

1 **Disruption of flour beetle microbiota limits experimentally evolved immune**  
2 **priming response, but not pathogen resistance**

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26 **ABSTRACT:**

27 Host-associated microbiota play a fundamental role in the training and induction of  
28 different forms of immunity, including inducible as well as constitutive components.  
29 However, direct experiments analysing the relative importance of microbiota during  
30 evolution of different immune functions are missing. We addressed this gap by using  
31 experimentally evolved lines of *Tribolium castaneum* that either produced inducible  
32 immune memory-like responses (immune priming) or constitutively expressed basal  
33 resistance (without priming), as mutually exclusive strategies against *Bacillus thuringiensis*  
34 infection. We disrupted the microbial communities in these evolved lines and estimated the  
35 impact on the beetle's ability to mount a priming response vs basal resistance. Populations  
36 that had evolved immune priming lost the ability to mount a priming response upon  
37 microbiota disruption. Microbiota manipulation also caused a drastic reduction in their  
38 reproductive output and post-infection longevity. In contrast, in pathogen-resistant beetles,  
39 microbiota manipulation did not affect post-infection survival or reproduction. The  
40 divergent evolution of immune responses across beetle lineages was thus associated with  
41 divergent reliance on the microbiome. Whether the latter is a direct outcome of differential  
42 pathogen exposure during selection or reflects evolved immune functions remains unclear.  
43 We hope that our results will motivate further experiments to understand the mechanistic  
44 basis of these complex evolutionary associations between microbiota, host immune  
45 strategies, and fitness outcomes.

46

## 47 INTRODUCTION:

48 Growing evidence reveals the critical role of microbiota in altering various aspects of host  
49 development, behaviour, and reproduction (Gould et al., 2018), as well as in training and  
50 induction of host immune responses (Zheng et al., 2020). In many species, including  
51 humans, the microbiota is required for successfully mounting different forms of immunity  
52 (e.g., innate vs adaptive) (Chudnovskiy et al., 2016; Karimi et al., 2009; Mazmanian et al.,  
53 2005; Muhammad et al., 2019), such that depletion or loss of microbial diversity can  
54 increase the vulnerability to pathogens (Dillon and Dillon, 2003; Engel and Moran, 2013).  
55 Gut bacteria can also influence tissues, cells and molecular pathways involved in  
56 gastrointestinal immunity, and changes in microbiome composition leads to overactive  
57 inflammatory responses causing bowel disorders (Kostic et al., 2014). Together, these  
58 results indicate an optimal association between host and microbiota forged over a long  
59 coevolutionary history (Lee and Mazmanian, 2010), to appropriately train and regulate  
60 immune responses (Belkaid and Hand, 2014; Thaïss et al., 2016). Recent studies also suggest  
61 a role for microbiota in inducing immune memory-like responses in insects (immune  
62 priming), whereby prior exposure to a low dose of infection improves survival against a  
63 lethal infection caused by the same pathogen later in life (Futo et al., 2015; Muhammad et  
64 al., 2019). Thus, host microbiomes appear to be generally important in shaping various  
65 forms of immunity across diverse taxa.

66 However, it is less clear whether microbiota are similarly important in shaping different host  
67 immune strategies. Host immune systems can evolve to new equilibrium states reflecting  
68 distinct immune strategies in response to different pathogen selection pressures (Mayer et  
69 al., 2016, Khan et al. 2017). Although experimental support is missing, host-associated  
70 microbiota, owing to their immunomodulatory role, might also exhibit correlated changes as  
71 host immune functions diverge (Zheng et al., 2020). However, although pathogen resistance  
72 is one of the major evolutionary advantages conferred by microbiota (McLaren and  
73 Callahan, 2020), there are no experiments to test whether or to what extent the role of  
74 microbiota varies across divergent forms of host immunity. We thus conducted a proof-of-  
75 principle study to analyse the impacts of microbiota in replicated experimental evolution  
76 lines of flour beetle *Tribolium castaneum* that separately evolved either constitutively  
77 expressed higher basal resistance, or inducible immune priming responses against their

78 natural pathogen *Bacillus thuringiensis* (Bt) (Khan et al., 2017; Prakash et al., 2022). We  
79 disrupted the microbiome of these evolved lines to test whether their evolved immune  
80 strategies and fitness traits depended on the host-associated microbiome.

## 81 **MATERIALS & METHODS**

82 We used previously described, replicate populations of *T. castaneum* that were infected in  
83 each generation with live Bt (strain DSM 2046), either with or without prior exposure to  
84 priming with heat-killed Bt cells to create distinct selection regimes (Khan et al., 2017; also  
85 see the supplementary information) as follows. (a) C populations: Control populations with  
86 no priming or infection; (b) PI populations: Priming with heat killed Bt, followed by infection  
87 with live Bt (PI); and (c) I populations: Mock priming (i.e., injected with insect Ringer),  
88 followed by infection with live Bt. Of the 4 original replicate populations per selection  
89 regime, in the present work, we analysed three replicates (total 9 populations). After 14  
90 generations of continuous selection, we found that I populations only evolved priming  
91 responses, whereas PI beetles had higher basal resistance (Prakash et al., 2022) as mutually  
92 exclusive responses— i.e., evolved populations either showed priming or resistance, but  
93 never produced both the responses together. In this study, we used the same beetle lines  
94 after another round of selection (i.e., 15 generations), then removed pathogen selection for  
95 two additional generations to minimize maternal or other epigenetic effects. We then  
96 collected “standardized” eggs to obtain experimental beetles with minimum non-genetic  
97 parental effects, to analyse the impact of disrupting microbiota on the already evolved  
98 immune responses (i.e., priming vs basal resistance).

### 99 **Experimental manipulation of the beetle microbiome and subsequent assays**

100 Previous work shows that the beetle microbiome is most likely acquired from the flour that  
101 the beetles inhabit and consume, and in which they also defecate and reproduce (Agarwal  
102 and Agashe, 2020). Beetles also derived significant fitness benefits from flour-acquired  
103 microbes, including higher fecundity and lifespan (Agarwal and Agashe, 2020). Thus, the  
104 easiest way to manipulate the beetle microbiome is to deplete the flour-associated  
105 microbial flora. We followed a previously published protocol in the lab (Agarwal and Agashe,  
106 2020), where thin layers of wheat flour were exposed to UV radiation (UV -C ~254nm) in a  
107 laminar airflow for 2h. This treatment significantly alters flour microbiome with drastic

108 depletion of the dominant bacterial taxa (also see Fig. S1 for reduction in CFUs on LB agar  
109 plates post UV-treatment). We then isolated single standardised eggs from each population  
110 in the wells of 96 well plates containing ~0.25 g of either UV-treated flour or normal wheat  
111 flour and reared them as virgins until adulthood. We did not track the sex of beetles in  
112 subsequent experiments (unless stated otherwise) because neither priming nor basal  
113 infection responses varied across sexes in our previous studies (Khan et al., 2017; Prakash et  
114 al., 2022). Below, we describe the assays performed with standardised beetles reared in  
115 normal vs UV-treated flour—

116 A. Evolved priming vs basal infection response: To prime and infect beetles, we  
117 used the septic injury method as described earlier (Khan et al., 2016; also see SI  
118 information). Briefly, 10-day old virgin I regime adults (24 beetles/priming  
119 treatment/ microbiota manipulation/ replicate population) were randomly  
120 assigned to one of the following treatments: beetles were either injected with  
121 insect Ringer solution (unprimed) or primed with heat-killed Bt cells adjusted to  
122  $10^{11}$  cells/100 $\mu$ l Ringer solution (primed). Six days later, we infected all beetles  
123 with live Bt ( $\sim 10^{10}$  cells in 75  $\mu$ l Ringer solution) and recorded their mortality for  
124 14 days. We did not assay priming for C and PI beetles since they never showed a  
125 priming response in our earlier experiments (see the assay in generation 14,  
126 (Prakash et al., 2022)). Instead, we compared 16-day old C and PI unhandled  
127 beetles directly for survival after infection with live Bt, across microbiota  
128 manipulations (n=24 beetles/treatment/dietary resource/replicate populations).  
129 This is because evolved basal resistance of PI is an estimate relative to post-  
130 infection survival of control C beetles which did not evolve against Bt. We did not  
131 observe any mortality in sham-infected beetles.

132  
133 We analysed priming and basal infection response data using a mixed effects Cox  
134 model (implemented in R, Therneau, 2015) with replicate population as a  
135 random effect, specified as: (1) Priming ~ Priming treatment (i.e., unprimed vs  
136 primed) x microbiota manipulation (i.e., UV-treated vs normal wheat flour) +  
137 (1|replicate population)] (2) Basal infection response ~ Selection regime (i.e., C &  
138 PI) x microbiota manipulation + (1|replicate population). A significant interaction

139 between priming treatment (or selection regime) and microbiota manipulation  
140 would indicate that the survival benefits of evolved priming (or evolved basal  
141 infection response) in I (or PI) populations vary significantly with disruption of  
142 microbiota. Further, to disentangle the changes in priming response of I beetles  
143 with vs without the microbiota manipulation, we analysed priming for each  
144 microbiota manipulation treatment separately, using a mixed effects Cox model  
145 specified as:  $\text{Priming} \sim \text{Priming treatment} + (1|\text{replicate population})$ , with  
146 priming treatment and replicate population as a fixed and random effect  
147 respectively.

148

149 B. Lifespan after priming: In a separate experiment, we collected virgin females  
150 reared in normal vs UV-sterilized wheat flour as described above (n= 12  
151 females/treatment/microbiota manipulation/replicate population) to estimate  
152 the long-term survival benefits of priming (same dose as mentioned above) and  
153 basal infection response against a lower dose of infection adjusted to  $10^6$  cells in  
154 75 $\mu$ l Ringer solution. We observed beetle mortality every 5 days until 90 days  
155 when most of them were dead. We analysed lifespan data using model  
156 specifications as described above.

157

158 C. Reproductive output: Finally, we measured the impact of microbiota  
159 manipulation on reproductive fitness of evolved PI and I beetles. We first paired  
160 10-day-old unhandled virgin males and females across selection regimes and  
161 microbiota manipulation. After two days of mating, we separated the females  
162 and allowed each to oviposit for 48h in 5g wheat flour (n=39-  
163 52/treatment/replicate population). After 4 weeks, we counted the total number  
164 of eggs laid per female as a proxy for reproductive fitness. At each step, beetles  
165 were either given access to UV-irradiated or untreated flour, according to their  
166 rearing condition. We analysed the data using a mixed effects Generalised Linear  
167 Model with Quasi-Poisson error, specified as:  $\text{Reproduction} \sim \text{Selection regime}$   
168 (i.e., C, I, PI) x microbiota manipulation + (1|replicate population). To disentangle  
169 the changes in each selection regime, we also analysed them separately.

170 For each analysis, we could pool the data across replicate populations since population  
171 identity did not show any significant main impact or interactions as a fixed factor  
172 ( $P < 0.05$ ).

173

174 **RESULTS:**

175 **I. Disruption of microbiota causes the loss of immune priming response but not**  
176 **basal resistance**

177 Here, we present results from data pooled across replicate populations of each selection  
178 regime, since we did not find a significant population effect (see Methods). Separate  
179 analyses and plots for each replicate population are shown in the supplementary materials.  
180 We first compared the priming response of I beetles (with an evolved priming response)  
181 reared in normal vs UV-treated flour. We found a significant interaction between priming  
182 treatment and microbiota manipulations (Table S1A). Priming improved beetle survival only  
183 in I populations reared in the standard diet (normal wheat flour with microbes), but not  
184 when they consumed UV-treated flour (Fig. 1A, S2A; Table S1B, S1C). Thus, there was a loss  
185 of evolved priming ability with disruption of the dietary source of microbiota.

186 Subsequently, we compared C vs PI beetles to estimate the changes in basal resistance as a  
187 function of the microbiota manipulation. We found a significant main effect of the selection  
188 regime (as expected, PI beetles had higher, evolved basal resistance), but microbiota  
189 manipulation had no impact (Table S2). Further, the lack of a significant interaction between  
190 selection regime and microbiota manipulations indicated that the higher post-infection  
191 survival of PI beetles was not affected by their microbiota (Fig. 1B, S2B; Table S2).

192 These results corroborate another independent experiment where selected beetles were  
193 infected with a relatively lower dose of Bt and their lifespan was recorded until 90 days  
194 post-infection. Primed I beetles lived significantly longer when they were reared in the  
195 standard diet, but this benefit of priming disappeared when beetles were fed with UV-  
196 treated flour (Fig. 1C; S3A; Tables S3A, S3B, S3C). Hence, the longevity effects of priming  
197 also relied on the presence of microbiota. As expected, PI beetles lived longer than C  
198 beetles, regardless of the UV treatment of their diet (Fig. 1D, S3B; Table S4), suggesting that  
199 longevity effects of basal resistance do not depend on dietary microbes.

200 **II. Disrupting dietary microbes affects the reproductive potential of beetles with**  
201 **evolved priming, but not that of resistant beetles**

202 Next, we analysed the effects of dietary microbe manipulations on reproductive output of  
203 beetle populations with divergent immune functions. Depletion of microbiota reduced



204 reproductive output in both C and I females, but not in PI females (Fig. 1E, S4; Table S5A).  
205 Interestingly, the negative effect of microbiome disruption was more pronounced in I  
206 beetles, with a steeper decline of fitness relative to C beetles (Fig. 1E). We also note that the  
207 results for pooled data of C populations (Fig. 1E) differ from individual replicate populations  
208 (Fig. S4; Table S5B), which separately did not show a significant impact of microbiota  
209 manipulation. However, all the populations showed a consistent trend towards lower  
210 reproductive output of C beetles in UV treated flour (Fig. S4).

211 Interestingly, C beetles (which evolved in the absence of pathogen selection) most closely  
212 represent the ancestral condition. Thus, starting from a baseline negative effect of  
213 microbiome loss on reproduction, in I populations the effect became more pronounced,  
214 whereas in PI populations the effect of microbiota was lost. Thus, the reproductive effects  
215 of microbiota potentially co-evolved with pathogen selection and host immune strategies.

## 216 **DISCUSSION:**

217 In the past few decades, we have learnt that host-associated microbial communities can  
218 have major impacts on the host immune system and may have co-evolved with their hosts  
219 over evolutionary time (reviewed in Zheng et al., 2020). However, we lack direct evidence  
220 for the impact of microbiota on different components of the immune system when evolving  
221 under strong pathogen selection. This is possibly due to the lack of a suitable experimental  
222 system where evolutionary trajectories of different immune responses can be clearly  
223 distinguished. Previously, we reported a unique set of experimentally evolved beetle lines  
224 where pathogen-imposed selection led to the rapid, parallel and divergent evolution of  
225 either strong basal pathogen resistance (PI populations) or immune priming (I populations)  
226 as mutually exclusive responses (Khan et al. 2017; Prakash et al., 2022). Here, we showed  
227 that evolved basal resistance vs priming ability also have varied functional dependence on  
228 the host beetle microbiota. The disruption of microbiota led to a complete loss of the  
229 survival benefit of priming, whereas basal resistance to Bt infection remained unaffected. In  
230 beetles that evolved priming ability, depletion of microbiota also revoked the benefit of  
231 longer lifespan after priming and reduced their reproductive output; but this was not the  
232 case in resistant PI beetles. Impacts of microbiota as a function of evolved immune  
233 responses might thus extend to multiple fitness traits. Moreover, the absence of  
234 reproductive effects in PI beetles starkly contrasts the observation that in unselected

235 control C beetles an intact microbiota was necessary to maintain reproductive output. PI  
236 beetles thus also gained independence from the reproductive fitness effects of microbiota  
237 during evolution of basal resistance against pathogens.

238 Why does the effect of microbiome differ across evolved immune strategies? We speculate  
239 that the effects may be determined by how the microbiome modulates specific immune  
240 pathways underlying priming or basal infection responses. For example, in flour beetles,  
241 prior priming improves post-infection survival by controlling pathogen growth (Khan et al.,  
242 2019), with the help of canonical resistance mechanisms such as increased phenoloxidase  
243 response (Ferro et al., 2019). However, if the disruption in microbiome composition  
244 interferes with the activation of bactericidal phenoloxidase response in primed I beetles,  
245 they may have a higher pathogen burden, thereby neutralizing the net beneficial effects of  
246 priming. Recent experiments with the moth *Plodia interpunctella* corroborate this  
247 hypothesis: removal of gut bacteria reduced phenoloxidase activity and concomitantly  
248 increased mortality after Bt infection (Orozco-Flores et al., 2017). Dietary microbes may also  
249 somehow modulate the priming effects of Bt cells introduced into the beetle haemolymph  
250 via septic injury in our experiments, with the exciting implication of cross-talk between the  
251 gut environment and priming responses produced in the haemolymph (Freitak et al., 2007;  
252 Kwong et al., 2013). In contrast, the evolved basal infection resistance of PI beetles might  
253 have been achieved by improving overall body condition (Prakash et al., 2022), which could  
254 have also increased their ability to withstand the effects of infection (.e., increased  
255 tolerance, Seal et al., 2021), without directly activating or involving the immune response.  
256 As a result, the immunomodulatory effects of microbiota might not be relevant for PI  
257 beetles anymore. Another possibility is that distinct sets of microbes may regulate the  
258 efficiency of evolved basal resistance vs priming, with the former being UV-resistant and the  
259 latter UV-sensitive.

260 Finally, we note that the potential divergence in microbiomes as well as beetle immune  
261 function may be unlinked, with each being driven independently by the specific selection  
262 regime. Whether this hypothesis is true, and if so, what is the direction of causality, remains  
263 to be determined. For instance, the beetle microbiome could be first rapidly altered by Bt  
264 infection (Li et al., 2020), due to infection-induced changes in host physiology. Since I vs. PI  
265 regimes involved differential exposure to Bt, the two regimes may have allowed for

266 divergent changes in the microbiome. Eventually, the altered microbiomes could have  
267 facilitated the subsequent evolution of beetle immune function. Alternatively, beetle  
268 immune function may have diverged first across regimes (Cherif et al., 2008), changing the  
269 resident microbiomes later as a by-product. To distinguish between these alternatives, one  
270 would need to analyse the time course of change in host immune function as well as  
271 microbiomes during evolution. We hope that our results revealing the possibility of  
272 divergent impacts of microbiota across immune strategies will spur further work to test  
273 whether or to what extent these changes in the host immune function and microbiome are  
274 causally linked, and if so, through what mechanism.

275

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## 281 **Author contributions**

282 IK conceived the experiment; IK, AP and DA designed the experiment; AP performed the  
283 experiment; AP and IK analyzed the data. IK & DA wrote the manuscript with inputs from  
284 AP.

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## 290 **Competing interests**

291 None

## 292 **References**

- 293 Agarwal, A., Agashe, D., 2020. The red flour beetle *Tribolium castaneum*: A model for host-  
294 microbiome interactions. *PLoS ONE* 15, e0239051.  
295 <https://doi.org/10.1371/journal.pone.0239051>
- 296 Belkaid, Y., Hand, T.W., 2014. Role of the microbiota in immunity and inflammation. *Cell*  
297 157, 121–41. <https://doi.org/10.1016/j.cell.2014.03.011>
- 298 Cherif, A., Rezgui, W., Raddadi, N., Daffonchio, D., Boudabous, A., 2008. Characterization  
299 and partial purification of entomocin 110, a newly identified bacteriocin from  
300 *Bacillus thuringiensis* subsp. *Entomocidus* HD110. *Microbiological Research* 163,  
301 684–692. <https://doi.org/10.1016/j.micres.2006.10.005>
- 302 Chudnovskiy, A., Mortha, A., Kana, V., Kennard, A., Ramirez, J.D., Rahman, A., Remark, R.,  
303 Mogno, I., Ng, R., Gnjatic, S., Amir, E. ad D., Solovyov, A., Greenbaum, B., Clemente,  
304 J., Faith, J., Belkaid, Y., Grigg, M.E., Merad, M., 2016. Host-Protozoan Interactions  
305 Protect from Mucosal Infections through Activation of the Inflammasome. *Cell* 167,  
306 444-456.e14. <https://doi.org/10.1016/j.cell.2016.08.076>
- 307 Dillon, R.J., Dillon, V.M., 2003. THE GUT BACTERIA OF INSECTS: Nonpathogenic Interactions.  
308 *Annual Review of Entomology* 49, 71–92.  
309 <https://doi.org/10.1146/annurev.ento.49.061802.123416>

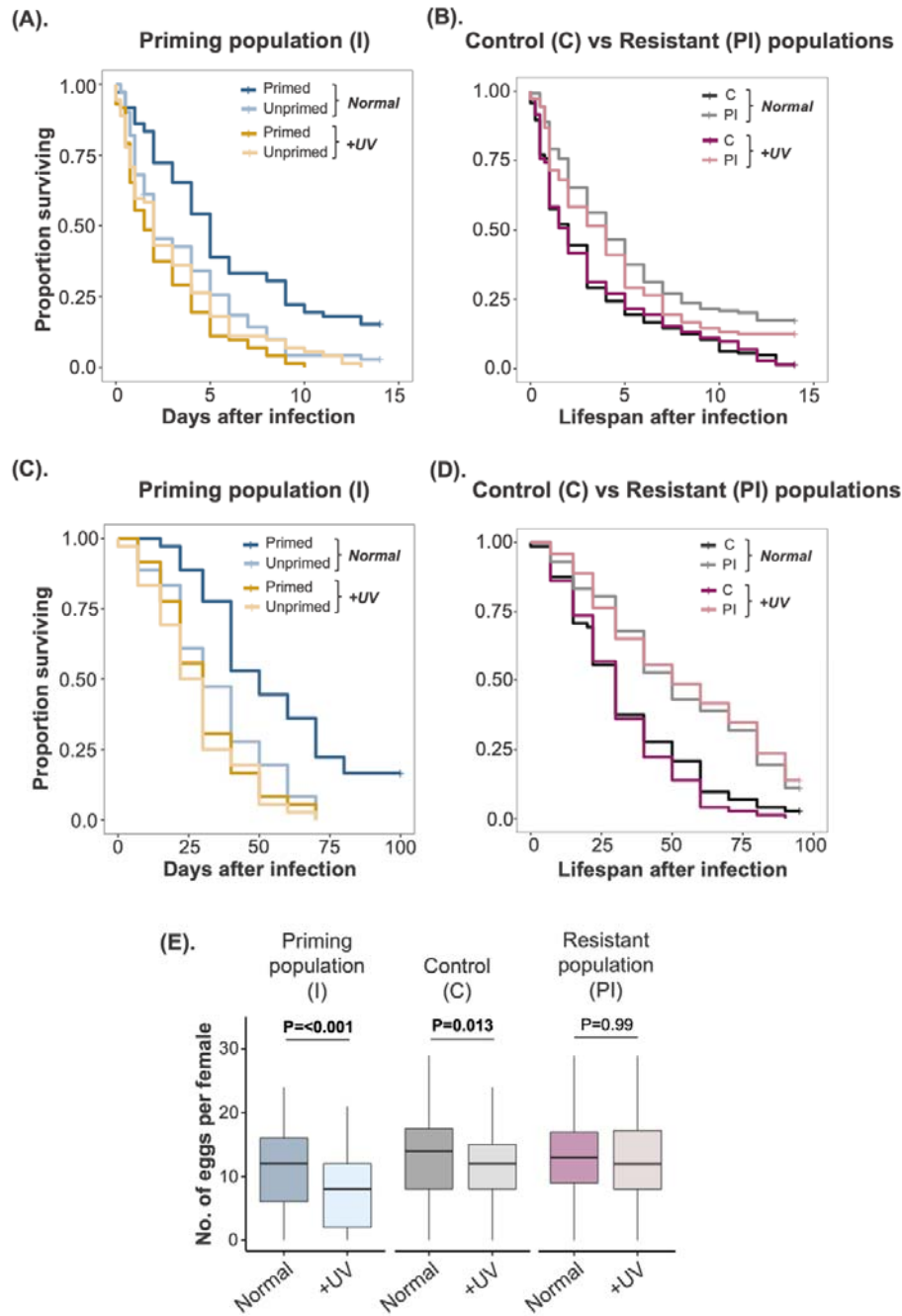
- 310 Engel, P., Moran, N.A., 2013. The gut microbiota of insects - diversity in structure and  
311 function. *FEMS Microbiology Reviews* 37, 699–735. [https://doi.org/10.1111/1574-](https://doi.org/10.1111/1574-6976.12025)  
312 [6976.12025](https://doi.org/10.1111/1574-6976.12025)
- 313 Ferro, K., Peuß, R., Yang, W., Rosenstiel, P., Schulenburg, H., Kurtz, J., 2019. Experimental  
314 evolution of immunological specificity. *Proc Natl Acad Sci USA* 116, 20598–20604.  
315 <https://doi.org/10.1073/pnas.1904828116>
- 316 Freitak, D., Wheat, C.W., Heckel, D.G., Vogel, H., 2007. Immune system responses and  
317 fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*.  
318 *BMC Biology* 5, 56. <https://doi.org/10.1186/1741-7007-5-56>
- 319 Futo, M., Armitage, S.A., Kurtz, J., 2015. Microbiota plays a role in oral immune priming in  
320 *Tribolium castaneum*. <https://doi.org/10.3389/fmicb.2015.01383>
- 321 Gould, A.L., Zhang, V., Lamberti, L., Jones, E.W., Obadia, B., Korasidis, N., Gavryushkin, A.,  
322 Carlson, J.M., Beerenwinkel, N., Ludington, W.B., 2018. Microbiome interactions  
323 shape host fitness. *Proceedings of the National Academy of Sciences* 115, E11951–  
324 E11960. <https://doi.org/10.1073/pnas.1809349115>
- 325 Karimi, K., Inman, M.D., Bienenstock, J., Forsythe, P., 2009. *Lactobacillus reuteri*-induced  
326 regulatory T cells protect against an allergic airway response in mice. *American*  
327 *Journal of Respiratory and Critical Care Medicine* 179, 186–193.  
328 <https://doi.org/10.1164/rccm.200806-951OC>
- 329 Khan, I., Prakash, A., Agashe, D., 2019. Pathogen susceptibility and fitness costs explain  
330 variation in immune priming across natural populations of flour beetles. *J Anim Ecol*  
331 88, 1332–1342. <https://doi.org/10.1111/1365-2656.13030>
- 332 Khan, I., Prakash, A., Agashe, D., 2017. Experimental evolution of insect immune memory  
333 versus pathogen resistance. *Proceedings. Biological sciences* 284, 20171583.  
334 <https://doi.org/10.1098/rspb.2017.1583>
- 335 Khan, I., Prakash, A., Agashe, D., 2016. Divergent immune priming responses across flour  
336 beetle life stages and populations. *Ecol Evol* 6, 7847–7855.  
337 <https://doi.org/10.1002/ece3.2532>
- 338 Kostic, A.D., Xavier, R.J., Gevers, D., 2014. The Microbiome in Inflammatory Bowel Disease:  
339 Current Status and the Future Ahead. *Gastroenterology, The Gut Microbiome in*  
340 *Health and Disease* 146, 1489–1499. <https://doi.org/10.1053/j.gastro.2014.02.009>
- 341 Kwong, W.K., Mancenido, A.L., Moran, N.A., 2013. Immune system stimulation by the native  
342 gut microbiota of honey bees. *Royal Society Open Science* 4, 170003.  
343 <https://doi.org/10.1098/rsos.170003>
- 344 Lee, Y.K., Mazmanian, S.K., 2010. Has the microbiota played a critical role in the evolution of  
345 the adaptive immune system? *Science* 330, 1768–1773.  
346 <https://doi.org/10.1126/science.1195568>
- 347 Li, S., de Mandal, S., Xu, X., Jin, F., 2020. The tripartite interaction of host immunity-bacillus  
348 thuringiensis infection-gut microbiota. *Toxins*.  
349 <https://doi.org/10.3390/toxins12080514>
- 350 Mayer, A., Mora, T., Rivoire, O., Walczak, A.M., 2016. Diversity of immune strategies  
351 explained by adaptation to pathogen statistics. *Proceedings of the National Academy*  
352 *of Sciences* 113, 8630–8635. <https://doi.org/10.1073/pnas.1600663113>
- 353 Mazmanian, S.K., Cui, H.L., Tzianabos, A.O., Kasper, D.L., 2005. An immunomodulatory  
354 molecule of symbiotic bacteria directs maturation of the host immune system. *Cell*  
355 122, 107–118. <https://doi.org/10.1016/j.cell.2005.05.007>

- 356 McLaren, M.R., Callahan, B.J., 2020. Pathogen resistance may be the principal evolutionary  
357 advantage provided by the microbiome. *Philosophical Transactions of the Royal*  
358 *Society B: Biological Sciences* 375, 20190592.  
359 <https://doi.org/10.1098/rstb.2019.0592>
- 360 Muhammad, A., Habineza, P., Ji, T., Hou, Y., Shi, Z., 2019. Intestinal Microbiota Confer  
361 Protection by Priming the Immune System of Red Palm Weevil *Rhynchophorus*  
362 *ferrugineus* Olivier (Coleoptera: Dryophthoridae). *Frontiers in Physiology* 10.  
363 <https://doi.org/10.3389/fphys.2019.01303>
- 364 Orozco-Flores, A.A., Valadez-Lira, J.A., Oppert, B., Gomez-Flores, R., Tamez-Guerra, R.,  
365 Rodríguez-Padilla, C., Tamez-Guerra, P., 2017. Regulation by gut bacteria of immune  
366 response, *Bacillus thuringiensis* susceptibility and hemolin expression in *Plodia*  
367 *interpunctella*. *Journal of Insect Physiology* 98, 275–283.  
368 <https://doi.org/10.1016/j.jinsphys.2017.01.020>
- 369 Prakash, A., Agashe, D., Khan, I., 2022. The costs and benefits of basal infection resistance vs  
370 immune priming responses in an insect. *Developmental & Comparative Immunology*  
371 126, 104261. <https://doi.org/10.1016/j.dci.2021.104261>
- 372 Seal, S., Dharmarajan, G., Khan, I., 2021. Evolution of pathogen tolerance and emerging  
373 infections: A missing experimental paradigm. *eLife* 10, e68874.  
374 <https://doi.org/10.7554/eLife.68874>
- 375 Thaïss, C.A., Zmora, N., Levy, M., Elinav, E., 2016. The microbiome and innate immunity.  
376 *Nature* 535, 65–74. <https://doi.org/10.1038/nature18847>
- 377 Therneau, T., 2015. Mixed Effects Cox Models, in: *Mixed Effects Cox Models*. CRAN  
378 repository.
- 379 Zheng, D., Liwinski, T., Elinav, E., 2020. Interaction between microbiota and immunity in  
380 health and disease. *Cell Res* 30, 492–506. [https://doi.org/10.1038/s41422-020-0332-](https://doi.org/10.1038/s41422-020-0332-7)  
381 [7](https://doi.org/10.1038/s41422-020-0332-7)
- 382

383

384 **FIGURES**

385 **Figure 1.** Effects of microbiota disruption by UV-irradiation of flour on **(A)** priming response  
386 (i.e., post-infection survival of primed vs unprimed individuals) of I beetles (with evolved  
387 priming); **(B)** basal resistance to Bt, i.e. post-infection survival of C (Control beetles) vs PI  
388 populations (with evolved higher basal resistance); **(C)** Lifespan of I beetles after priming  
389 and low dose of Bt; **(D)** Lifespan of C vs PI beetles after low dose of infection; **(E)**  
390 Reproductive fitness of naïve beetles from C, PI and I populations. In panel E, asterisks  
391 indicate significantly different groups. C= Control populations; I (or PI) = Replicate  
392 populations that evolved priming (or strong basal resistance); Normal= untreated wheat  
393 flour; +UV= UV-irradiated wheat flour; ns= not significant. Each panel represents pooled  
394 data across replicate populations (see Figs S2-4 for individual replicate populations). For  
395 panels A and B, n=24 beetles/priming and infection treatment/microbiota  
396 manipulation/replicate population; for panels C and D, n=12 females/priming and infection  
397 treatment/microbiota manipulation/replicate population; for panel E, n=39-52/microbiota  
398 manipulation/replicate population.



399

400



401 **SUPPLEMENTARY INFORMATION**

402

403 **Supplementary methods**

404

405 **I. Priming and infection protocol**

406 We used a strain of *Bacillus thuringiensis* (Bt - DSM 2046), isolated from a Mediterranean flour moth  
407 (Roth et al., 2009), as a model bacterial pathogen to prime and infect adult beetles (see Khan et al.,  
408 2017). To prime beetles, we pricked them between their head and thorax with a 0.1 mm insect pin  
409 (Fine Science Tools, CA) dipped in heat-killed bacterial slurry adjusted to  $10^{11}$  cells/100 $\mu$ l Ringer  
410 solution, prepared from freshly grown overnight Bt culture at 30°C (optical density OD<sub>600</sub> = 0.95). We  
411 used insect Ringer solution as mock priming (unprimed). The priming with heat-killed Bt cells can  
412 activate the immune response without imposing any direct cost of infection. Six days after priming,  
413 we infected both primed and unprimed individuals with a live bacterial culture adjusted to  $\sim 10^{10}$   
414 cells in 75  $\mu$ l insect Ringer solution. To measure lifespan after mounting a priming response, we used  
415 a milder dose of live bacterial culture adjusted to  $\sim 10^6$  cells in 75  $\mu$ l insect Ringer solution which does  
416 not cause any immediate mortality (within 7 days).

417

418 **II. Experimental evolution protocol (see Khan et al., 2017 for detailed protocol)**

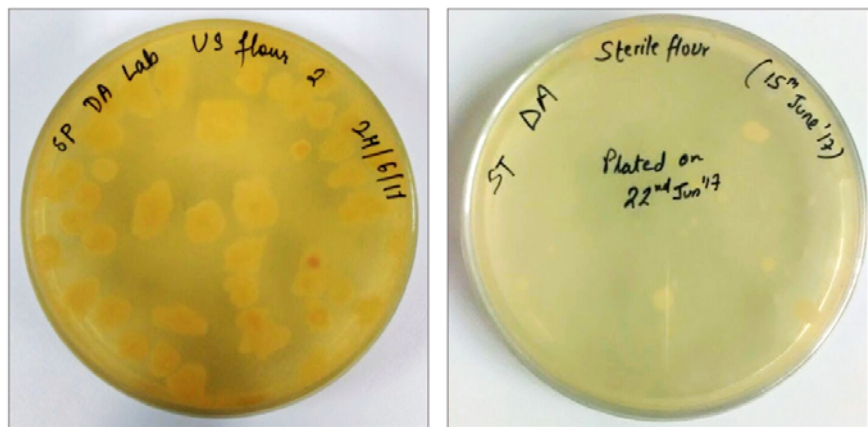
419 Briefly, at every generation of experimental evolution, we primed 10-day-old virgin PI adults from  
420 each replicate population with heat-killed Bt, as described above. Simultaneously, we also mock-  
421 primed 10-day-old virgin adult C and I beetles with sterile insect Ringer solution. After six days, we  
422 challenged individuals from I and PI regimes with high dose of live Bt infection as described above,  
423 whereas C beetles were just pricked with sterile insect ringer solution (mock challenge). Hence, we  
424 had two infection regimes where populations were challenged with a high dose of Bt infection, with  
425 (PI populations) or without (I Populations) the opportunity of priming; and a control regime (C  
426 populations) where beetles were never exposed to Bt antigen. Following the priming and infection  
427 treatments, we combined 60 pairs of surviving males and females from each replicate population  
428 and allowed females to oviposit for 5 days to initiate the next generation. We repeated the same  
429 protocol for 15 generations, and then allowed two generations of relaxed selection (i.e., no  
430 pathogen exposure) before we commenced the assays described in this study.

431

432 **Supplementary figures**

433 **Figure S1.** Representative LB agar plates to show the depletion of culturable microbiota after UV-  
434 irradiation of wheat flour

435

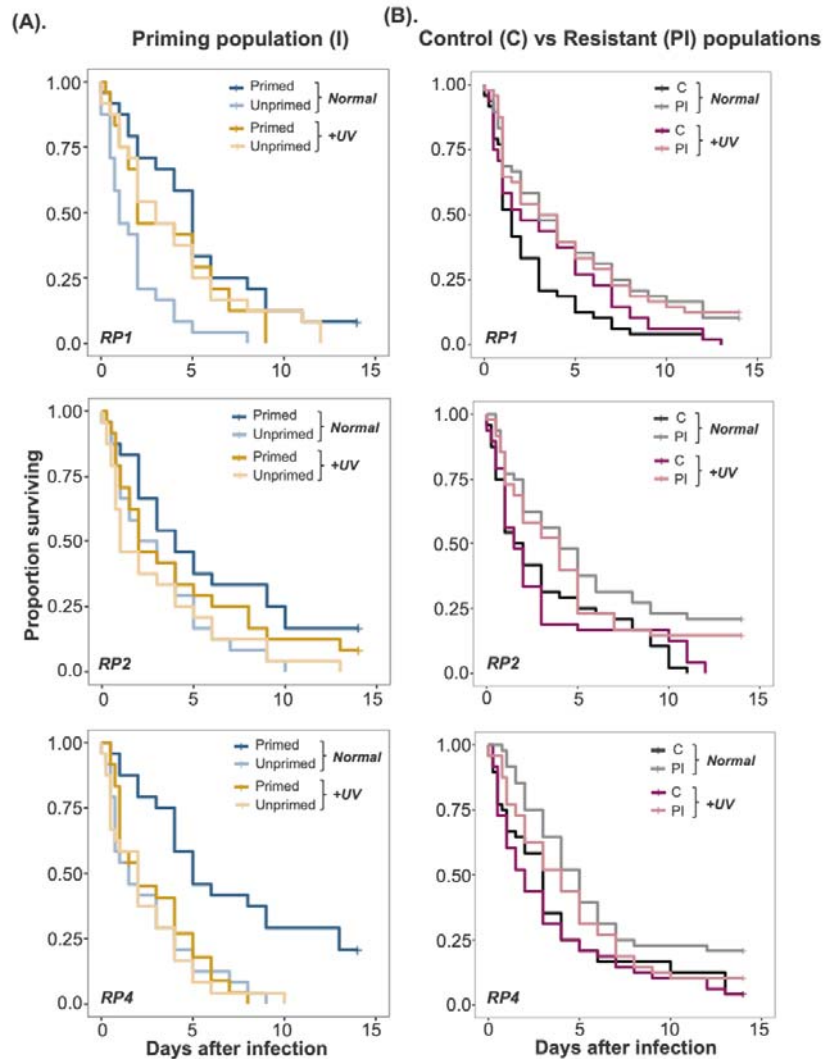


436 Normal wheat flour

437 UV-irradiated flour

438 **Figure S2.** Effects of microbiota disruption by UV-irradiation on (A) priming response (measured as  
439 the difference between post-infection survival of primed vs unprimed individuals) of each beetle  
440 replicate populations that evolved priming (I1, I2, I4 replicate populations) (n=24 beetles/priming  
441 and infection treatment/microbiota manipulation/replicate population); (B) Post-infection survival  
442 of beetles from each replicate control population (C1, 2 & 4 populations) vs beetles that evolved  
443 strong basal resistance (PI1, 2, 4 populations) (n=24 beetles/infection treatment/ microbiota  
444 manipulation/replicate populations). C1 and PI1, C2 and PI2, and C3 and PI3 were handled together  
445 during the experiment. Normal= Normal wheat flour; +UV= UV-irradiated wheat flour.

446

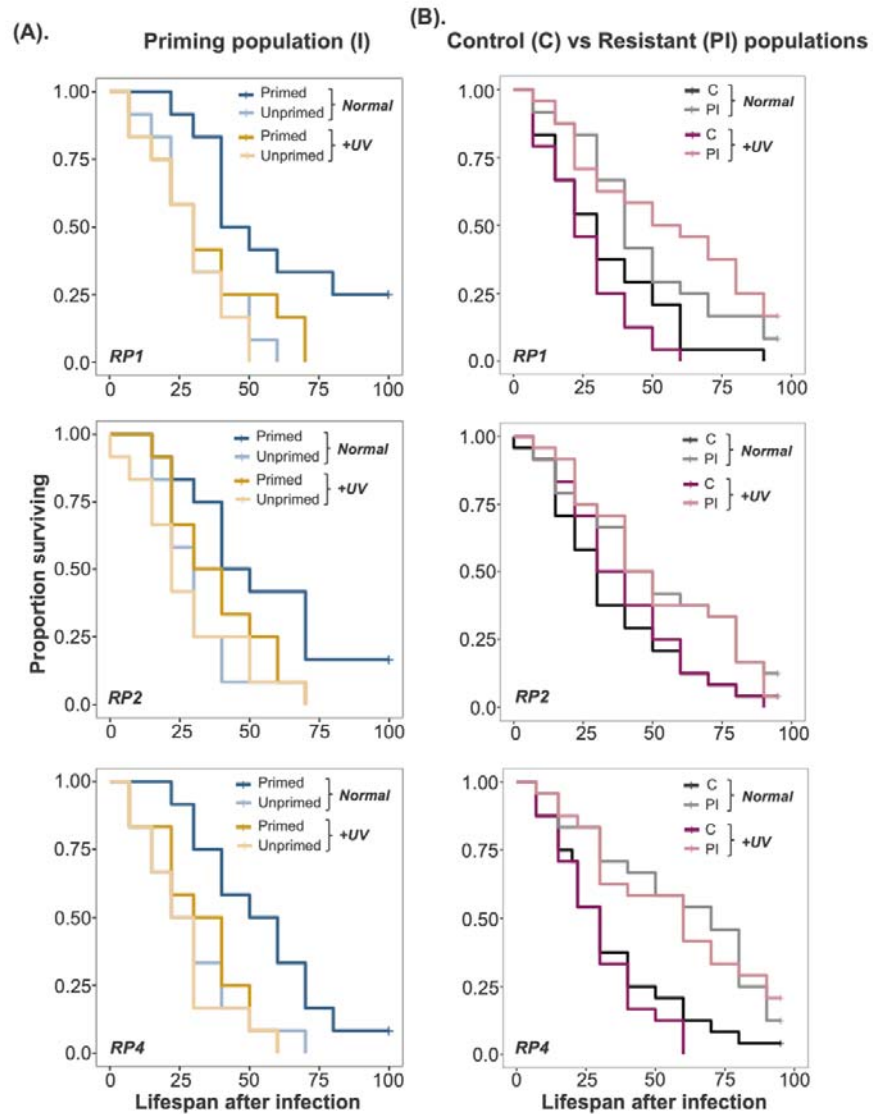


447

448

449 **Figure S3:** Effects of microbiota disruption by UV-irradiation on (A) lifespan after priming response  
450 (measured as the difference between post-infection lifespan of primed vs unprimed individuals)  
451 of each beetle replicate populations that evolved priming (I1, I2, I4 populations) (n=24 beetles/priming  
452 and infection treatment/ microbiota manipulation/replicate population); (B) post-infection lifespan  
453 of each replicate populations of control beetles (C1, 2 & 4 populations) vs beetles that evolved  
454 strong basal resistance (PI1, 2, 4 populations) (n=24 beetles/infection treatment/ microbiota  
455 manipulation/replicate populations). C1 and PI1, C2 and PI2, and C3 and PI3 were handled together  
456 during the experiment. Normal= Normal wheat flour; +UV= UV-irradiated wheat flour.

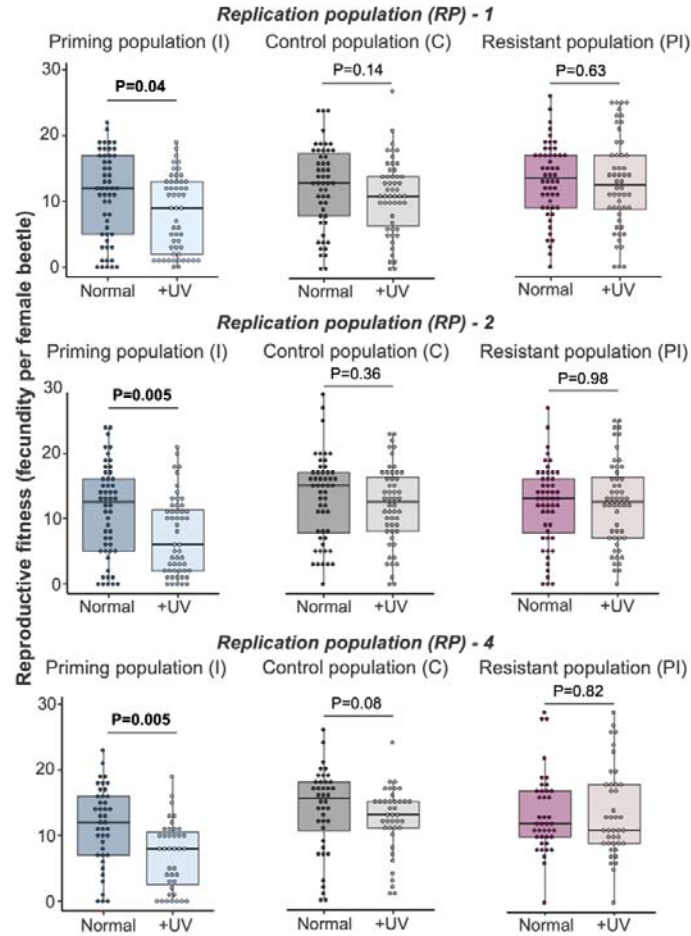
457



458

459 **Figure S4:** Effects of microbiota disruption by UV-irradiation on reproductive fitness of naïve beetles  
460 from each replicate populations of C, PI, and I populations (n=39-52 females/treatment/replicate  
461 population). Normal= Normal wheat flour; +UV= UV-irradiated wheat flour.

462



463

464 **Supplementary tables**

465

466 **Table S1.** Summary of a mixed effects Cox model analysis to estimate the changes in priming  
 467 response of I (experimentally evolved priming) population (**A**) as a function of microbiota disruption.  
 468 We specified the model as: Priming response ~ Priming treatment (P) x microbiota manipulation (M)  
 469 + (1|replicate population (RP)), with 'P' and 'M' as fixed effects, and RP as a random effect; (**B**)  
 470 separately across microbiota manipulations (i.e., normal vs UV-irradiated flour). For each microbiota  
 471 manipulation type, we specified the model as: Priming response ~ Priming treatment (P) +  
 472 1|replicate population (RP)], with 'P' as a fixed effect, and RP as a random effect; (**C**) Summary of a  
 473 Cox proportional hazard analysis for priming response in each of the replicate I populations after  
 474 disruption of dietary microbes.

475

A. Selection regime	Source	loglik	chiSq	df	p
I population	Priming treatment (P)	-1307.1	23.72	1	<0.001
	Microbiota manipulation (M)	-1306.1	1.965	1	0.16
	P x M	-1300.3	11.61	1	<0.001
	<i>Random effects</i>	<i>Std dev</i>			
	<i>Replicate population (RP)</i>	0.0053			

476

B. Selection regime	Resource	Source	loglik	chiSq	df	p
I population	Normal wheat	P	-542.05	31.004	1	<0.001
		<i>Random effects</i>	<i>Std dev</i>			
		<i>RP</i>	0.12			
	UV-irradiated flour	P	-560.02	1.7872	1	0.18
<i>Random effects</i>		<i>Std dev</i>				
	<i>RP</i>	0.10				

477

C. Replicate population	Resource	df	Chi. Sq.	p
1	Normal wheat	1	12.547	<b>0.0004</b>
	UV-irradiated flour	1	0.1684	0.68
2	Normal wheat	1	4.1869	<b>0.04</b>
	UV-irradiated flour	1	1.5243	0.21
4	Normal wheat	1	11.905	<b>0.0006</b>
	UV-irradiated flour	1	0.445	0.50

478

479

480 **Table S2.** Summary of a mixed effects Cox model analysis on survival data of beetles from control (C)  
 481 vs resistant (PI) populations as a function of microbiota manipulation. We specified the model as:  
 482 Post-infection survival ~ Selection regime (SR) x microbiota manipulation (M) + (1|Replicate  
 483 population (RP)), with 'SR' and 'M' as fixed effects, and RP as a random effect.

484

A. Comparison	Trait	loglik	chiSq	df	p
---------------	-------	--------	-------	----	---

C vs PI regime	Selection regime (SR)	-2934.2	36.32	1	<b>&lt;0.001</b>
	Microbiota manipulation (M)	-2933.9	0.758	1	0.38
	SR x M	-2933.1	1.651	1	0.19
	<i>Random effects</i>	<i>Std dev</i>			
	<i>Replicate population</i>	<i>0.004</i>			

485

486

487 **Table S3.** Summary of a mixed effects Cox model analysis to estimate the changes in lifespan of I  
 488 beetles after priming response (A) as a function microbiota disruption. We specified the model as:  
 489 Lifespan ~ Priming treatment (P) x Microbiota manipulation (M) + (1|Replicate population (RP)),  
 490 with 'P' and 'M' as fixed effects, and RP as a random effect; (B) separately across microbiota  
 491 manipulations (i.e., normal vs UV-irradiated flour). For each microbiota manipulation type, we  
 492 specified the model as: Lifespan ~ Priming treatment (P) + 1|Replicate population (RP)], with 'P' as a  
 493 fixed effect, and RP as a random effect; (C) Summary of a Cox proportional hazard analysis for  
 494 lifespan after priming response in each of the replicate I populations after disruption of dietary  
 495 microbes.

496

A.	Regime	Source	loglik	chiSq	df	p
	I population	Priming treatment (P)	-558.28	20.40	1	<b>&lt;0.001</b>
		Microbiota manipulation (M)	-554.30	7.953	1	<b>0.004</b>
		P x M	-551.68	5.237	1	<b>0.02</b>
		<i>Random effects</i>	<i>Std dev</i>			
		<i>Replicate population (RP)</i>	<i>0.0043</i>			

497

B.	Selection regime	Resource	Source	loglik	chiSq	df	p
	I population	Normal wheat	P	-222.52	19.755	1	<b>&lt;0.001</b>
			<i>Random effects</i>	<i>Std dev</i>			
			<i>RP</i>	<i>0.009</i>			
		UV-irradiated flour	P	-237.55	2.8548	1	0.09
			<i>Random effects</i>	<i>Std dev</i>			
			<i>RP</i>	<i>0.009</i>			

498

C.	Replicate population	Resource	Df	Chi. Sq.	p
	1	Normal wheat	1	5.1395	<b>0.023</b>
		UV-irradiated flour	1	0.7086	0.39
	2	Normal wheat	1	4.1535	<b>0.041</b>
		UV-irradiated flour	1	0.7907	0.37
	4	Normal wheat	1	4.9412	<b>0.02</b>
		UV-irradiated flour	1	0.3982	0.52

499

500

501 **Table S4.** Summary of a mixed effects Cox model analysis on lifespan data of beetles from control (C)  
 502 vs resistant (PI) populations after a mild infection dose, as a function of microbiota manipulation.

503 We specified the model as: Lifespan ~ Selection regime (SR) x Microbiota manipulation (M) +  
 504 (1|Replicate population (RP)), with ‘SR’ and ‘M’ as fixed effects, and RP as a random effect.

505

Comparison	Source	loglik	chiSq	df	p
C vs PI population	Selection regime (SR)	-1281.9	44.83	1	<0.001
	Microbiota manipulation (M)	-1281.8	0.185	1	0.66
	SR x M	-1281.2	1.206	1	0.27
<i>Random effects</i>		<i>Std dev.</i>			
<i>Replicate population</i>		0.02			

506

507

508 **Table S5: A.** Summary of a generalized linear mixed effects model best fitted to Quasi-Poisson  
 509 distribution for changes in the reproductive output across selection regimes (C, I and PI) as a  
 510 function of microbiota manipulation (i.e., Normal vs UV-irradiated wheat). We specified the model  
 511 as: Reproductive output ~ Selection regime x Microbiota manipulation + (1|Replicate population),  
 512 with ‘selection regime’ and ‘Microbiota manipulation’ as fixed effects and ‘replicate population’ a  
 513 random effect.

514

A.	Source	chiSq	df	p
	Selection regime (SR)	167.406	2	<0.001
	Microbiota manipulation (M)	56.454	1	<0.001
	SR x M	55.698	1	<0.001
<i>Random effects</i>		<i>Std error</i>		
<i>Replicate population</i>		0.01		

515

516 **Supplementary references**

517

518 Khan, I., Prakash, A., Agashe, D., 2017. Experimental evolution of insect immune memory versus  
 519 pathogen resistance. Proc. R. Soc. B. 284, 20171583.

520 <https://doi.org/10.1098/rspb.2017.1583>

521 Roth, O., Sadd, B.M., Schmid-Hempel, P., Kurtz, J., 2009. Strain-specific priming of resistance in the  
 522 red flour beetle, *Tribolium castaneum*. Proceedings. Biological sciences / The Royal Society

523 276, 145–51. <https://doi.org/10.1098/rspb.2008.1157>

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