

1 **BASAL RESISTANCE AGAINST A PATHOGEN IS MORE BENEFICIAL THAN IMMUNE**
2 **PRIMING RESPONSES IN FLOUR BEETLES**

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17 **KEYWORDS**

18 *Bacillus thuringiensis*, evolutionary costs, immune priming, pathogen selection, *Tribolium castaneum*

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24 **ABSTRACT**

25 Insects exhibit various forms of immune responses, including basal resistance to pathogens and a form of
26 immune memory (“priming”) that can act within or across generations. The evolutionary drivers of such
27 diverse immune functions remain poorly understood. Previously, we found that in the beetle *Tribolium*
28 *castaneum*, both resistance and priming evolved as mutually exclusive strategies against the pathogen
29 *Bacillus thuringiensis*. However, since evolved resistance improved survival far more than priming, the
30 evolution of priming in some populations was puzzling. Was resistance more costly in these populations,
31 or did priming provide added benefits? To test this, we revisited our evolved beetles and analyzed the costs
32 and benefits of evolved priming vs. resistance. Surprisingly, resistant beetles increased reproduction after
33 infection, with no measurable costs. In contrast, mounting a priming response reduced offspring early
34 survival, development rate and reproduction. Even added trans-generational survival benefits of evolved
35 priming could not tilt the balance in favor of priming. Hence, resistance is consistently more beneficial than
36 priming; and the evolution and persistence of costly priming rather than resistance remains a mystery.
37 Nevertheless, our work provides the first detailed comparison of the complex fitness consequences of
38 distinct insect immune strategies, opening new questions about their evolutionary dynamics.

39

40 INTRODUCTION

41 Until recently, it was assumed that insect immunity is nonspecific and cannot build immune memory against
42 previously encountered pathogens, since insects lack the immune cells responsible for adaptive immunity
43 in vertebrates (Cooper & Eleftherianos 2017). Now, growing evidence contradicts this traditional view:
44 priming with a sub-lethal exposure to a pathogen protects against a subsequent exposure to the same
45 pathogen. This survival benefit of priming is observed both in later life stages of primed individuals
46 (“within-generation immune priming”; henceforth WGIP), and in their offspring (“trans-generational
47 immune priming”; henceforth TGIP), in a range of insect species (reviewed in Milutinović et al. 2016)
48 including Dipterans (Pham et al. 2007; Ramirez et al. 2015, 2017), Coleopterans (Roth et al. 2009, 2010;
49 Khan et al. 2016), Lepidopterans, and Hymenopterans (Sadd & Schmid-Hempel 2006). Theoretical studies
50 also highlight the importance of priming in reducing infection prevalence and regulating population size,
51 stability and age structure during infection (Tate & Rudolf 2012; Best et al. 2013). Thus, it appears that
52 under pathogen pressure, priming should be selectively favored. Recently, we directly demonstrated this
53 adaptive value of WGIP and showed that it is a distinct immune strategy that can evolve independently of
54 basal pathogen resistance in the flour beetle *Tribolium castaneum* (Khan et al. 2017a). However, a striking
55 result of this study was that although the net survival benefit of evolved resistance was higher than that of
56 priming (80% vs. 50% survival after infection; Khan *et al.* 2017a), resistance against pathogens did not
57 evolve in all populations (**Fig. 1**).

58 One reason for this observation could be that resistance imposes higher costs than priming. For instance,
59 several studies suggest that resistance is associated with overexpression of fast acting immune responses
60 that impose large physiological costs (e.g. Sadd and Siva-Jothy 2006; Khan et al. 2017b). A general
61 mathematical model predicts that such costs of constitutively expressed basal resistance can be outweighed
62 by its benefit only under frequent lethal pathogenic infections, maximising the population’s growth rate
63 (Mayer et al. 2016). However, the cost of resistance may be larger when pathogens are encountered
64 infrequently. This is perhaps one reason why our beetle populations infected with a single large dose of
65 infection every generation evolved priming, whereas resistance could evolve only in populations that were
66 exposed repeatedly to the pathogen (primary exposure with heat-killed bacteria followed by live bacterial
67 infection) (Khan et al. 2017a). However, few studies have actually measured the fitness consequences of
68 evolved resistance, and these were equivocal: while some found significant costs (Ma et al. 2012; Ye et al.
69 2009), several others did not (Faria et al. 2015; Gupta et al. 2016). Costs of pathogen resistance may also
70 manifest as widespread tradeoffs with other life-history parameters (Reviewed in Sheldon & Verhulst 1996;
71 Lochmiller & Deerenberg 2000; Norris & Evans 2000; Rolff & Siva-Jothy 2003). In contrast, the impact
72 of immune priming on multiple various fitness parameters has only recently been tested: primed mosquitoes

73 (Contreras-Garduño *et al.* 2014), tobacco hornworms (Trauer & Hilker 2013) and flour beetles (Khan *et al.*
74 2019) show reduced fecundity, and primed mealworm beetle mothers produced progeny that develop
75 slowly (Zanchi *et al.* 2011) and have reduced competitive ability (Koella & Boete 2016). Although these
76 experiments tested for correlations between immune priming and fitness related traits, the direct costs of
77 evolved priming in response to pathogen pressure have not been measured. Hence, it remains unclear
78 whether a larger cost of evolved resistance could explain the evolution of priming under infrequent
79 pathogen exposure.

80 A second possibility of why resistance did not evolve in all populations is that evolved priming may confer
81 added survival benefits that manifest across generations (i.e. TGIP), enhancing its net fitness impacts and
82 facilitating its spread in populations. Although no direct experiments have tested whether such trans-
83 generational benefits evolve simultaneously with WGIP, theory offers some important clues. A model by
84 Tidbury and coworkers suggests that since TGIP has a lower ability to reduce infection prevalence,
85 selection should favor WGIP (Tidbury *et al.* 2012). On the other hand, Tate and Rudolf suggested that the
86 stage-specific effects of infection are important: TGIP is more beneficial when an infection affects juvenile
87 stages, whereas WGIP is more effective if adults incur higher infection costs than larvae (Tate & Rudolf
88 2012). The model also predicts that selection can strongly favor both WGIP and TGIP when the pathogen
89 affects larvae and adults equally (Tate & Rudolf 2012). Our previous experimental results suggest that this
90 hypothesis is relevant at least for flour beetles: both WGIP and TGIP were equally beneficial in beetles
91 infected with the general insect pathogen *Bacillus thuringiensis* (Bt), which imposed similar infection costs
92 in both life stages (Khan *et al.* 2016). Although these results represent an interesting correlation, the causal
93 link between the pathogen's impact on the host and its role in determining relative investment in different
94 priming responses is not yet confirmed.

95 Thus, our understanding of the selective pressures and fitness effects that directly impact the evolution of
96 diverse priming responses vs. basal resistance is incomplete. To fill these gaps, we used previously
97 described, evolved replicate populations of the red flour beetle *T. castaneum* that were infected in each
98 generation with Bt, either with or without the opportunity of priming with heat-killed Bt cells (see C, P, PI,
99 I populations; Khan *et al.* 2017a). Previously, we had analyzed evolved immune responses of these
100 populations after 11 generations of evolution (Khan *et al.* 2017a). Here, we re-tested the same populations
101 after a further 3 generations of evolution. We first confirmed that populations (I) where unprimed beetles
102 were injected directly with a high dose of live Bt still retained a strong WGIP response, whereas beetle
103 populations (PI) that were both primed and infected every generation showed evolved basal resistance.
104 Subsequently, to disentangle their respective fitness costs and adaptive benefits, we compared the fitness
105 effects of evolved immune strategies for critical fitness related traits of such as offspring development, early

106 reproduction and early survival. We also measured the impact of evolved immune functions on beetle
107 lifespan under starvation and normal conditions. Although these traits not directly relevant for our specific
108 selection lines (since the imposed generation time was much shorter than the beetles' expected lifespan),
109 these traits are known predictors of body condition in the wild, and often trade off with immunity (Hoang
110 2001; Jacot *et al.* 2004). Astonishingly, despite the higher survival benefits, resistance did not impose any
111 costs, contradicting our expectation that it would show strong fitness trade-offs. Instead, we found that the
112 maintenance and deployment of priming was costly, reducing multiple fitness parameters of I beetles. We
113 also found that WGIP in I populations was associated with evolved trans-generational priming (TGIP); but
114 the combined benefit of evolved priming was still lower than that of increased resistance. We were thus
115 unable to explain why priming was favored in I populations. Nevertheless, our present work provides the
116 first systematic analysis of the evolutionary cost and benefit structure influencing parallelly evolved,
117 divergent insect immune responses.

118 **MATERIALS AND METHODS**

119 **Experimental evolution**

120 We used laboratory-adapted populations of *T. castaneum* to initiate four distinct selection regimes: control
121 (C; untreated), priming only (P), primed and infected (PI) and infection only (I), each with 4 independent
122 replicate populations (Khan et al. 2017a). In the present study, for logistical reasons, we only analyzed three
123 replicates from each selection regime (C 1, 2 & 4; P 1, 2 & 4; PI 1, 2 & 4; I 1, 2 & 4). On different days,
124 we handled only one replicate population from each selection regime together – e.g. C1, P1, PI1, I1 or C2,
125 P2, PI2, I2 or C4, P4, PI4, I4). The detailed protocol for the experimental evolution is described in Khan et
126 al. (2017a). Briefly, every generation, we first primed 10-day-old virgin P and PI adults from each replicate
127 population with heat-killed bacterial slurry (see supplementary information for priming protocol).
128 Simultaneously, we also pricked virgin C and I beetles with sterile insect Ringer solution (mock priming).
129 Six days later, we challenged individuals from I and PI regimes with live Bt, whereas C and P beetles were
130 pricked with sterile insect ringer solution (mock challenge) (see supplementary information for infection
131 protocol). We thus created two infection regimes where populations were challenged with a high dose of
132 infection, with (PI) or without (I) the opportunity of priming; and two control regimes where beetles were
133 either pricked with Ringer (C) or heat-killed bacteria (P), but never exposed to live infection. Following the
134 priming and infection treatments, we randomly isolated 60 pairs of live virgin males and females from each
135 replicate population and provided them with 300g wheat to mate and oviposit for 5 days to initiate the next
136 generation. After 14 generations of continuous selection, we isolated a subset of individuals from each
137 replicate population to maintain them under relaxed conditions for two generations without priming or

138 infection (unhandled). The relaxed selection is expected to generate standardized experimental beetles with
139 minimum non-genetic parental effects.

140 **Joint assays of evolved priming and resistance, and their impacts on reproduction**

141 We designed our experimental framework to compare survival benefits and reproductive effects of evolved
142 priming vs. resistance (see **Fig. 2** for experimental design). Besides measuring survival after priming and
143 infection, we measured female reproductive output both before and after infection. This allowed us to
144 estimate the direct impact of experimental evolution with pathogens vs. the actual impact of inducing each
145 type of immune response. Simultaneously, we also tested for the evolution of TGIP, to compare relative
146 survival and reproductive effects of different priming responses.

147 To this end, we first collected pupae from each standardized population and isolated them into 96-well
148 microplate wells with ~0.25g wheat flour, for eclosion. We randomly assigned 10-day old virgin males and
149 virgin females from each population to one of the following primary exposure treatments: (a) naïve (or
150 unhandled) (b) primed (injected with heat-killed Bt) and (c) unprimed (i.e. injected with Ringer). After 24
151 hours of primary exposure, we formed mating pairs using males and females from each population and
152 treatment combination in 1.5ml micro-centrifuge tubes with 1g of wheat flour (n = 12 mating-pairs per
153 replicate population per selection regime). We allowed them to mate for 48 hours and then isolated the 12-
154 day-old females to oviposit for another 48 hours in 5 g whole wheat flour (oviposition plate), whereas males
155 were returned to 96-well microplates. After oviposition, we also returned the 14-day-old females to 96-well
156 microplates. Two days later (total six days after primary exposure), we infected males and females with
157 live Bt. We recorded male survival every 6 hours for 1 day and then every 24 hours for 7 days post-infection
158 (same as the selection window during experimental evolution; Khan et al 2017a). We tracked female
159 survival similarly, except that a day later, we again allowed 48-hour oviposition to estimate the impact of
160 infection and induction of any priming responses on reproduction. Here, we note that since bacterial
161 infection imposed significant mortality across regimes, the replicate size for our fitness assays was lower
162 than expected. Although more beetles were alive in PI regime during the experimental window of
163 reproductive assay, we did not find any significant difference in proportion of live beetles that reproduced
164 and assayed across different treatments and selection regime (**Table S1**). We also conducted mock
165 challenge for a subset of unprimed beetles as a procedural control for survival assay, but not for reproductive
166 output. We did not find any mortality in uninfected beetles within the experimental window of 7 days.

167 We allowed eggs laid by naïve, unprimed and primed females (both before and after infection) to develop
168 for 21 days at 34°C and counted the total number of progeny (mostly pupae). We retained the offspring
169 from the first round of oviposition (without infection). At this time, most offspring were pupae, and the few

170 adults we observed had pale body coloration indicating that they were not sexually mature and hence,
171 unlikely to be mated (Sokoloff 1977). We isolated these pupae and adults in 96-well plates with ~0.2g flour,
172 to obtain virgin beetles for future assays to measure trans-generational priming and offspring reproduction.
173 We only included offspring from mothers that produced more than 8 female and 4 male offspring (n= 8-10
174 mothers/ priming / replicate population/ selection regime), enabling us to sample enough beetles to test for
175 a correlation between offspring post-infection survival (a proxy of trans-generational priming) and
176 reproduction of each parental pair, as described below.

177 After 10 days, we allowed a subset of female offspring from each parental pair (n=4 offspring/ parental
178 pair/ replicate population/ selection regime) to mate with 10 day old virgin males from standard laboratory
179 stock population into a single mating pair for 48 hours and then allowed to oviposit as described before.
180 This procedure enabled us to measure the impact of parental priming on offspring reproductive output
181 across populations. On day 16, we infected females and then again assayed their reproductive output as
182 described above. On the same day, we also infected the remaining 16 day old virgin male and female
183 offspring from each parental pair with live Bt (n= 4 offspring/ sex/ parental pair) and noted their survival
184 every 6 hours for 2 days and then every 24 hours until all of them were dead. This experimental design not
185 only allowed us to jointly estimate the survival and reproductive effects of WGIP vs. TGIP for each parental
186 pair, but also analyze the impact of each immune response relative to evolved resistance. We did not find
187 any mortality in sham infected offspring within the experimental window.

188 We calculated the survival benefit of within-generation priming as the estimated hazard ratio of unprimed
189 infected versus primed infected groups, using Cox proportional hazard survival analysis conducted
190 separately for males and females from each standardized replicate population (with priming treatment as a
191 fixed factor). We noted individuals that were still alive at the end of the survival experiment as censored
192 values. A hazard ratio significantly greater than one indicates higher risk of mortality in the unprimed group
193 relative to primed individuals; hence, a significant survival benefit of within-generation priming.
194 Separately, we also estimated the hazard ratio of naïve infected beetles from P, PI or I regime versus naïve
195 infected C beetles to quantify evolved resistance. A hazard ratio significantly lesser than one indicates lower
196 risk of mortality, or increased resistance relative to C beetles.

197 To measure TGIP, we recorded survival of 4 male and 4 female offspring from each parental mating pair
198 assayed earlier for within-generation priming. We first calculated their mean lifespan as the unit of analysis
199 and then compared group means using a mixed model ANOVA with selection regime, parental priming
200 status and offspring sex as fixed factors across replicate populations. We noted that residuals of mean
201 lifespan data were not normally distributed (verified with Shapiro-Wilk tests). Therefore, we first
202 transformed the data into their square root values that fit a normal distribution. Since we noted a significant

203 main effect of replicate population identity, we then separately analyzed selection regimes that were
204 handled together using a 3-way ANOVA with selection regime, parental priming status and offspring sex
205 as fixed factors. We tested for pairwise differences between selection regimes and treatments after
206 correcting for multiple comparisons using Tukey's HSD.

207 To compare the relative survival benefits of TGIP versus WGIP, we also analyzed group mean male and
208 female offspring survival data using Cox proportional survival analysis to calculate the estimated hazard
209 ratio of offspring from unprimed parents versus primed parents. Subsequently, we used non-parametric
210 Wilcoxon Rank Sum tests compare hazard ratios from TGIP versus WGIP for each population.

211 We noted that the residuals of pre-infection reproductive output data of both parents and offspring were
212 non-normally distributed, and could not be transformed to a normal distribution. We therefore used non-
213 parametric Wilcoxon Rank Sum tests to analyze the impact of selection regime and priming treatment (for
214 replicate populations of C, P, PI and I that were handled together). We also used Wilcoxon tests to analyze
215 the impact of bacterial infection on the reproductive output of parents and offspring, separately for each
216 replicate population across selection regimes and treatments. Since residuals of reproductive output data
217 after infection were normally distributed, we analyzed these data using a 3-way ANOVA with selection
218 regime and treatment as fixed factors crossed with replicate populations, providing an overall estimate of
219 each effect. Further, to disentangle the effects of each type of evolved immune response (TGIP, WGIP and
220 resistance), we compared reproductive data from each selection regime separately with that of control
221 beetles. We used Tukey's HSD to test for pairwise differences between selection regimes and treatments,
222 as described above.

223 **Quantifying development and survival under starvation and with food, in evolved lines**

224 In separate experiments, we measured the direct impacts of evolved priming responses and resistance on
225 other fitness components of naïve beetles.

226 (1) Impact on lifespan under starvation and with food: We first isolated 10 day old naïve virgin males and
227 females from each population in 96-well microplate wells without food ($n = 20$ beetles/ sex/
228 population). We noted mortality every 12 hours (10 am & 10pm ± 1 hour) for the next 12 days until all
229 beetles died. In a separate experiment, we similarly distributed naïve virgin females into 96-well
230 microplates, but with access to food. We noted their survival every 5 days for 95 days to estimate the
231 long-term survival costs of evolved immune responses. We did not assay males for long-term survival
232 costs due to logistical challenges. We analysed survival data under starvation and with food for each
233 replicate population and sex separately, using Cox proportional hazard test with the original selection
234 regime as a fixed factor.

235 (2) Quantifying early survival, development and viability costs in evolved lines: We next estimated the
236 impact of evolved immune responses on aspects of early survival and development. We allowed 12 day
237 old mated females from each population ($n = 60$) to oviposit in 150g of doubly sifted flour (using sieves
238 with pore size of 50μ to remove large flour particles; Diager USA) for 24 hours. We discarded the
239 females, and isolated 96 randomly chosen eggs into 96-well microplate wells with ~ 0.2 g flour. This
240 method is designed to minimize competition during larval development. After 10 days, we sifted the
241 flour from each microplate to count live larvae and measure egg hatchability. Following this, we
242 returned the live larvae to 96-well plates and provided fresh flour. In our standard stock beetle
243 populations, pupation and adult emergence begins around 3-4 weeks after oviposition. Therefore, we
244 estimated the proportion of pupae and adults after 3 and 4 weeks post-egg collection respectively, as
245 proxies for time to pupation and adult emergence. We repeated this experiment three times. We did not
246 assay P beetles due to logistical challenges. We analysed data using a 2-way ANOVA with selection
247 regime and replicate experiments as fixed factors, and tested for pairwise differences using Tukey's
248 HSD.

249

250 RESULTS

251 Our previous work demonstrated that lethal Bt infection can rapidly select for divergent immune strategies
252 in PI and I beetles, within 11 generations (Khan et al. 2017a). Populations (I regime) that were directly
253 infected with a single large dose of Bt evolved within-generation priming, whereas PI populations where
254 beetles were injected first with heat-killed and then live Bt evolved high resistance. We also found that
255 resistance provides higher survival benefits than priming, and yet I populations evolved priming instead of
256 resistance.

257 Here, we reanalyzed the same beetle populations after 14 generations of experimental evolution to directly
258 test whether higher costs of evolving resistance could explain this surprising pattern of evolved immune
259 responses. As observed after 11 generations (Khan et al. 2017a), we found evolved priming responses only
260 in males and females from I populations (~ 3 -fold increase in their survival relative to control beetles) (**Fig.**
261 **3A & S1, Table S2-S3**); whereas PI beetles had higher basal resistance (3 to 28-fold increase in the survival
262 of naïve PI beetles relative to control beetles) (**Fig. 3A, Table S4**). We also found that whereas the survival
263 of I beetles after Bt infection was still 50%, PI beetle survival had increased to $\sim 85\%$ (**Fig. S2**). Replicate
264 populations from the C or P regimes where beetles were not exposed to live infection did not evolve any
265 priming ability or higher resistance to infection.

266 **Evolved immune responses do not incur reproductive costs**

267 We first measured the impact of evolved immune responses on beetle reproduction, and found complex
268 fitness effects that varied substantially with priming type and infection. Evolved priming or resistance had
269 no impact on the reproduction of naïve unhandled beetles or uninfected beetles pricked with Ringer solution
270 or heat-killed bacteria (**Fig. 3B**, naïve treatment before infection; **Table S5**). Thus, the maintenance of
271 priming or resistance does not impose a reproductive cost. However, infection with live pathogen reduced
272 beetle reproduction in most populations, except PI beetles (with evolved resistance) where the impact of
273 infection was inconsistent across treatments and replicate populations (**Fig. 3B**, naïve treatment after
274 infection; **Table S6**). Only a few PI populations showed reduced reproductive output after infection,
275 whereas others showed no impact (**Table S6**). Overall, the average post-infection reproductive cost of
276 evolved resistance was lower than that of evolved priming (compare PI vs. I populations in **Fig 3B**, naïve
277 treatment after infection; **Table S6**).

278 Subsequently, we analyzed the impact of experimental priming (mimicking selection regimes during
279 experimental evolution) on the reproductive output of infected beetles. We expected that after infection,
280 beetles in the priming treatment would reflect reproduction of PI beetles during experimental evolution;
281 when compared to beetles from the C (control) regime, these data would inform about the impact of evolved
282 resistance on reproduction. Similarly, after infection, beetles in the unprimed treatment would mimic I
283 beetles during experimental evolution, and in comparison to C beetles, provide an estimate of the
284 reproductive cost (or benefit) of evolved WGIP. A mixed model ANOVA followed by separate
285 comparisons with control beetles (e.g. PI vs. C; I vs. C; P vs. C) revealed main effects of both priming
286 treatment and original selection regime, as well their interaction, in each case (**Table S7**). Evolved
287 resistance were beneficial for reproduction, but only in naïve or unprimed beetles (compare PI and P
288 regimes vs. C regime after infection, **Fig. 3B**). However, experimental priming also increased the
289 reproduction of C beetles, revealing that I beetles (with evolved priming) pay a relative reproductive cost
290 compared to PI and C beetles (compare primed beetles after infection, **Fig. 3B**). Overall, this suggests that
291 I lines (which evolved priming) paid a reproductive cost of their increased survival benefits after mounting
292 within-generation priming responses; but PI lines (which evolved resistance) could alleviate this
293 reproductive cost. Thus, evolved resistance is better than priming not only in terms of their survival benefit,
294 but also in terms of reproduction.

295 **Evolved priming reduces early survival and extends development time**

296 In separate experiments, we tested the direct impacts of evolved priming and resistance on other fitness
297 traits such as survival under starvation or normal condition and features of early survival such as egg
298 hatchability and total number of viable offspring at various developmental stages. We also measured the
299 proportion of pupae and adults at week 3 and 4, as proxies of development rate. An analysis of survival

300 data under starvation using Cox proportional hazard test (**Table S8**) revealed that males and females across
301 all selection regimes had similar lifespan under starvation (**Fig. S3**). Similarly, we also analyzed long-term
302 survival data of naïve females under normal condition up to 95 days from all the selection treatments. None
303 of the selection treatments had any consistent impact on long-term survival (**Fig S4, Table S9**).

304 In contrast, we found significant effects of selection regime on egg hatchability, total number of viable
305 offspring and proportion of adult offspring at week 4 (but not on the proportion of pupae at week 3) (**Fig.**
306 **4A-D**). Since we also observed significant impacts of replicate experiments, we analyzed each replicate
307 experiment separately. In all replicate experiments, we found that the number of viable offspring at week 4
308 was drastically reduced in beetles from the I regime (**Fig. 4D, Table S10**). This is perhaps due to significant
309 early mortality during egg to larval development in I beetles: while ~75% C, P and PI eggs hatched into
310 larvae, only 55% I eggs survived (**Fig. 4A, Table S10**). In addition, the proportion of adults at week 4 was
311 lowest in I regime, suggesting delayed development (**Fig. 4C, Table S10**). Overall, these results suggest
312 that maintenance of priming imposed considerable costs of reduced early survival and slower development
313 in I beetles. In contrast, evolved basal resistance did not appear to impose a substantial cost with respect to
314 these traits.

315 **Evolved within-generation priming (WGIP) is associated with trans-generational priming (TGIP)**

316 Finally, we asked whether evolved priming conferred added trans-generational benefits, increasing its
317 overall fitness impacts. To do this, we used a mixed model ANOVA (randomized across replicate
318 populations) to analyze the mean post-infection survival of offspring from beetles assayed above as a
319 function of selection regime, parental priming status and offspring sex (**Table S11**). Both selection regime
320 and parental priming status had significant impacts, but offspring sex did not affect survival. Here too, we
321 found that overall, offspring of PI beetles had the highest survival, though they did not show effects of
322 parental priming. In contrast, parental priming increased offspring survival in the I regime, suggesting that
323 TGIP benefits are solely restricted to I beetles. Since we also observed a significant impact of replicate
324 population identity, we next separately analyzed selection regimes that were handled together (**Table S12**).
325 Parental priming increased female offspring's post-infection survival in all I populations (I1, I2 & I4),
326 whereas male offspring had longer lifespan only in replication populations I1 and I2 (**Fig. 5A**). Male
327 offspring from primed I4 parents also appeared to survive longer than offspring of unprimed parents, but
328 the difference was not statistically significant ($P > 0.05$). We also tested whether the relative survival benefits
329 of TGIP were equal to that of WGIP. We used Cox proportional hazard analysis of the grouped mean
330 offspring survival data for each parental mating pair from I populations, and calculated the strength of
331 evolved TGIP as the estimated hazard ratio for offspring from unprimed vs. primed parents. We found a
332 significant TGIP response in offspring from replicate populations I1 and I2 (**Table S13**). In contrast, primed

333 and unprimed offspring from replicate population 4 had similar survival. Interestingly, the survival benefit
334 of TGIP and WGIP was also similar across replicate populations ($p > 0.05$; **Fig. S5A, Table S14**), supporting
335 the hypothesis that Bt-imposed selection favors the evolution of both types of priming to a similar extent
336 (**Fig. S5B, Table S13**).

337 As found with mothers (above), evolved priming and resistance did not consistently affect the reproductive
338 output of naïve or uninfected offspring (**Fig 5B, Table S14**), but infection generally reduced offspring
339 reproductive output in all selection regimes except PI beetles (**Table S15**). A full factorial mixed model
340 ANOVA revealed significant main effects of only selection regime, whereas priming and replicate
341 populations had no impact (**Table S16**). Offspring of PI beetles again reproduced more than other beetles,
342 regardless of their parental priming status; whereas TGIP had no impact on the reproduction of I offspring.
343 Overall, it is surprising that although multiple forms of priming jointly evolved in I populations, their
344 combined effects were still not as high as resistance, and I beetles (without priming) were still highly
345 susceptible to infection, suffering a large relative fitness loss each generation.

346 **DISCUSSION**

347 Previously, we showed that priming and resistance against *B. thuringiensis* infection evolve as mutually
348 exclusive strategies in flour beetles (Khan et al. 2017a). However, since evolved resistance conferred a
349 greater survival benefit than priming, it was puzzling why some populations evolved priming instead of
350 resistance. We had speculated that resistance might incur hidden fitness costs that we had not been able to
351 measure. Here, we revisited our beetle lines to systematically test this hypothesis. Conversely, we also
352 asked whether priming confers additional, trans-generational fitness benefits that may facilitate its fixation.
353 To our surprise, we did not find any evidence for a cost of evolved resistance: it did not impact development,
354 reproduction, or survival during starvation and normal conditions, contradicting the traditional view of
355 immunity-fitness trade-offs (Ye et al. 2009; Ma et al. 2012). Instead, our data add to the growing body of
356 work that suggest only a weak role for life-history trade-offs during the evolution of pathogen resistance
357 (Faria et al. 2015; Gupta et al. 2016). Interestingly, we also found that WGIP (within-generation immune
358 priming) was associated with the evolution of TGIP (trans-generation immune priming) in females from all
359 replicate populations, and in males from two of the three replicate populations that we tested. However, the
360 combined benefit of these two forms of priming (~50% survival after Bt infection) was still lower than that
361 conferred by increased baseline resistance to Bt (~85% survival). Hence, the peculiar patterns of the
362 evolution of various immune responses remain a mystery.

363 Most surprisingly, we found that although infection reduced reproduction in all regimes, the effect was less
364 pronounced in PI beetles (which had evolved increased resistance), and hence, evolved basal resistance was

365 also associated with a relative reproductive advantage. Interestingly, P (priming only) beetles also had
366 higher reproduction than control beetles after infection, which is counterintuitive because these beetles
367 never experienced live infection during experimental evolution. Note that this relative reproductive
368 advantage would be important during experimental evolution, since beetles reproduced for 5 days after
369 infection in each generation (see methods). How do we interpret these apparent reproductive fitness benefits
370 in PI and P beetles? First, the reduced cost of infection in these beetles might represent evolved tolerance,
371 whereby beetles do not invest in directly clearing pathogens via canonical resistance mechanisms, allowing
372 greater reproductive investment during an infection (Ayres and Schneider 2012). Second, these results
373 could reflect a trade-off between early vs. late reproduction. In other words, increased reproduction might
374 represent terminal investment in P and PI populations, whereas C and I populations instead suppress
375 immediate reproduction after infection to maintain survival and somatic maintenance later in life (Luu and
376 Tate 2017). Although we could not test these hypotheses here, our results suggest that divergent immune
377 responses can have important consequences for reproductive success, and deserve further attention.

378 Our results also contradict our prior hypothesis that at a low pathogen frequency (experienced by I beetles),
379 priming may be more favorable than resistance due to its low maintenance costs (Khan et al. 2017a).
380 Instead, we found that overall maintenance of priming responses is costly. Although evolved priming did
381 not affect lifespan or survival under starvation, it directly reduced egg hatchability, offspring viability and
382 development rate in naïve I beetles compared to control beetles. However, priming had variable effects on
383 reproduction. For instance, mounting a within-generation priming response helped C beetles to increase
384 their reproduction after infection; whereas infected I beetles, despite evolving survival benefits, could not
385 improve their reproduction. These results mirror our recent observations with wild-caught populations,
386 where primed and infected females with increased post-infection lifespan produced fewer offspring (Khan
387 et al. 2019) and vice versa. We thus speculate that a hidden trade-off with reproduction might constrain the
388 survival benefits of within-generation priming responses at a much lower level than resistance. Mounting
389 trans-generational priming responses, on the other hand, had no effect on offspring reproduction, suggesting
390 that fitness effects are not uniform across different priming responses. Our results broadly corroborate other
391 work showing the negative effects of priming on various fitness parameters (Trauer & Hilker 2013;
392 Contreras-Garduño *et al.* 2014). However, these studies primarily used phenotypic manipulations within a
393 single generation, whereas ours is the first study to directly measure the complex fitness costs associated
394 with evolved priming across multiple generations of pathogen exposure.

395 Our experiments provide the first empirical evidence that insects can evolve multiple priming responses
396 simultaneously. Interestingly, both transgenerational and within-generation priming provided almost
397 equivalent fitness benefits, corroborating our prior work showing similar benefits of WGIP and TGIP across

398 10 distinct wild-caught beetle populations (Khan et al. 2016). Such parallel results from natural and
399 laboratory-evolved populations indicate that pathogens such as Bt may serve as a potent source of selection
400 favoring the evolution of diverse immune responses in insects. As discussed earlier, Bt reduces the survival
401 of flour beetle larvae and adults equally (Khan et al 2016), which should favor the simultaneous evolution
402 of WGIP and TGIP (Tate and Rudolf 2012). However, during experimental evolution we only infected
403 adult beetles, which should have restricted host-pathogen interaction to adults. It is possible that infected
404 adults directly transmitted Bt to eggs, imposing selection favoring TGIP. Alternatively, infected adults
405 could have transmitted Bt (or antigen) to larvae via the flour, either through infected beetle cadavers (~10-
406 15% mortality during oviposition period in I beetles) or excreta (Argôlo-filho & Loguercio 2014). Another
407 possibility is that ancestral beetle populations may have already coevolved with Bt in their natural habitat
408 before they were brought into the lab. Consequently, despite being infected only as adults during
409 experimental evolution, the beetle immune system could perhaps readily recognize Bt as a risk across life
410 stages, due to their shared evolutionary history. Finally, if WGIP and TGIP involve shared molecular
411 pathways, direct pathogen pressure on adults could result in simultaneous evolution of both types of
412 priming. While the molecular details responsible for immune priming are still unclear (Cooper &
413 Eleftherianos 2017), recent data hint at shared immune pathways between different priming types. For
414 instance, both within- (Pham et al. 2007) and trans-generationally primed honeybees (Barribeau et al. 2016)
415 show increased expression of Toll signaling pathways. Further experiments to carefully compare the
416 molecules underlying different immune responses can help distinguish between the above hypotheses.

417 In closing, we note that the relative importance of priming vs. general resistance has long been debated,
418 primarily because it was unclear whether (a) diverse priming types (within- vs. trans-generational) together
419 constitute distinct strategies, separate from basal resistance (b) their costs vs. benefits differ substantially,
420 and (c) they involve different or overlapping sets of immune pathways. Our work represents one of the first
421 steps to address the first two problems, demonstrating distinct costs and benefits of multiple priming
422 responses vs. resistance evolving simultaneously in response to selection imposed by the same pathogen
423 (also see Khan et al. 2017a). While these results highlight the remarkable diversity and flexibility of insect
424 innate immune adaptation against infections, they also suggest that the early survival vs. reproductive costs
425 of priming can constrain their adaptive evolution, much more so than resistance. However, it remains a
426 mystery why putative resistance alleles either did not arise or failed to outcompete putative priming alleles,
427 despite their large selective advantage in I beetles. We hope that our results will motivate further
428 experiments to address this problem. Specifically, we look forward to detailed mechanistic studies to test
429 whether host-pathogen interactions at low frequency of infection not only favor the evolution of priming,
430 but involve immune pathways that mechanistically preclude more beneficial resistance alleles from fixing
431 in host populations.

432 **AUTHOR CONTRIBUTIONS**

433 IK conceived experiments; IK, AP and DA designed experiments; AP carried out experiments; IK and AP
434 analyzed data; IK and DA acquired funding; IK and DA wrote the manuscript with inputs from AP. All
435 authors gave final approval for publication.

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442 **COMPETING INTERESTS**

443 We have no competing interests

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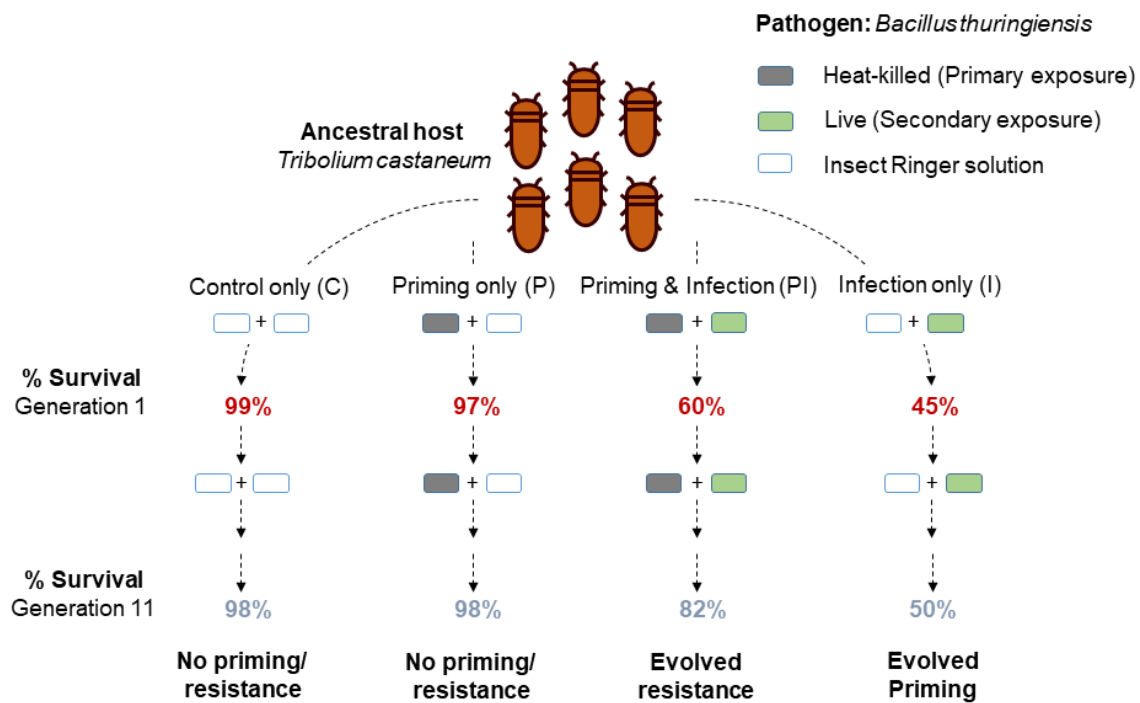
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- 524

525 **FIGURES**

526 **Figure 1.** Summary of the design and outcome of experimental evolution of *Tribolium castaneum* flour
527 beetles against the bacterial pathogen *Bacillus thuringiensis*, previously described in Khan et al 2017a. The
528 schematic indicates beetle survival before and after 11 generations of experimental evolution, as well as the
529 evolved immune response (resistance or priming) observed in all populations of each regime. Every
530 generation, 10-day-old virgin beetles were either injected with heat-killed bacterial slurry (P & PI) or sterile
531 insect Ringer solution (C & I) (primary exposure). After six days, individuals from I and PI regimes were
532 challenged with live Bt, whereas C and P beetles were pricked with sterile insect ringer solution (secondary
533 exposure). Each selection regime included 4 independent replicate populations. In the current study, we
534 analyzed 3 replicate populations from each regime.



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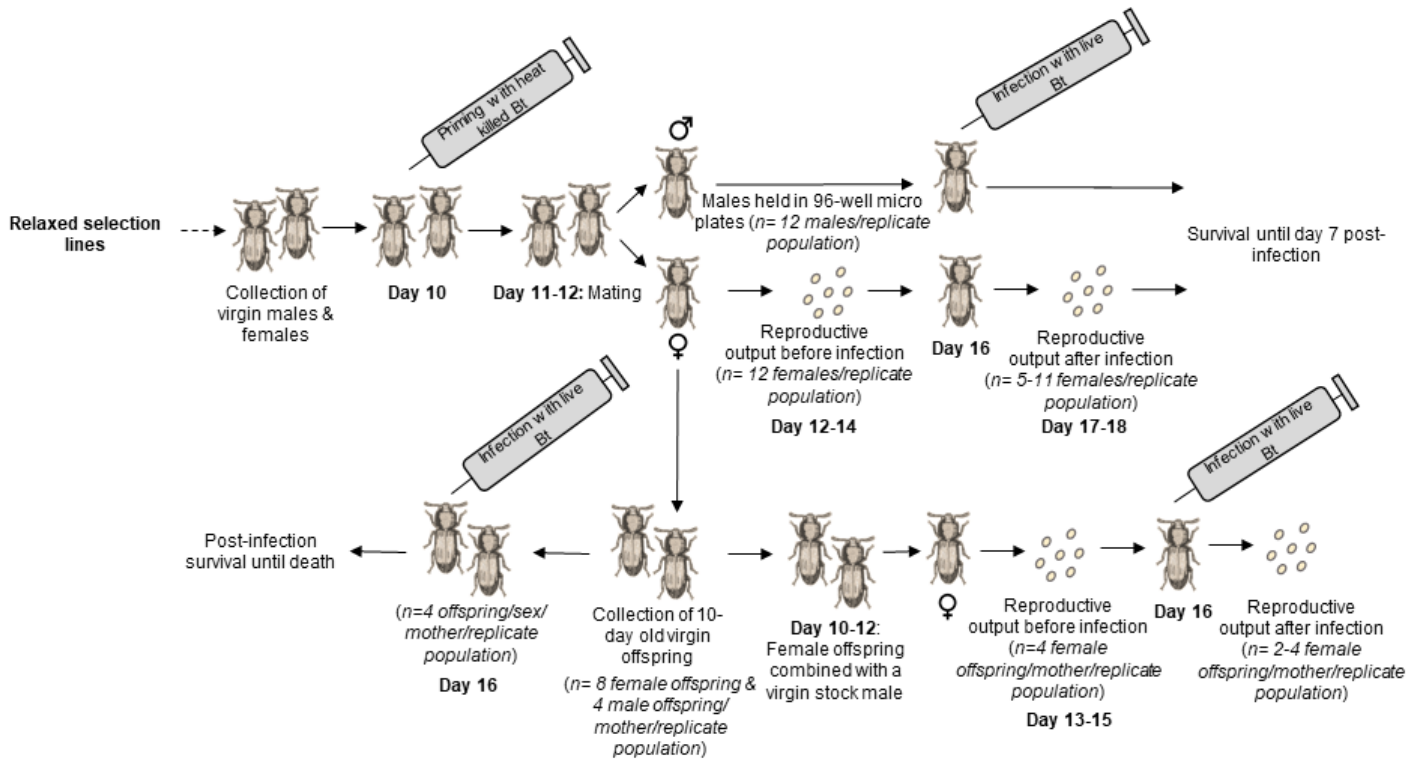
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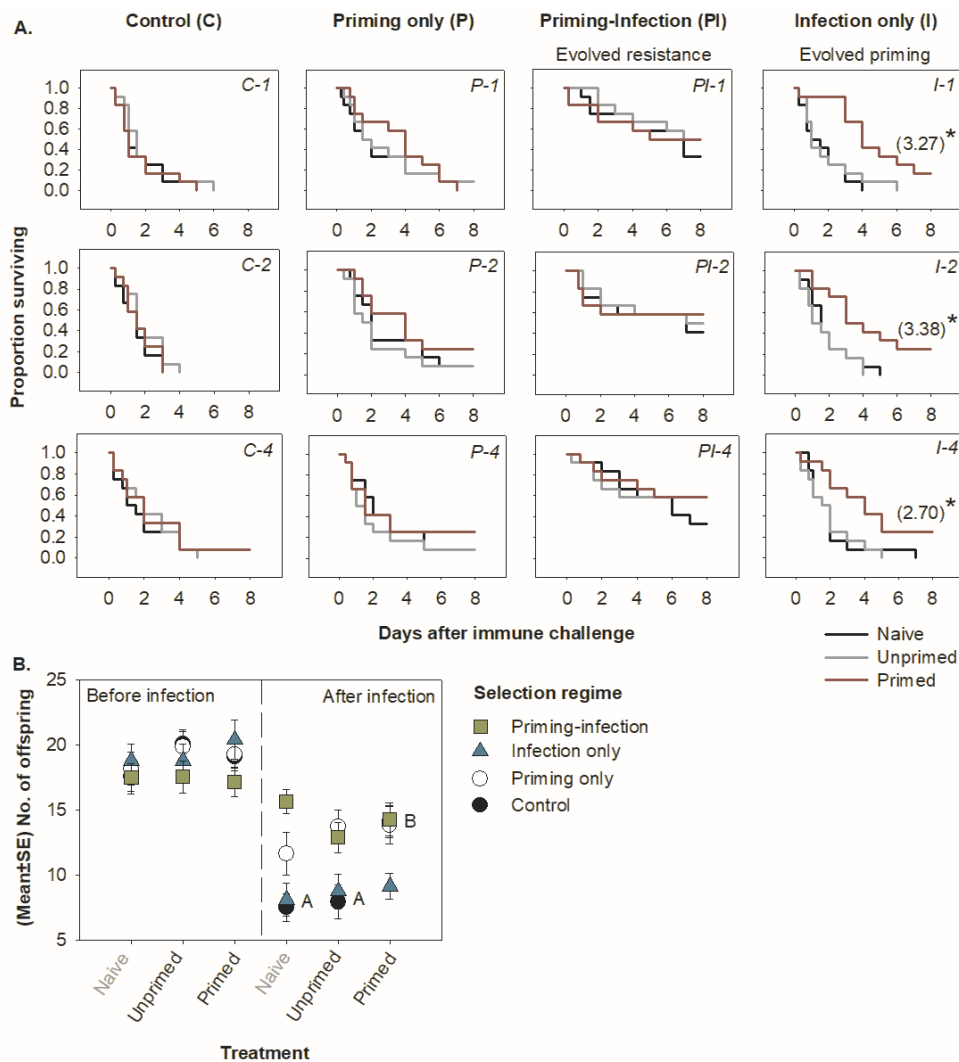
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542 **Figure 2.** Design of joint experiments to assay evolved immune responses and their impacts on beetle
543 reproduction.



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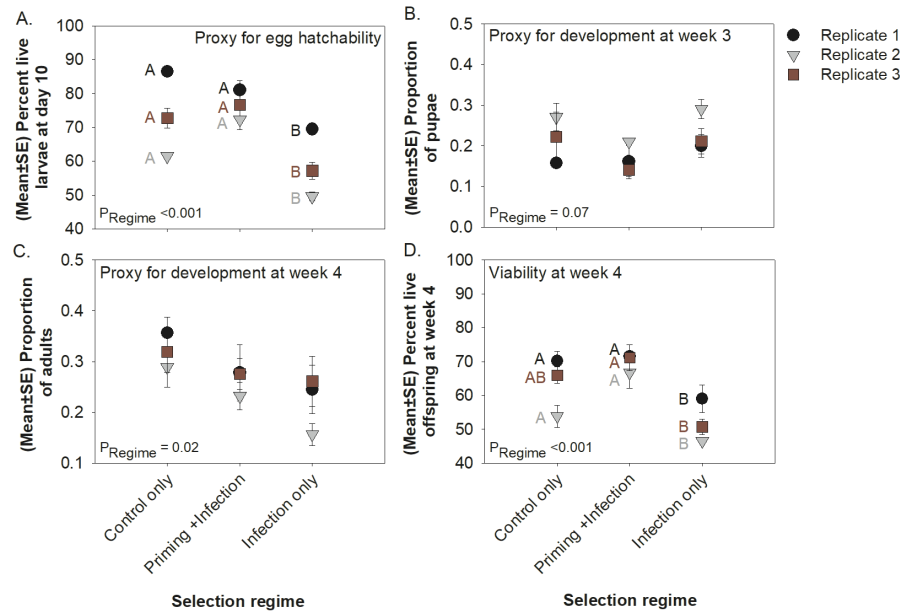
545 **Figure 3.** (A) Survival curves for within-generation priming and resistance in females (n= 12
 546 females/treatment/selection regime/replicate population) after 14 generations of selection. Asterisks and the
 547 numbers in parentheses for I beetles denote the hazard ratios calculated from survival curves for priming
 548 that are significantly greater than 1 (p<0.05; a greater hazard ratio indicates higher benefit of priming) (B)
 549 Impact of evolved within-generation priming (WGIP) and resistance on female reproductive output, both
 550 before (n=12 females/treatment/selection regime/replicate population) and after bacterial infection (n=5-11
 551 females/treatment/selection regime/replicate population). Alphabets indicate significant changes in C
 552 beetles' post-infection reproduction after mounting a within-generation priming response.



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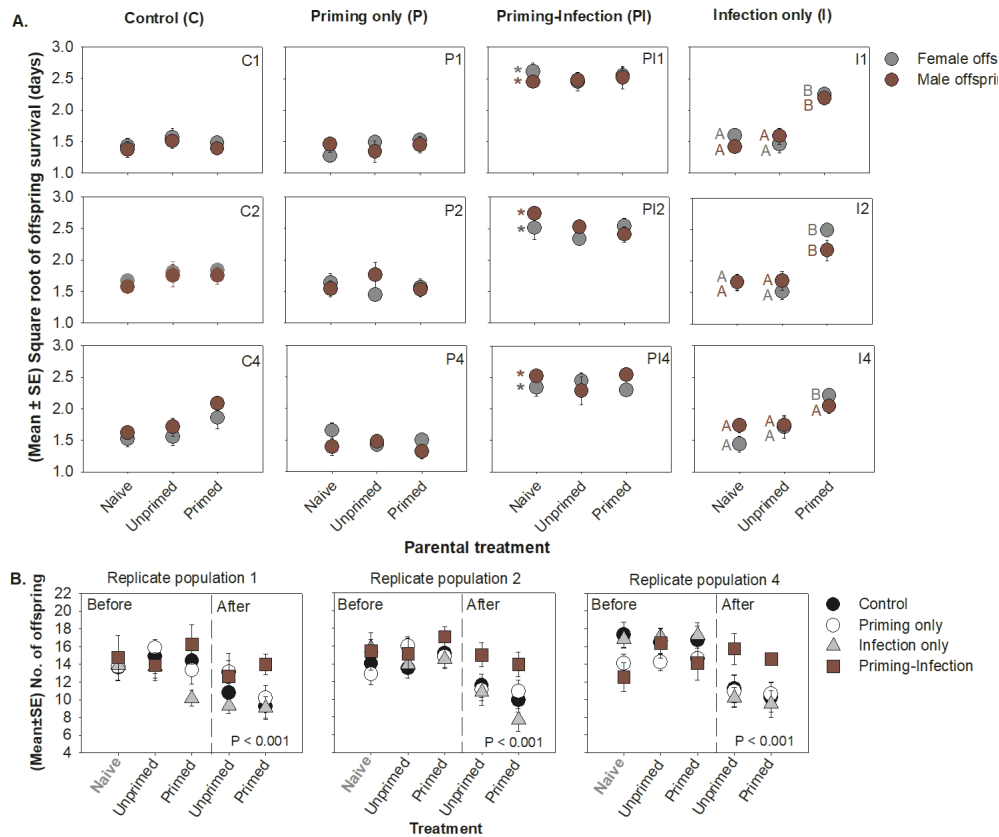
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555 **Figure 4.** Impact of evolved immune responses on (A) total number of eggs that hatched into larvae (egg
556 hatchability); proportion of (B) pupae at week 3 and (C) adults at week 4 as proxies for developmental rate;
557 (D) total number of viable offspring, including larvae, pupae and adults, at week 4; (n=3 females/selection
558 regime/replicate experiment). P values for the impact of selection regime are reported in each panel.
559 Significantly different groups are indicated by distinct alphabets, based on Tukey's HSD.



560

561 **Figure 5.** (A) Offspring survival after trans-generational immune priming (TGIP) and infection (group
 562 mean survival of 4 offspring from 8-11 parental pairs/ treatment/ selection regime/ offspring sex). TGIP
 563 increased offspring survival only in I regime, indicated by distinct alphabets, based on Tukey's HSD.
 564 Asterisks indicate significant increase in post-infection survival (resistance) of naïve PI beetles compared
 565 to naïve C beetles. (B) Impact of evolved trans-generational priming on offspring's reproductive output,
 566 both with and without infection, for replicate populations that were handled together (group mean survival
 567 of 2-4 offspring from 8-11 parental pair/ treatment/ selection regime/ offspring sex). P values for the impact
 568 of selection regime on post-infection reproductive output are reported in each panel.



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