[78–81],which are not the
442 focus of this study.

443 The improved response of adapted flies to exposure to damage-inducing substrate can be
444 explained by: 1) an increased capacity to withstand damage provoked by their own immune

445 response, 2) virulence factors present in inactivated bacterial supernatant, or 3) a correlation with
446 the previously identified tighter immune repression. Disentangling the relative contribution of
447 these mechanisms to the overall adapted phenotype would need further empirical characterization.

448

449 In sum, we were able to identify resistance and disease tolerance mechanisms as targets
450 of experimental evolution for increased survival to *P. entomophila* oral infection. Our
451 observations align with previous work that identifies a positive genetic correlation between these
452 two distinct immune defense strategies[82]. The increased survival of our experimentally adapted
453 population to oral infection directly relates to its capacity to decrease bacterial loads after
454 infection, independently of bacterial feeding rate, gut renewal capacity and bacterial defecation.
455 Previous work found resistance to be targeted by the selection of immune response in *D.*
456 *melanogaster*[79,83]. Our work reports one additional case of resistance evolution embedded in a
457 much wider ecological immunity context that exhaustively approaches the different processes by
458 which immunity may evolve in populations fighting off persistent infections. Indeed, in addition
459 to resistance, we could find a quantifiable role for disease tolerance in the evolved response of
460 BactOral, revealing the complex nature of this adaptative process[82], which is likely to rely also
461 on improved tissue repair coupled with a more controlled deployment of damage-inducing
462 immune effectors, such as AMPs[84,85]. Future work will focus on identifying the specific
463 mechanisms of disease tolerance and their physiological mode of action and interactions with
464 resistance.

465

466 # Materials and Methods

467 ## Maintenance of Drosophila populations under relaxed selection

468 Experimentally evolved Control and BactOral populations were initially derived from a
469 highly outbred population of *D. melanogaster*, as described in previous studies [19,46]. After
470 selection was terminated, populations were kept at high census (1500-2000 individuals per cage),
471 allowing for maintenance of high genetic variability and optimal larval development. For all
472 generations, flies were kept under constant temperature (25°C), humidity (55–65%) and light-
473 darkness cycle (12:12), and fed with a standard cornmeal-agar medium, consisting of 4.5%
474 molasses, 7.5% sugar, 7% corn-flower, 2% granulated yeast extract, 1% agar and 0.25% nipagin,
475 mixed in distilled water.

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

481 ## Pathogen culture and infections

482 Oral infection protocol with *P. entomophila* was adapted from the one used in Martins
483 and Faria et al[19]. Briefly, single bacterial colonies were grown in kick-start cultures (5mL of LB)
484 for approximately 8 hours after which they were transferred into larger volumes (1 L of LB) for
485 overnight growth, both periods at 29ºC. After centrifugation and resuspension of bacterial pellets,
486 concentration was adjusted to OD_{600}= 100 in PBS and finally diluted 1:1 with a 5% sucrose
487 solution. For infection, 3-5 days-old flies were separated by sex into groups of twenty with the
488 use of CO_2 (at least 24 hours before the start of the experiments) and allowed to feed on a filter
489 disk embedded with the bacterial solution described above for 24 hours (for survival assays) or
490 for the amount of time specific to each experiment (see Results). After this period, infected flies
491 were flipped onto clean food and survival was scored daily. Controls were fed a 1:1 PBS:5%
492 sucrose solution.

493 For the artificial immune activation experiment, a solution of *P. entomophila* at OD_{600}=
494 100 was previously heat-killed for 1 hour at 55ºC, mixed 1:1 with a 5% sucrose solution and fed
495 to flies for 2 hours, in a similar way as described above for regular oral infection. After this time,
496 flies were transferred into tubes containing a live solution of *P. entomophila* for 24 hours, after
497 which they were changed back to clean food and their survival scored.

498 For the experiment with prolonged exposure to heat-killed *P. entomophila* a similar
499 bacterial preparation and heat-killing protocol were performed as described above, but the final
500 dead bacteria suspension was mixed 1:1 with standard fly food and dispensed into fly vials. To
501 the final solution, extra agar powder was added to account for viscosity and to optimize texture
502 and left to cool down and solidify for at least 2 hours, before being fed to flies. The same protocol
503 was performed for the control treatment, in which fly food was mixed with sterile 1X PBS.
504 Exposure to these treatments was constant throughout the experiment, during which replicates of
505 15 females and 15 males were co-housed in vials, exclusively feeding on the dead-bacteria or
506 PBS mixtures. Survival scoring began at the start of exposure and lasted 14 days. During the
507 treatment, flies were frequently flipped onto fresh food mixture and survival scoring continued
508 daily.

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

518 immunocompetence (Fig. 1)- *coxme*(~Population*Treatment*Sex+(1|Replicate));

519 priming (Fig. 5)- *coxme*(~Population*Treatment*Sex+(1|block/Replicate)) and

520 immune over-activation (Fig. 6)– *coxme*(~Population*Treatment*Sex+(1|Replicate), data =
521 survival_HKexposure).

522 Bacterial defecation assay

523 To have a measure of the rate of gut purge/defecation a change was introduced to the
524 bacterial infection protocol, whereby the feeding period was reduced to a 3-hour period. After
525 this time, flies were surface sterilized and separated individually into plastic spectrophotometry
526 cuvettes, previously filled with fly food. After 6 hours (6 hours.), flies were changed into fresh
527 cuvettes where they remained for an additional 12 hours (18 hours.), after which they were
528 discarded. Bacterial quantification was performed on the interior surface of the cuvettes, for both
529 timepoints, as described below.

530 Statistical analysis on number of defecated bacteria was done by fitting a zero-inflated on
531 the CFU counts and zeros (using the *zeroinfl* function[90] from the *pscl* package[91]). This model
532 allowed for the fitting of a negative-binomial distribution on the counts data and of a binomial
533 distribution on the zeros (*zeroinfl*(formula = Counts ~ Population * Sex * Timepoint | Population
534 * Timepoint, dist = "negbin")). To assess differences between specific pairwise comparisons we
535 used *emmeans* with *tukey* adjustments, on both distributions of the zero-inflated model. In the
536 instances where flies died during either timepoint, their count at that time was removed from
537 further analysis and the linear regression was not included in Figure 2B.

538 Quantification of bacterial loads

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

548 Analysis of the time-course of infection progression was performed by dividing the data
549 obtained from the bacterial quantification into "counts" and "zeros" and using different models
550 to examine their dynamics in our evolved populations. For the counts, the modelling of the mean
551 of the load dynamics during the time-course was done using a sigmoidal function that considers
552 four parameters: a the log (initial load), k the timepoint at which the load is half of the maximum
553 value (in this case, a), h the slope at the k timepoint (steepest slope of the curve), and b the log
554 (final load). A linear model was applied to each of the parameters which was fitted using a Monte
555 Carlo Markov Chain approach. To estimate the impact of Sex and Population on the likely
556 distribution of the parameters, a normal error structure was assumed, using Control females as
557 reference. For the "zeros", a modified logistic model with four parameters was ran: a_0 initial
558 fraction of zeros, k_0 rate of zero disappearance, k_1 rate of zero re-appearance, t_0 timepoint at which
559 zeros re-appear and a_1 final fraction of zeros. Similarly, to the "counts" analysis, linear models
560 were used to assess the impact of Sex and Population on all four parameters.

561 Quantification of food intake by CApillary FEeder Assay - CAFE

562 To measure the amount of bacterial solution ingested by single flies, an adapted CAFE[47]
563 assay was performed. We substituted the food of small fly vials with agarose, to ensure humidity,

564 and placed a glass capillary in each lid, filled with 5% sucrose solution. We allowed individual
565 flies to habituate to this experimental setup for 24 hours before the infection. After this period,
566 capillaries filled with *P. entomophila* solution (prepared as described before) were given to
567 individual females to feed on and consumption estimated, at different timepoints. To facilitate
568 scoring in capillaries, the bacterial solution was mixed with blue food colouring. Controls were
569 fed dyed solution with equivalent dilution but without bacteria.

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

577 RNA extractions and quantitative PCR

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

584 For quantification of gene expression, qPCRs were performed using SYBR™ Green
585 Master Mix (Thermo Scientific®) and reactions ran on 384-well plates (Applied Biosystems®).
586 The PCR conditions used in all experiments were: initial denaturation/ enzyme activation, 95ºC
587 for 10'; followed by 45 cycles of denaturation, 95ºC for 10"; annealing, 60ºC for 10"; extension,
588 72ºC for 30". Sequences of primers used for qPCRs are shown in Supp. Table. 1. Either EIF2
589 (eIF-2alpha) or Rpl32 were used as reference.

590 Analysis of gene expression differences was done with the relative quantification
591 technique (ΔΔCt)[95]. Briefly, to the average of technical replicates for each candidate gene we
592 subtracted the average of the Ct values of the respective sample's house-keeping gene (EIF2 or
593 Rpl32) (ΔCt) and normalized this value to the ΔCt of the respective reference condition. For the
594 time-course analysis, the reference was the "Unchallenged" timepoint while for comparisons
595 between "Unchallenged" conditions among populations, Control was used as reference. Finally,

596 we used reverse logarithm to transform the final gene expression values into fold change levels
597 ($2^{-\Delta\Delta Ct}$) and analysed the Log_2(foldchange) differences across Populations and/or Timepoints.

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

605 Measurement of gut length and crop size

606 Females were individually dissected in PBS 1X, and their guts removed for imaging by
607 adapting a protocol described in[96]. Dissections started by fixing flies in the head with a Minutien
608 pin and gradually separating the gut from the remaining tissues, in a posterior-to-anterior
609 direction. After the gut was exposed, we added a few drops of a solution of 5% Acetic Acid and
610 50%EtOH, which slightly whitens the tissues, allowing for better visualization and a slight tissue
611 fixation. After having isolated the guts, they were individually transferred to a coverslip and
612 photographed using a uEye camera installed on an Olympus SZX7 stereoscope.

613 Images were processed in FIJI v2.0.0 and measurements were collected by drawing
614 consecutive transects along the total length of guts, as illustrated in Supplementary figure 1A.
615 Each measurement was repeated 3 times, to account for errors in the manual drawing of the
616 transects and averaged to obtain a final gut length in millimetres (mm).

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

621 Statistical analysis and graphical representations

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

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

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FIGURES AND TABLES

Figure 1.

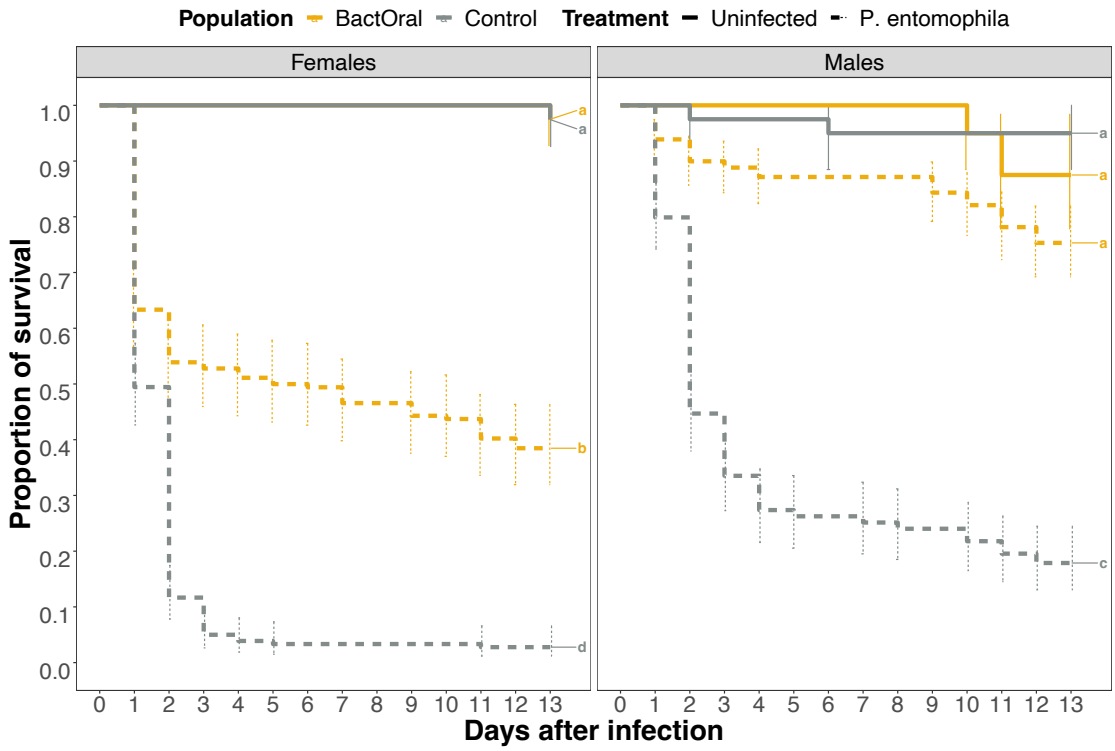
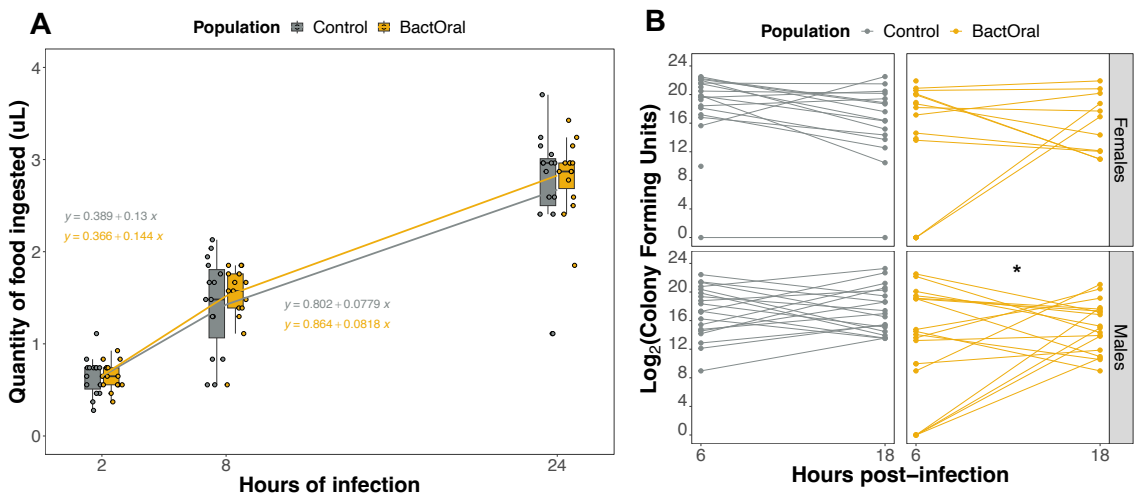
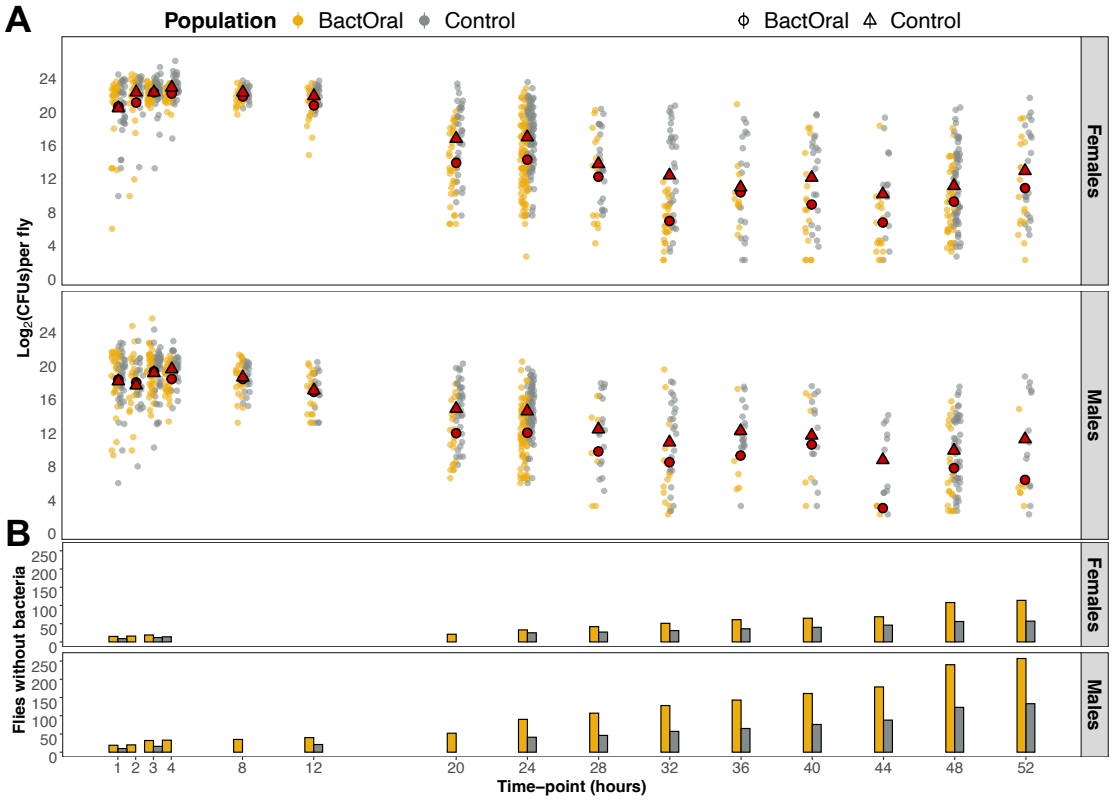


Figure 2.



984 **Figure 3.**



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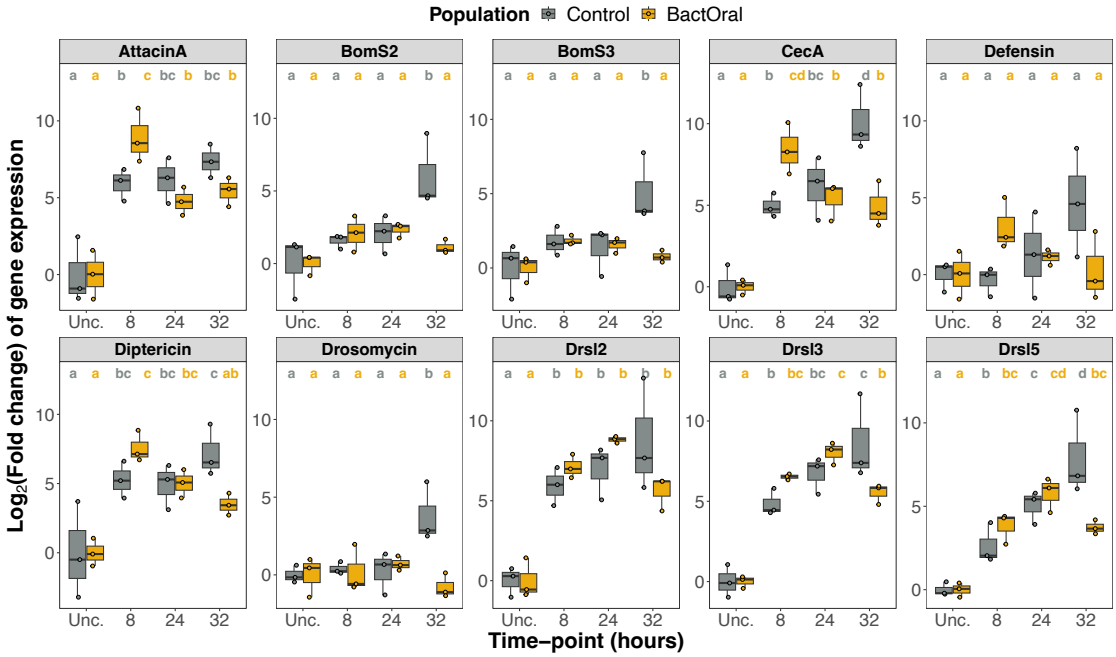
989 **Figure 4.**



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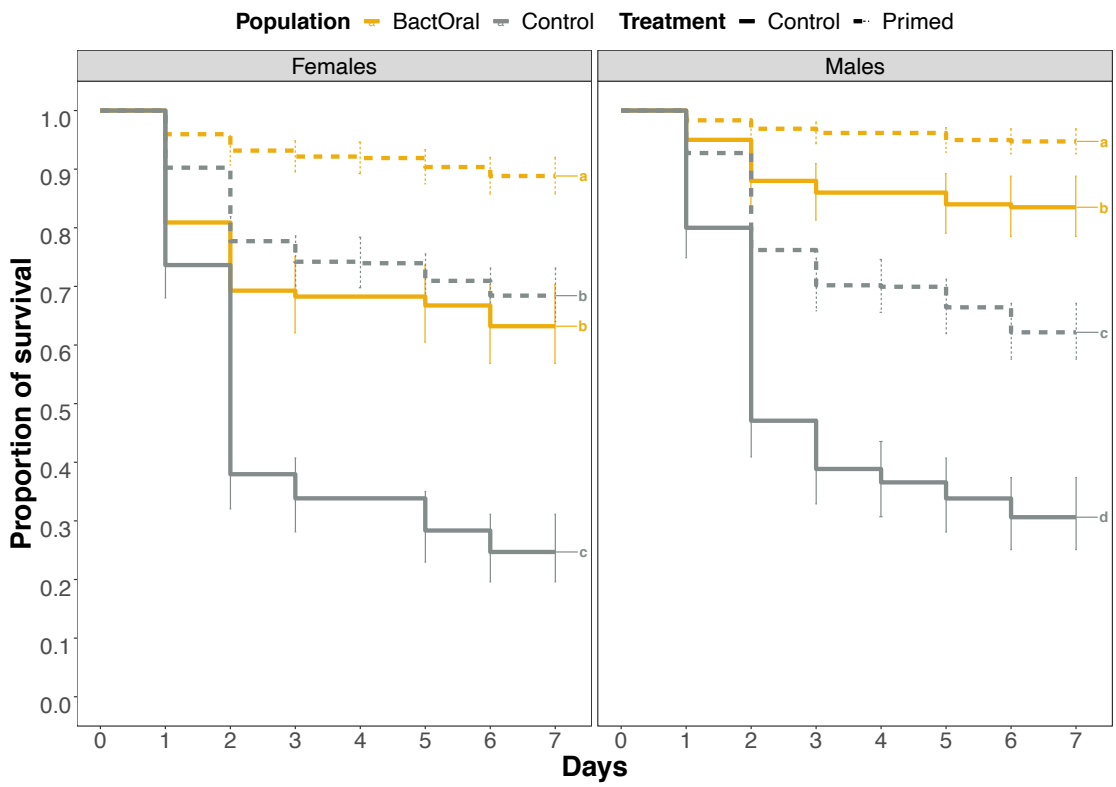
992 **Figure 5.**



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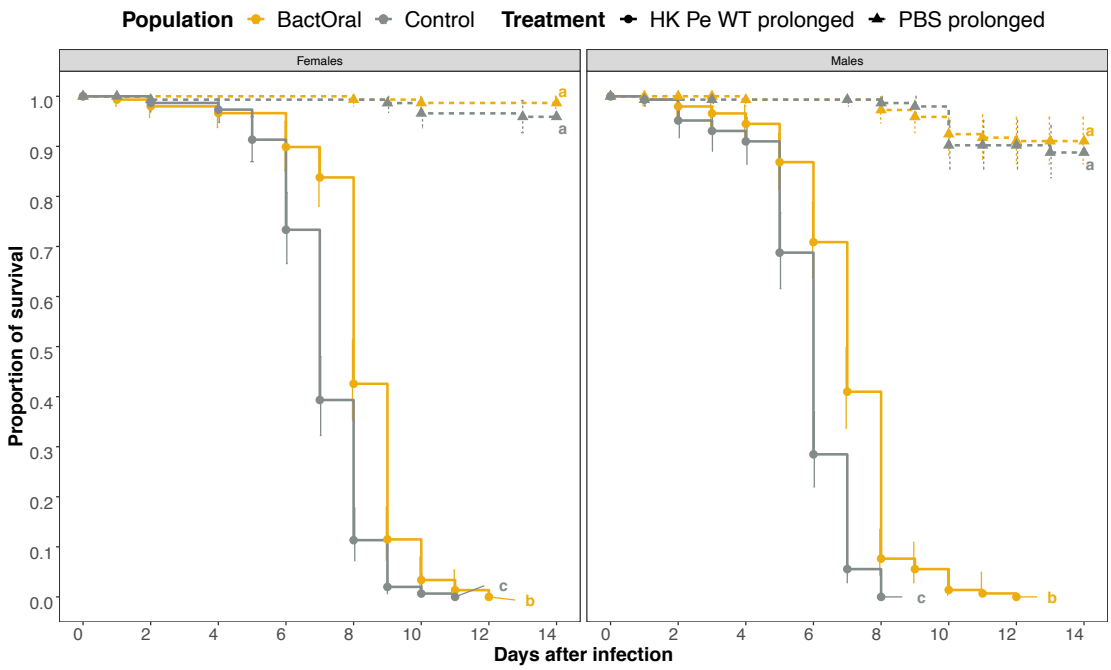
996 **Figure 6.**



997 **Table 1.**

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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

1000 1001 1002 1003 1004 1005 1006 1007

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.

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1017 **Table 2.**

1018

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

1019

Table 2. Parameter estimates for the model logistic function fitted to the zero counts of the time-course of bacterial counts. An adapted logistic function of 4 parameters (a_0, a_1, k_0, k_1) was fitted to the progression of zero counts shown in Fig. 3B), for quantification of bacterial clearance differences between Control and BactOral. Single estimates were generated for the contribution of each variable (Sex: females and males; Population: Control and BactOral) to the model parameters. Effects were considered significant when was considered when the mean estimate ± standard deviation did not overlap for the same parameter between each variable, which is the case for a_1 when considering Population and Sex. High-density intervals of 3% and 97% show that all our estimated parameters have a high-density probability (i.e. credibility) as they are all included in them.

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1024 # FIGURE LEGENDS

1025

1026 **Figure 1. Survival upon infection with *P. entomophila.***

1027 Females (top) and males (bottom) of Control (grey) and BactOral (yellow) populations were
1028 orally infected with a culture of *P. entomophila* OD_{600}=50 (dashed lines and circles) or a control
1029 solution (solid lines and squares) for 24 hours, after which they were flipped onto clean food and
1030 their survival measured daily. We identify a significantly higher survival after infection in
1031 BactOral (in both sexes) as compared with Control, in a treatment-dependent manner. Results
1032 from multiple comparisons are shown as letters in the plots.

1033

1034

1035 **Figure 2. Evolved and control populations eat and defecate equivalent amounts of bacteria.**

1036 **A)** CAFE assay. Females of Control and BactOral populations were fed separately *P.*
1037 *entomophila* solution through a graduated glass capillary and the amount of bacterial solution
1038 ingested (in μL) per fly was registered at 2, 8 and 24 hours. Cumulative number of μL ingested
1039 per fly showed no statistically significant difference in amounts ingested by either population at
1040 the different time-points. Linear regressions between averaged values eaten by each population
1041 between time-points show a decrease in feeding as infection progresses (equations shown in plot
1042 for the periods, 2–8 hours (above lines) and 8–24 hours (below lines). **B)** Defecation assay.
1043 Females (top) and males (bottom) from Control (grey) and BactOral (yellow) populations were
1044 fed *P. entomophila* for 3 hours, after which the number *P. entomophila* defecated was estimated
1045 after 6 hours and 12 hours. Lines connecting the two points provide a sense of individual
1046 defecation rate within this time window. Linear regressions between the two counts of the same
1047 fly show a significant decrease in the number of bacteria defecated during the initial 6 hours and
1048 the following 12 hours by males of BactOral (shown with *). Additionally, there is a significant
1049 difference in the number of BactOral flies that defecated below our detection threshold (zero CFU
1050 counts) at 6 hours compared to 18 hours and compared to Control for both timepoints.

1051

1052 **Figure 3. Bacterial load dynamics.** Individual flies from Control (grey) and BactOral (yellow)
1053 were collected upon exposure to *P. entomophila* at different timepoints for assessment of the
1054 number of Colony Forming Units (CFUs) inside their bodies. After the end of the infection period
1055 (24 hours), flies were changed onto clean food and collection continued until 52 hours post-
1056 infection. **A)** Log_2 of the number of CFUs found for females (top) and males (bottom) of either
1057 Control (grey) or BactOral (yellow) populations, with the average value at each time-point
1058 evidenced in red (triangles for Control and circles for BactOral). There are no detectable

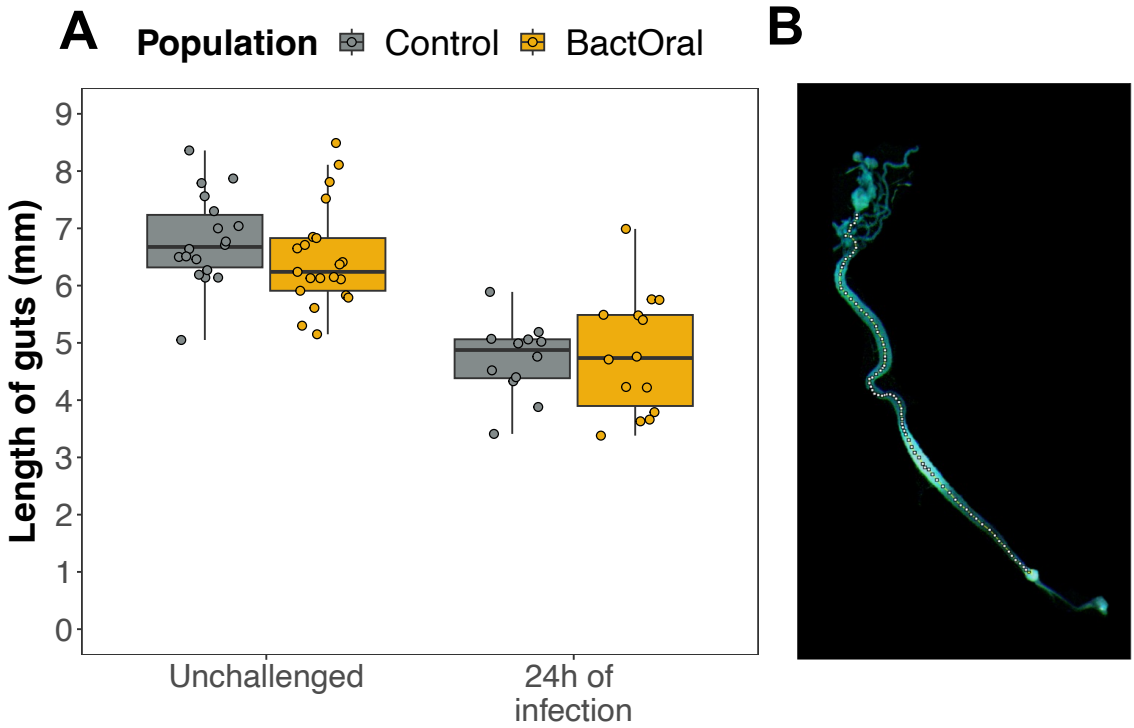
1059 differences in bacterial loads between both populations until 20 hours of infection, when BactOral
1060 starts to evidence a bigger decrease in bacterial counts. **B)** Cumulative number of individual flies
1061 from which no CFUs were obtained increases as time progresses, with the BactOral reaching
1062 significantly higher final values.
1063
1064 **Figure 4. AMP expression differs between populations.**
1065 Guts from Control (grey) and BactOral (yellow) female flies were dissected before exposure
1066 ("Unc.") and at different timepoints after oral infection (8, 24 and 32 hours) with *P. entomophila.*
1067 Relative gene expression, normalized to housekeeping gene *EIF2,* was performed for a
1068 representative panel of 10 AMPs. We detected a significantly higher up-regulation in BactOral
1069 compared to Control for *AttacinA* and *CecropinA* at 8-hours of infection. Results from these
1070 comparisons are shown in the letters above the plots.

1071
1072
1073 **Figure 5. Control flies primed with heat-killed bacteria phenocopy BactOral survival to oral**
1074 **infection with *P. entomophila*.** Control (grey) and BactOral (yellow) females (left) and males
1075 (right) were fed a solution of heat-killed bacteria for 2 hours before oral infection (dashed lines)
1076 or directly infected with live *P. entomophila* (full lines) for 24 hours, after which they were
1077 changed onto clean food and their survival measured daily. Previous exposure to heat-killed
1078 bacteria before infection increased survival in both populations, leading additionally Control
1079 females to phenocopy BactOral survival levels upon infection. Results from multiple comparisons
1080 are shown as letters in the plots.

1081
1082
1083 **Figure 6. BactOral displays higher survival than Control upon immune system activation**
1084 **without bacterial proliferation.** Daily survival was measured for Control (grey) and BactOral
1085 (yellow) populations fed a mixture of food with heat-killed bacteria or PBS continuously BactOral
1086 females and males exhibit a slower mortality compared to Control flies until around day 10. This
1087 difference fades away for both sexes after 14 days. Survival comparisons between populations
1088 and treatments are shown as lower-case letters.
1089

1090 SUPPLEMENTARY FIGURES AND TABLES

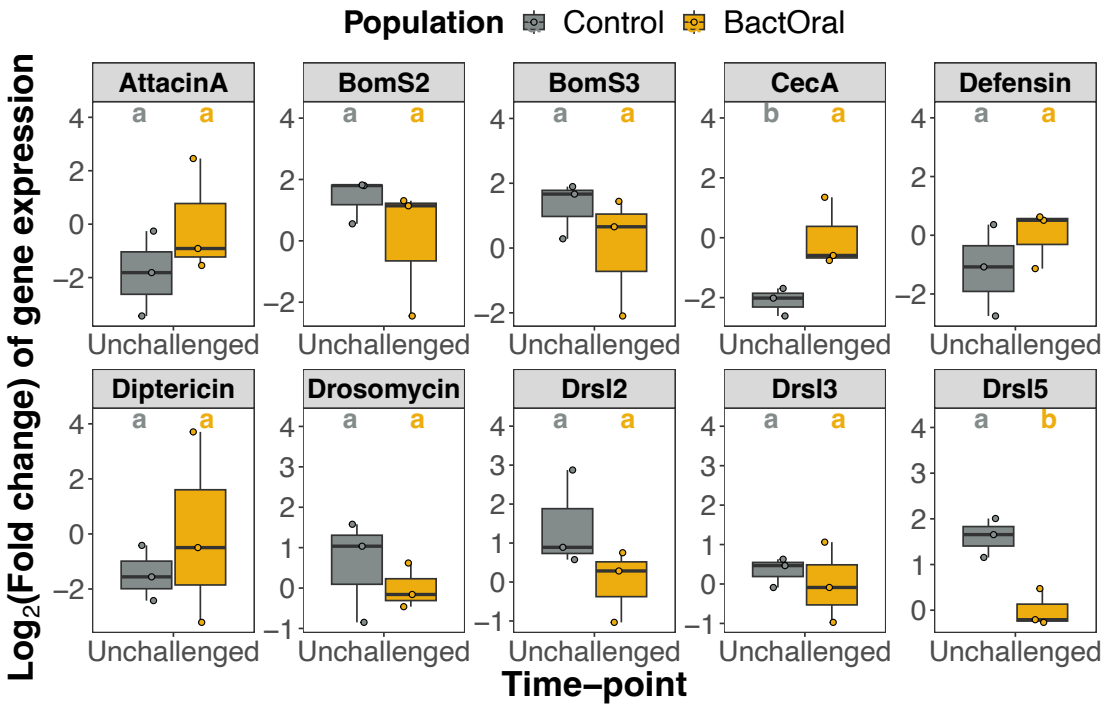
1091



1092

1093 **Supplementary Figure 1.**

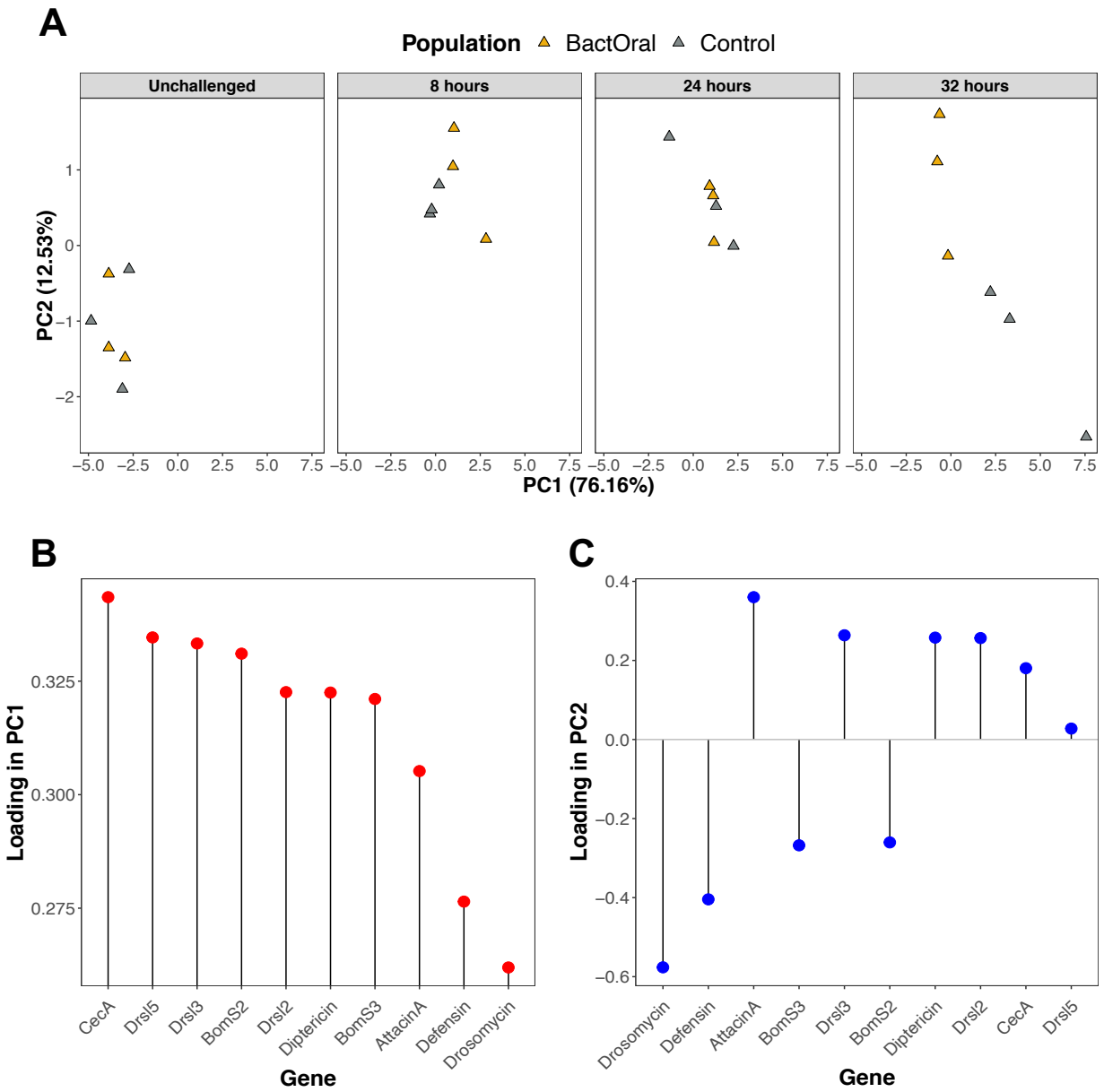
1094 **No detectable differences in gut structure between Control and BactOral after oral infection**
1095 **with *P. entomophila*.** Females of Control (grey) and BactOral (yellow) were fed normal food
1096 ("Without infection") or orally infected with *P. entomophila* for 24 hours, after which their **A)**
1097 guts or **B)** crops were dissected and photographed. Gut length (in mm) was measured by drawing
1098 consecutive longitudinal transects along the guts thrice and averaging those measurements to
1099 reach final length per sample. Crop perimeter was measured in a similar way, by drawing
1100 segments around the border of each crop. **A)** Exposure to bacteria shortens the guts significantly
1101 in both populations (Control before infection *versus* Control 24 hours post-infection: *t.ratio* =
1102 6.274, p <0.0001; BactOral before infection *versus* BactOral 24 hours post-infection: *t.ratio* =
1103 5.453, p <0.0001). No differences were detected in gut lengths between populations within each
1104 timepoint (Without infection: BactOral *versus* Control – p = 0.6719; after 24 hours of infection –
1105 p = 0.9925), although a significant effect of treatment was observed in both Control (Without
1106 infection *versus* after 24 hours of infection – p < 0.0001) and BactOral (p < 0.0001), showing that
1107 gut shortening due to infection affected both regimes in a similar manner. **B)** Dissected guts of
1108 females from Control and BactOral populations were photographed and scaled for posterior
1109 length quantification. Manual transects were drawn longitudinally along the guts (as shown
1110 above) and total size of the cumulative transects was calculated in FIJI. Three independent
1111 measurements were done on each gut and averaged to obtain a final value per sample (Fig. 2 A)).



Supplementary Figure 2.

AMP expression differences between populations under homeostatic conditions. Guts from Control (grey) and BactOral (yellow) female flies were dissected at different timepoints after oral infection with *P. entomophila* and relative quantification of gene expression was performed. Expression levels of each gene in BactOral samples were normalized to expression levels of Control samples. Log_2(FoldChange) of these differences were fitted on a linear model with Population as factor and followed by multiple comparisons (*emmeans*) to uncover pairwise differences. Results from these comparisons are shown in the letters above the plots. There are significantly lower levels of CecropinA (CecA) (*t.ratio*= 2.892, p = 0.0445) and significantly higher levels of *Drosomycin-like 5* (Drsl5) (*t.ratio*= -4.686 , p = 0.0094) being expressed in guts of BactOral flies under unchallenged conditions.

1127



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1129 **Supplementary Figure 3.**

1130 **Principle Component Analysis on relative gene expression levels in evolved populations. (A)**
1131 Relative AMP expression levels of Control and BactOral flies upon oral infection with *P.*
1132 *entomophila* showed significant differences at various timepoints (see Fig. 4 and Supp. Fig. 2).
1133 **(B-C)** Relative influence of each AMP to the establishment of PC1 and PC2 (see Supp. fig. 3 A).
1134 Importantly, Cecropin A1 represents the largest contributor to PC1 (**B**) whereas Drosomycin
1135 represents the largest contributor, although negatively correlated, to the variation in PC2 (**C**).

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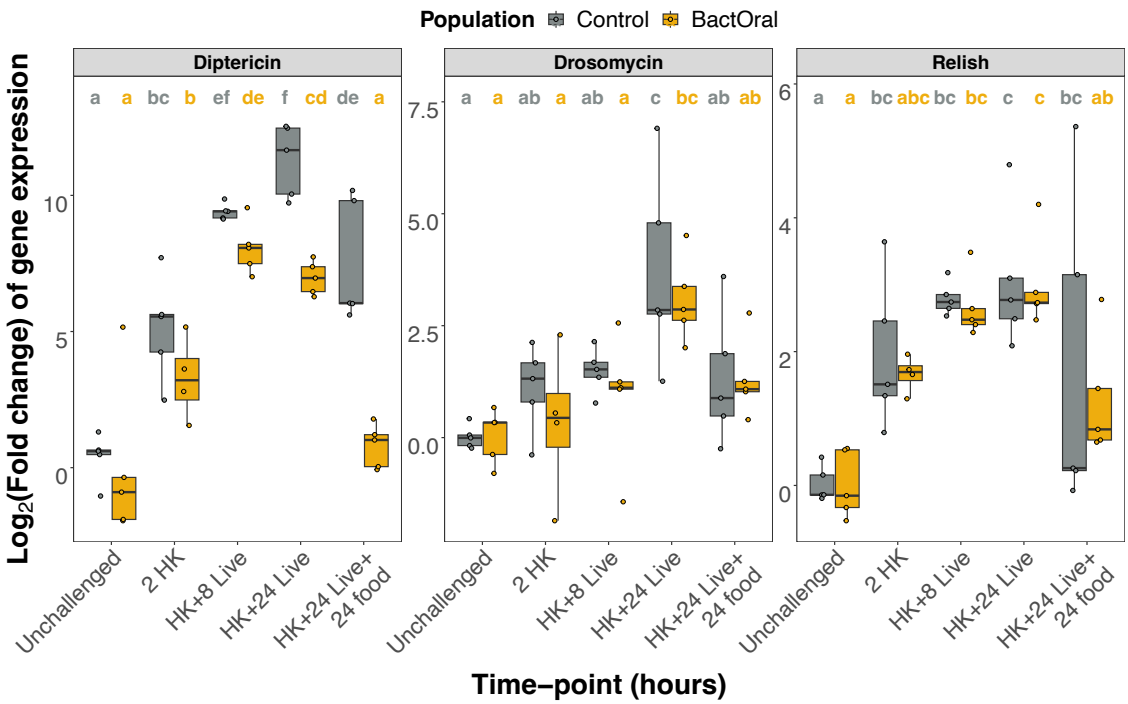

Supplementary Figure 4.

Quantification of immunity-related gene expression upon artificial immune stimulation and infection. To test for individual pairwise differences, relative log2 (FoldChange) expression levels of each gene were fitted onto a linear model with Population and Timepoints as factors and followed by multiple comparisons (*emmeans*). No differences are observed between non-challenged individuals or between individuals after 2 hours of exposure. However, for Diptericin Unchallenged versus 2 HK show differences in both populations (Control: *t.ratio*= -4.778 , *p*= 0.0001; BactOral: *t.ratio*= -3.122, *p*= 0.0052).