THE COSTS AND BENEFITS OF BASAL INFECTION RESISTANCE VS DIVERSE IMMUNE PRIMING RESPONSES IN AN INSECT

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HIGHLIGHTS

1. Divergent costs and benefits of experimentally evolved immune priming vs resistance
2. Increased reproduction and lifespan in resistant populations
3. No other hidden costs of resistance
4. In contrast, reduced juvenile fitness and reproduction in primed populations
5. First evidence for experimentally evolved trans-generational immune priming

ABSTRACT

In insects, basal pathogen resistance and immune priming can evolve as mutually exclusive strategies, with distinct infection outcomes. However, the evolutionary drivers of such diverse immune functions remain poorly understood. Here, we addressed this key issue by systematically analyzing the differential fitness costs and benefits of priming vs. resistance evolution in *Tribolium* beetle populations infected with *Bacillus thuringiensis*. Surprisingly, resistant beetles had increased post-infection reproduction and a longer lifespan under both starving as well as fed conditions, with no other measurable costs. In contrast, priming reduced offspring early survival, development rate and reproduction. Priming did improve post-infection survival of offspring, but this added trans-generational benefit of immune priming might not compensate for its pervasive costs. Resistance was thus consistently more beneficial. Our work represents the first report of experimentally evolved trans-generational priming, and a detailed comparison of the complex fitness consequences of evolved priming vs resistance.

KEY-WORDS

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

46 **1. INTRODUCTION**

47 Until recently, it was assumed that insects have nonspecific immunity and cannot build immune memory
48 against previously encountered pathogens, since they lack the immune cells responsible for adaptive
49 immunity in vertebrates (Cooper and Eleftherianos, 2017). Now, growing evidence contradicts this
50 traditional view: in addition to basal resistance mechanisms against infections (Rolff and Siva-Jothy,
51 2003), priming with a sub-lethal exposure to a pathogen often protects against subsequent exposure to
52 the same pathogen. This survival benefit of priming is observed both in later life stages of primed
53 individuals ("within-generation immune priming"; henceforth WGIP), and in their offspring ("trans-
54 generational immune priming"; henceforth TGIP), in a range of insect species (reviewed in Milutinović et
55 al., 2016) including Dipterans (Pham et al., 2007; Ramirez et al., 2015; Ramirez et al., 2017),
56 Coleopterans (Roth et al., 2009; Roth et al., 2010; Khan et al., 2016), Lepidopterans (Fallon et al., 2011)
57 and Hymenopterans (Sadd and Schmid-Hempel, 2006). Theoretical studies also highlight the importance
58 of priming in reducing infection prevalence and regulating population size, stability and age structure
59 during infection (Tate and Rudolf, 2012; Best et al., 2013). Thus, it appears that under pathogen
60 pressure, priming responses should be selectively favoured. Recently, we also, for the first time, directly
61 demonstrated the adaptive value of priming within the same generation and showed that it is a distinct
62 immune strategy that can evolve independently of basal resistance (without priming) to infection
63 susceptibility in the flour beetle *Tribolium castaneum* (Khan et al., 2017a). Also, a striking result of this
64 study was that the net survival benefit of evolved priming was considerably lower than that of resistance
65 (50% vs 80% survival after infection; Khan et al., 2017a), suggesting their inherently different effects on
66 overall infection outcomes. However, the selective forces influencing the evolution of these diverse
67 insect immune strategies and infection outcomes remain largely unclear.

68 Do different evolutionary forces shape insect priming vs basal resistance to infection? The potentially
69 divergent costs of maintenance and deployment of various immune responses can shed some light here

70 (Norris and Evans, 2000; Sheldon and Verhulst, 1996). Several studies suggest that resistance is
71 associated with overexpression of fast-acting immune responses that impose large physiological costs
72 (e.g. Sadd and Siva-Jothy, 2006; Khan et al., 2017b). A general mathematical model predicts that such
73 costs of constitutively expressed basal resistance can be outweighed by its benefit only under frequent
74 lethal pathogenic infections, maximising the population's growth rate (Mayer et al., 2016). However, the
75 cost of resistance may be larger when pathogens are encountered infrequently. This is perhaps one
76 reason why our beetle populations infected with a single large dose of infection every generation
77 evolved priming (Khan et al., 2017a). In contrast, resistance could evolve only in populations that were
78 exposed repeatedly to the pathogen within the same generation (primary exposure with heat-killed
79 bacteria followed by live bacterial infection) (Khan et al., 2017a). However, few studies have actually
80 measured the fitness consequences of evolved resistance in insects, and these were equivocal: e.g.
81 while some found significant costs in terms of reduced longevity, poor larval competitive ability and
82 delayed development (Kraaijeveld and Godfray, 1997; Ye et al., 2009; Ma et al., 2012), several others did
83 not (Faria et al., 2015; Gupta et al., 2016). In general, costs of pathogen resistance may manifest as
84 widespread tradeoffs with other life-history parameters as well, including reproduction (Reviewed in
85 Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000; Rolff and Siva-Jothy, 2003; Schwenke et
86 al., 2016). In contrast, the impact of immune priming-mediated protection on various fitness parameters
87 has only recently been tested: e.g., primed mosquitoes (Contreras-Garduño et al., 2014), tobacco
88 hornworms (Trauer and Hilker, 2013) and flour beetles (Khan et al., 2019) show reduced fecundity or
89 primed mealworm beetle mothers produced progeny that developed slowly (Zanchi et al., 2011).
90 Although these experiments revealed diverse fitness impacts of mounting priming responses using
91 phenotypic manipulations, the actual costs of evolving, maintaining and deploying priming responses in
92 populations while facing persistent pathogen-imposed selection have never been measured in detail
93 (also see Ferro et al., 2019).

94 We also considered the potential added fitness benefits of evolved priming, where survival benefits
95 manifest not only in the primed individuals (i.e., WGIP) but also across generations (i.e., TGIP). Besides
96 enhancing the net fitness impact of priming via WGIP, TGIP might also facilitate the evolution and
97 spread of priming ability in populations. Although no direct experiments have tested whether such
98 trans-generational benefits evolve simultaneously with WGIP, theory offers some important clues. A
99 model by Tidbury and coworkers suggests that since TGIP has a lower ability to reduce infection
100 prevalence, selection should favour WGIP more (Tidbury et al., 2012). On the other hand, Tate and
101 Rudolf suggested that the stage-specific effects of infection are important: TGIP is more beneficial when
102 an infection affects juvenile stages, whereas WGIP is more effective if adults incur higher infection costs
103 than larvae (Tate and Rudolf, 2012). The model also predicts that selection can strongly favour both
104 WGIP and TGIP when the pathogen affects larvae and adults equally (Tate and Rudolf, 2012). Our
105 previous experimental results suggest that this hypothesis might be relevant at least for flour beetles:
106 both WGIP and TGIP were equally beneficial in beetles infected with the general insect pathogen
107 *Bacillus thuringiensis* (Bt), which imposed similar infection costs across life stages (Khan et al., 2016).
108 Although these results represent an interesting correlation, the causal link between the pathogen's
109 impact on the host and its role in determining relative investment in different priming responses is not
110 yet confirmed.

111 To understand the selective pressures and fitness effects that directly impact the evolution of diverse
112 priming responses vs basal infection resistance, we used previously described, evolved replicate
113 populations of the red flour beetle *T. castaneum* that were infected in each generation with Bt (DSM
114 2046), either with or without the opportunity of priming with heat-killed Bt cells (described in Khan et
115 al., 2017a; also see **Fig.** 1A and Supplementary methods). Earlier, we had analysed evolved infection
116 responses of these populations after 11 generations of evolution (Khan et al., 2017a): (i) post-infection
117 survival benefits of primed individuals (relative to their unprimed counterparts) as effects of immune

118 priming, and (ii) the inherent ability to survive after infection (without prior priming with heat-killed
119 cells) with respect to sham infected controls, as a proxy for basal resistance against infections. In the
120 present study, we re-tested the same populations after a further 3 generations of experimental
121 evolution to analyse the underlying fitness costs and benefits of their evolved responses. We measured
122 a range of critical fitness-related traits in these populations such as offspring development, early
123 reproduction and early survival, as well as adult lifespan under starvation and normal conditions. We
124 also tested whether the evolution of priming ability was associated with transgenerational survival
125 benefits during infection.

126 Note that we did not measure the direct changes in immune function or pathogen clearance in our
127 experiments. Hence, the resistance, estimated from post-infection survival data alone, can be
128 confounded by the potential evolution of tolerance, whereby survival might increase by reducing the
129 cost of infection or immune responses, not because of more efficient pathogen killing mechanisms
130 (Ayres and Schneider, 2012). Nonetheless, our work provides the first systematic analysis of the
131 evolutionary cost and benefit structure influencing parallelly evolved, divergent survival responses to
132 pathogens infections, by using possibly different immune strategies. We also provide the first report of
133 the experimental evolution of TGIP in insects.

134 **2. METHODS**

135 **2.1 Experimental evolution**

136 We used laboratory-adapted populations of *T. castaneum* to initiate four distinct selection regimes,
137 described in (Khan et al., 2017a): control (previously C ; henceforth "no_evol_C"), priming only
138 (previously P; henceforth "no_evol_P"), primed and infected (previously PI; henceforth
139 "evol_resistance_PI") and infection only (previously I; henceforth "evol_priming_I"), each with originally
140 4 independent replicate populations (also outlined in **Fig.** 1). In the present study, for logistical reasons,

141 we could only analyze three replicates from each selection regime (no_evol_C 1, 2 & 4; no_evol_P 1, 2 &
142 4; evol_resistance_PI 1, 2 & 4; evol_priming_I 1, 2 & 4). On different days, we handled one replicate
143 population from each selection regime together. The detailed protocol for the experimental evolution is
144 described in (Khan et al., 2017a) (also see Supplementary methods), where we quantified evolved
145 responses after 11 generations of selection (**Fig.** 1A). In the current study, we reanalyzed these evolved
146 lines after three further generations of selection (**Fig.** 1B). Thus, after 14 generations of continuous
147 selection, we isolated a subset of individuals from each replicate population and propagated them
148 without any selection for two generations, i.e., without priming or infection (unhandled). This relaxing of
149 selection is expected to generate standardised experimental beetles with minimum non-genetic
150 parental effects.

151 **2.2 Joint assays of evolved priming and resistance, and their impacts on reproduction**

152 We designed our experimental framework to jointly compare survival benefits and reproductive effects
153 of evolved priming vs. resistance against Bt infection (see **Fig.** 2A for experimental design). Besides
154 measuring survival after priming and infection, we measured female reproductive output both before
155 and after infection. This allowed us to estimate the direct impact of experimental evolution with
156 pathogens vs. the actual impact of inducing each type of immune response. Simultaneously, we also
157 tested for the evolution of trans-generational immune priming (TGIP), to compare relative survival and
158 reproductive effects of different priming responses.

159 2.2.1 <u>Confirmation of evolved immune responses, and measurement of their reproductive effects (see</u>
160 <u>**Fig.** 2A for experimental outline):</u>

161 We first collected pupae from each standardised population and isolated them into 96-well microplate
162 wells with ~0.2g wheat flour, for eclosion. We randomly assigned 10-day old virgin males and virgin
163 females from each population to one of the following primary exposure treatments: (a) naïve (or

164 unhandled) (b) primed (injected with heat-killed Bt) and (c) unprimed (i.e., injected with insect Ringer)
165 (see **SI** methods for various priming and infection treatments). After 24 hours of primary exposure, we
166 formed mating pairs using males and females within the same primary exposure treatment combination
167 from each population in 1.5ml micro-centrifuge tubes with 1g of wheat flour (n = 12 mating-pairs/
168 priming treatment/ replicate population/ selection regime). We allowed them to mate for 48 hours and
169 then isolated the 12-day-old females to oviposit for another 48 hours in 5 g whole wheat flour
170 (oviposition plate), whereas males were returned to 96-well microplates. After oviposition, we also
171 returned the 14-day-old females to 96-well microplates. Two days later (total six days after primary
172 exposure), we infected males and females with live Bt. We recorded male survival every 6 hours for 1
173 day and then every 24 hours for 7 days post-infection (same as the selection window during
174 experimental evolution; Khan et al., 2017a). We tracked female survival similarly, except that a day after
175 infection, we again allowed 48-hour oviposition to estimate the reproductive impact of infection and
176 induction of any priming responses. Here, we caution that since bacterial infection imposed significant
177 mortality across regimes, the replicate size for our fitness assays was lower than expected (n=5-12
178 females/ priming treatment/ replicate population/ selection regime; **Table** S1; resulting analyses can
179 thus be relatively underpowered compared to the reproductive assays performed before infection). We
180 also note that although more beetles were alive in the evol_resistance_PI regime during the
181 reproductive assay, we did not find a significant difference in the proportion of live beetles that
182 reproduced across different treatments and selection regimes (**Table** S1). We also conducted a mock
183 challenge for a subset of unprimed beetles as a procedural control for the survival assay, but not for
184 reproductive output. We did not find any mortality in uninfected beetles within the experimental
185 window of 7 days.

186 To test whether reproductive costs of evolved resistance manifest under stressful conditions, we
187 compared the reproductive output of naïve unhandled evol_resistance_PI beetles with no_evol_C

188 beetles reared in a poor-quality diet, corn (Agashe et al., 2011). To generate experimental adults, we
189 allowed females from standardized no_evol_C and evol_resistance_PI populations (replicate
190 populations 1 and 2) to oviposit in cornflour and collected their offspring to measure reproductive
191 output as described above (n=36 females/replicate population/selection regime).

192 2.2.2 Generation of beetles (progeny) for transgenerational priming (TGIP) assay:

193 We used eggs laid by naïve, unprimed and primed females (assayed to estimate the effects of WGIP,
194 described above) to also measure TGIP. We allowed the eggs to develop for 21 days at 34ºC and
195 counted the total number of progeny (mostly pupae). Particularly, we retained the offspring from the
196 first round of oviposition (without infection) (See **Fig.** 2A). At this time, most offspring were pupae, and
197 the few adults we observed had pale body coloration indicating that they were not sexually mature and
198 hence, unlikely to be mated (Sokoloff, 1977). We isolated these pupae and adults in 96-well plates with
199 ~0.2g flour, to obtain virgin beetles for assays to measure trans-generational priming and offspring
200 reproduction. We included offspring from mothers that produced more than 8 female and 4 male
201 offspring (n= 8-10 mothers/ priming treatment / replicate population/ selection regime), enabling us to
202 sample enough beetles to jointly test offspring post-infection survival (a proxy of trans-generational
203 priming; described below) and reproduction of each parental pair.

204 2.2.3 Assaying transgenerational priming response and its reproductive effects (See **Fig.** 2A for
205 experimental outline):

206 After 10 days, we combined a subset of female offspring from each parental pair (n=4 offspring/
207 parental pair/ priming treatment/ replicate population/ selection regime) with 10-day old virgin males
208 from standard laboratory stock population into a single mating pair for 48 hours and then allowed them
209 to oviposit. This enabled us to measure the impact of parental priming on offspring reproductive output
210 across populations. On day 16, we infected females and then again assayed their reproductive output as

211 described above. We could only compare the post-infection reproduction of primed and unprimed
212 females, but not naïve females as we lost them accidentally. On the same day, we also infected the
213 remaining 16-day old virgin male and female offspring from each parental pair with live Bt (n= 4
214 offspring/ sex/ parental pair/ priming treatment/ replicate population/ selection regime) and noted
215 their survival every 6 hours for 2 days and then every 24 hours until all of them were dead. This
216 experimental design not only allowed us to jointly estimate the survival and reproductive effects of
217 WGIP vs. TGIP for each parental pair, but also to analyse the impact of each type of immune response
218 relative to evolved resistance. We did not find any mortality in sham infected offspring within the
219 experimental window.

220 **2.3 Quantifying development and survival under starvation and with food, in evolved lines**

221 In separate experiments, we measured the direct impacts of evolved priming responses and resistance
222 on other fitness components of naïve unhandled beetles (without priming and challenge), such as early
223 survival, development, starvation resistance and lifespan.

224 2.3.1 Impact on lifespan under starvation and with food (See **Fig**. 2B for experimental outline):

225 We first isolated 10-day old naïve virgin males and females from each population in 96-well microplate
226 wells without food (*n* = 20 beetles/ sex/ replicate population/ selection regime). We noted mortality
227 every 12 hours (10 am & 10 pm ±1 hour) for the next 12 days until all beetles died (**Fig.** 2B). In a separate
228 experiment, we similarly distributed naïve virgin females into 96-well microplates, but with access to
229 food (*n* = 48 females/ replicate population/ selection regime). We noted their survival every 5 days for
230 95 days to estimate the long-term survival costs of evolved immune responses. We could not assay
231 males for long-term survival costs due to logistical challenges.

232 2.3.2 Quantifying early survival, development and viability costs in evolved lines (See **Fig**. 2C for
233 experimental outline):

234 We next estimated the impact of evolved immune responses on aspects of early survival and
235 development. We allowed 12-day old, mated females from each population (n = 60/ replicate
236 population/ selection regime) to oviposit in 150g of doubly sifted flour (using sieves with pore size of
237 50µ to remove large flour particles; Diager USA) for 24 hours. We had 3 replicates for each population.
238 We discarded the females, and isolated 96 randomly chosen eggs into 96-well microplate wells with ~0.2
239 g flour (n= 3 microplates/ replicate population/ selection regime). This method was designed to detect
240 intrinsic patterns of several developmental features across populations, without the confounding effects
241 of density-dependent competition at the juvenile stage. After 10 days, we sifted the flour from each
242 microplate to count live larvae and measure egg hatchability. Following this, we again returned the live
243 larvae to 96-well plates and provided fresh flour. In our standard stock beetle populations, pupation and
244 adult emergence begins around 3-4 weeks after oviposition. Therefore, we estimated the proportion of
245 pupae and adults after 3- and 4-weeks post-egg collection respectively, as proxies for time to pupation
246 and adult emergence. Finally, we calculated the percentage of surviving larvae, pupae and adults at
247 week 4, as a proxy for overall viability. Since no_evol_P beetles did not evolve priming or resistance, we
248 excluded them from the assay.

249 **2.4 Data analysis**

250 2.4.1 Within generation priming response and resistance in parents: We first analysed survival data for
251 all selection regimes using a mixed effects Cox model using the R package 'coxme' (Therneau, 2015). We
252 fit separate models for females and males, since they were assayed on different days. In each case, we
253 specified the model as survival ~ selection regime × priming treatment + (1|selection regime/ replicate
254 population), with selection regime and priming treatment as fixed effects, and replicate populations
255 nested within the selection regime as a random effect. A significant interaction between priming
256 treatment and selection regime would indicate that the effects of priming varied across selection
257 regimes. We note that although this analysis provides an overall estimate of each effect, complex

258 interactions can prevent us from making meaningful comparisons between selection regimes. To
259 understand the differences across priming treatments (i.e., unprimed vs primed) and selection regimes
260 in detail, we therefore separately analysed survival data for each standardised replicate population,
261 using Cox Proportional Hazard survival analysis. We noted individuals that were still alive at the end of
262 the survival experiment as censored values and estimated the survival benefit of priming as the hazard
263 ratio of unprimed versus primed groups. A hazard ratio significantly greater than one indicates a higher
264 risk of mortality in the unprimed group relative to primed individuals; hence, a significant survival
265 benefit of WGIP.
266 Separately, we also estimated the hazard ratio of naïve infected beetles from the no_evol_C regime
267 against naïve infected beetles from all other regimes, to quantify evolved resistance against Bt infection.
268 While a hazard ratio significantly greater than one indicates a higher risk of mortality in no_evol_C
269 beetles, it also implies increased evolved resistance in selected regimes relative to no_evol_C beetles.
270 2.4.2 Transgenerational priming response in offspring: To measure TGIP, we recorded the survival of 4
271 male and 4 female replicate offspring from each parental mating pair assayed earlier for within-
272 generation priming. We calculated the mean lifespan for both the sexes as the unit of analysis but noted
273 that residuals of lifespan data were not normally distributed (verified with Shapiro-Wilk tests; $p<0.01$).
274 Therefore, we transformed the data into square root values that fit a normal distribution, and then
275 compared group means of male and female offspring survival using a linear mixed effects model (R
276 package 'lme4', Bates et al., 2012) with selection regime, parental priming status and offspring sex as
277 fixed factors and replicate populations as a random effect. We tested for pairwise differences between
278 selection regime and treatment after correcting for multiple comparisons using Tukey's HSD (honest
279 significant difference), using the R package 'lsmeans' (Lenth, 2017). We performed model reduction
280 through stepwise removal of nonsignificant terms.

281 In addition to detecting the overall TGIP response in evol_priming_I beetles, we also wanted to compare
282 their relative survival benefit with that of WGIP. To this end, for each replicate population, we first
283 separately analysed the lifespan of male and female offspring from each parental pair, using a mixed
284 effects Cox model to calculate the estimated hazard ratio of offspring from unprimed parents versus
285 primed parents. Since we recorded the survival of 4 male and 4 female offspring from each parental
286 pair, we specified the model as: survival ~ parental priming status + (1| parental mating pair/ replicate
287 offspring), with parental priming status as a fixed effect and male (or female) replicate offspring nested
288 within each parental mating pair as a random effect. Since all beetles died within the experimental
289 window, we did not have any censored beetles in the analysis.
290 Subsequently, we used non-parametric Wilcoxon Rank Sum tests to compare hazard ratios calculated
291 from TGIP versus WGIP for each population.
292 2.4.3 Reproductive effects: We found that the residuals of pre-infection reproductive output data of
293 both parents and offspring were non-normally distributed and could not be transformed to a normal
294 distribution. We, therefore, used non-parametric Wilcoxon Rank Sum tests to analyse the impact of
295 selection regime and priming treatment (for replicate populations of no_evol_C, no_evol_P,
296 evol_resistance_PI and evol_priming_I that were handled together). We also used Wilcoxon tests to
297 analyse the impact of bacterial infection on the reproductive output of parents and offspring, separately
298 for each replicate population across selection regimes and priming treatments.
299 We analysed post-infection reproductive fitness data using a linear mixed effects model with selection
300 regime and priming treatment as fixed factors and replicate populations as a random effect [Model: No.
301 of offspring~ Priming treatment × Selection regime + (1 | Replicate population); fitted separately for
302 parents and offspring]. To understand the complex interactions between treatment and selection
303 regime and to disentangle the effects of each type of evolved immune response (i.e., priming in
304 evol_priming_I or resistance in evol_resistance_PI), we also compared post-infection reproductive data

305 of parents from each selection regime separately with that of control beetles (no_evol_C), wherever
306 needed. We used Tukey's HSD to test for pairwise differences between selection regimes and
307 treatments. As described above, we removed the nonsignificant effects stepwise to determine the best
308 reduced model.

309 2.4.4 Survival under starvation and fed conditions: We analysed survival data under starvation and with
310 food, using mixed effects Cox models as described below (best reduced model, wherever needed):

311 I. Lifespan under starvation: survival ~ selection regime + sex + (1| selection regime/ replicate
312 population), with selection regime and sex as fixed effects, and replicate populations nested
313 within selection regimes as a random effect.

314 II. Lifespan with food: survival ~ selection regime + (1| selection regime/ replicate population),
315 with selection regime as a fixed effect, and replicate populations nested within selection
316 regimes as a random effect.

317 2.4.5 Early survival, development and viability costs: We analysed data using a linear mixed effects
318 model with selection regime as fixed factor and replicate population as a random effect. In each case,
319 we described the model as: Trait ~ Selection regime + (1|Replicate population). We tested for pairwise
320 differences using Tukey's HSD.

321 **3. Results**

322 Our previous work demonstrated that lethal Bt infection rapidly selects for divergent post-infection
323 survival responses (potentially via divergent immune strategies in beetles), within 11 generations (Khan
324 et al., 2017a). Populations that were directly infected with a single large dose of Bt evolved within-
325 generation priming response (evol_priming_I regime), whereas populations, where beetles were
326 injected first with heat-killed and then live Bt, evolved basal resistance to infection (evol_resistance_PI)
327 (**Fig.** 1A). Our results after 14 generations of selection were consistent with these prior observations
328 (Khan et al., 2017a) (**Fig.** 1B). A mixed-effects Cox model fitted separately to male and female survival

329 data revealed a significant interaction between selection regime and treatment, suggesting that effects
330 of priming and infection varied across selection regimes (**Fig.** S1-2, **Table** S2-3).

331 To understand these complex effects in detail, we next, analysed survival data for each population with
332 a Cox proportional hazard analysis. We found evolved priming responses only in males and females from
333 evol_priming_I populations (~3-fold increase in their survival relative to control beetles) (**Fig.** S1-2, **Table**
334 S2-S3); whereas evol_resistance_PI beetles had higher basal resistance (3 to 28-fold increase in the
335 survival of naïve evol_resistance_PI beetles relative to control beetles) (**Fig.** S1-2, **Table** S4). We also
336 found that while the survival of evol_priming_I beetles after Bt infection was still 50%,
337 evol_resistance_PI beetle survival had increased to ~85% (**Fig.** S3). As expected, populations from the
338 no_evol_C or no_evol_P regimes (where beetles were not exposed to live infection) did not evolve any
339 priming ability or higher resistance to infection.

340 **3.1 Maintenance of evolved immune responses do not incur reproductive costs**

341 We measured the impact of evolved immune responses on beetle reproduction and found complex
342 fitness effects that varied substantially with priming type and infection status. Evolved priming or
343 resistance had no significant impact on the reproduction (**Fig.** 3, **Table** S5). There were also no general
344 effects of priming treatment on beetle reproduction (**Table** S5). Thus, the maintenance of evolved
345 priming or resistance does not impose a reproductive cost. However, we noticed that females infected
346 with live pathogen later in their life (see methods) had reduced reproductive output in most
347 populations, except those from evol_resistance_PI regime where the effect was relatively mild (compare
348 across selection regimes and treatments; **Fig.** 3, **Table** S6). Thus, most evol_resistance_PI beetles,
349 regardless of their priming status, generally did not exhibit reproductive costs of infection. Also, the
350 average post-infection reproductive cost of evolved priming appeared higher than that of evolved
351 resistance (compare beetles from evol_resistance_PI vs evol_priming_I populations in **Fig.** 3, **Table** S6),

352 highlighting contrasting outcomes of live infection across evolved regimes. These patterns were further
353 confirmed in subsequent analyses where we separately compared the reproductive fitness of naïve and
354 unprimed females after infection, using a Wilcoxon rank sum test (data was not normally distributed;
355 Shapiro-Wilk test: $P<0.05$). We did not include primed beetles in these analyses, because we wanted to
356 measure the effect of live infection without any interference from previous antigen exposure (i.e., heat-
357 killed bacteria during priming). Overall, there was no effect of the experimental treatment (naïve vs
358 unprimed females after infection: df=1, $\chi2= 0.021$, $P=0.87$), but overall infected evol_resistance_PI
359 beetles produced significantly more offspring than no_evol_C (df=3, $\chi2= 12.458$, $P<0.001$) and
360 evol_priming_I beetles (df=3, $\chi2= 14.837$, $P<0.001$) beetles.

361 Next, we analysed the full dataset (including naïve, unprimed & primed females) to study the impact of
362 experimental priming (mimicking selection regimes during experimental evolution) on the reproductive
363 output of infected beetles. We expected that after infection, beetles in the priming treatment would
364 reflect reproduction of evol_resistance_PI beetles; whereas beetles in the unprimed treatment would
365 mimic evol_priming_I beetles during experimental evolution. In comparison to no_evol_C beetles, they
366 would thus provide an estimate of the reproductive effects of mounting evolved resistance and priming
367 response respectively during actual infection. A linear mixed effects model with reproductive output
368 data from all regimes revealed significant main effects of both selection regime and treatment as well as
369 their interaction (**Table** S7 I). Beetles from the evol_resistance_PI ($P=0.04$) and no_evol_P ($P=0.02$)
370 regimes appeared to produce more offspring than no_evol_C beetles, though these effects were
371 perhaps confounded by their priming status. Since our experiments were primarily designed to
372 disentangle the reproductive effects of separately evolved responses with respect to control no_evol_C
373 beetles, we also compared each selection regime individually with no_evol_C control beetles (e.g.,
374 evol_priming_I vs no_evol_C, or evol_resistance_PI, vs no_evol_C) (**Table** S7 II-IV). In contrast to results
375 obtained from the full dataset (**Table** S7 I), these comparisons did not show consistent effects of the

376 selection regime, but revealed significant effects of the priming treatment and its interaction with
377 selection regime in each case (**Table** S7 III-IV). In both analyses (i.e., full dataset and pairwise
378 comparisons), experimental priming induced a large increase in reproduction only in no_evol_C regime
379 after infection (primed vs unprimed no_evol_C; Tukey's HSD: P=0.005), but not in beetles from other
380 selection regimes (Tukey's HSD: p>0.9) (**Fig.** 3). Surprisingly, primed evol_priming_I beetles failed to
381 increase their reproduction despite increased post-infection survival benefits (after mounting WGIP),
382 suggesting a fitness cost. This was in contrast to evol_resistance_PI lines that could apparently alleviate
383 this reproductive cost by maintaining higher reproduction than no_evol_C beetles regardless of their
384 priming or infection status (discussed above). Thus, evolved resistance is better than priming not only in
385 terms of its survival benefit, but also in terms of reproduction. We also verified these significant
386 differences by calculating confidence intervals (2.5%, 97.5%) [no_evol_C: naïve (4.633, 10.366),
387 unprimed (5.528, 10.391), primed (11.595, 16.665) vs. evol_ priming_I: naïve (5.595, 10.665), unprimed
388 (6.108, 11.415), primed (6.977, 11.345) vs. evol_resistance_PI: naïve (13.269, 18.038), unprimed
389 (10.719, 15.087), primed (12.046, 16.486)].

390 Finally, in a separate experiment, we demonstrated that evol_resistance_PI beetles reared in poor
391 quality diet corn produced an equal number of offspring to that of no_evol_C beetles (**Fig**. S4; **Table** S8:
392 p=0.34), indicating that evolved resistance might not impose reproductive costs even under stressful
393 conditions.

394 **3.2 Evolved resistance increases survival under both starvation and fed conditions**

395 We analysed the direct impacts of evolved priming and resistance on survival under starvation or fed
396 conditions, using a mixed effects Cox model. In both cases, survival was significantly higher in
397 evol_resistant_PI beetles compared to no_evol_C beetles (P<0.04 in each assay), but not in
398 evol_priming_I or no_evol_P beetles (P>0.05 in each assay) (**Fig**. S5-6; **Table** S9-10). Males and females

399 from all selection regimes had similar lifespan under starvation (**Fig.** S5, **Table** S9), suggesting no sex-
400 specificity in starvation resistance.

401 **3.3 Evolved priming reduces early survival and extends the development time**

402 We measured features of early survival such as egg hatchability and the total number of viable offspring
403 (i.e., live larvae, pupae and adults) within the first 4 weeks after oviposition. We also measured the
404 proportion of pupae and adults at week 3 and 4, as proxies of development rate. We found significant
405 effects of selection regime on the egg hatchability, the total number of viable offspring and proportion
406 of adult offspring at week 4, but not on the proportion of pupae at week 3 (**Fig.** 4). The number of viable
407 offspring at week 4 was drastically reduced in beetles from the evol_priming_I regime (**Fig.** 4D, **Table**
408 S11). This was perhaps due to significant early mortality during egg to larval development in
409 evol_priming_I beetles: while ~75% no_evol_C, no_evol_P and evol_resistance_PI eggs hatched into
410 larvae, only 55% evol_priming_I eggs survived (**Fig.** 4A, **Table** S11). Besides, the proportion of adults at
411 week 4 was lowest in the evol_priming_I regime, suggesting delayed development (**Fig.** 4C, **Table** S11).
412 Overall, these results indicate that maintenance of priming imposed considerable costs in terms of
413 reduced early survival and slower development in evol_priming_I beetles. In contrast, evolved basal
414 resistance did not appear to impose a significant cost with respect to these traits.

415 **3.4 Evolved WGIP is also associated with TGIP**

416 Finally, we asked whether evolved priming conferred added trans-generational benefits, increasing its
417 overall fitness impacts. We used a linear mixed effects model to analyse the mean post-infection
418 survival of offspring from beetles assayed above as a function of selection regime, parental priming
419 status and offspring sex. The selection regime and parental priming status had significant main effects as
420 well as an interaction effect, whereas offspring sex had no impact (P>0.05) (**Fig.** 5A, **Table** S12). Here
421 too, we found that overall, offspring of evol_resistance_PI beetles had the highest survival (compared

422 with no_evol_C beetles across parental priming status, all comparisons with Tukey's HSD: $p<0.001$),
423 though they did not show effects of parental priming. In contrast, parental priming increased offspring
424 survival in the evol_priming_I regime (Tukey's HSD: $p<0.001$), suggesting that TGIP benefits are
425 restricted to evol_priming_I beetles.

426 Separately, we also tested whether the relative survival benefits of TGIP were equal to that of WGIP. For
427 each replicate population of evol_priming_I beetles and offspring sex, we thus used a mixed effects Cox
428 model to analyze the individual offspring survival data nested within each parental mating pair, and
429 calculated the strength of evolved TGIP as the estimated hazard ratio for offspring from unprimed vs.
430 primed parents. We found a significant TGIP response in offspring from all replicate populations, except
431 for male offspring from evol_priming_I4 parents (**Fig.** S7A, **Table** S13). Primed evol_priming_I4 male
432 offspring appeared to survive more than their unprimed counterparts, but the difference in their
433 survival was not statistically significant (**Fig.** S7A, **Table** S13). Nonetheless, the net survival benefit of
434 TGIP and WGIP was not different across replicate populations (Wilcoxon Rank Sum Test: $p>0.05$),
435 possibly supporting the hypothesis that Bt-imposed selection favours the evolution of both types of
436 priming to a similar extent (**Fig**. S7B).

437 **3.5 Evolved trans-generational priming (TGIP) does not impose a separate reproductive cost**

438 As found with mothers (above), evolved priming and resistance did not consistently affect the
439 reproductive output of naïve or uninfected offspring (**Fig.** 5B, **Table** S14); but infection generally reduced
440 offspring reproductive output in all selection regimes except evol_resistance_PI (**Table** S15). A linear
441 mixed effects model revealed significant main effects of selection regime and parental priming status
442 (**Table** S16). Evol_resistance_PI beetles produced more offspring than control beetles (no_evol_C vs.
443 evol_resistance_PI: $P=0.009$) (**Fig.** 5B); but the reproductive output of no_evol_P and evol_priming_I
444 beetles did not differ from control (all $P>0.05$, Tukey's HSD). Interestingly, parental priming reduced

445 reproductive output in all beetles across selection regimes. However, since there was no significant
446 interaction effect between selection regime and parental priming, we inferred that transgenerational
447 survival benefits in evol_priming_I beetles did not impose any added reproductive costs, relative to
448 no_evol_C beetles (**Fig.** 5B).

449 4. **DISCUSSION**

450 Previously, we showed that priming response and basal resistance against *Bt* infection evolve as
451 divergent mutually exclusive strategies in flour beetles (Khan et al., 2017a). However, it was unclear if
452 selective parameters underlying their evolution, and potential trade-offs, were also different. Here, we
453 revisited these beetle lines to identify whether they actually incurred divergent fitness costs.
454 Concomitantly, we also asked whether priming confers additional, trans-generational fitness benefits.
455 To our surprise, we did not find any evidence for a cost of evolved resistance: it did not impact
456 development and reproduction, and even increased survival during both starvation and normal
457 conditions, contradicting the traditional view of immunity-fitness trade-offs (Ye et al., 2009; Ma et al.,
458 2012). Instead, our data add to the growing body of work that suggests only a weak role for life-history
459 trade-offs during the evolution of pathogen resistance (Faria et al., 2015; Gupta et al., 2016). Contrary to
460 our expectation, evolved priming imposed diverse fitness costs such as reduced offspring early survival,
461 development rate and reproduction. Interestingly, we found that priming ability was also associated
462 with trans-generational benefits, representing the first evidence of experimentally evolved TGIP.
463 However, the combined benefit of these two forms of priming (~50% survival after Bt infection) was still
464 lower than that conferred by increased basal resistance to Bt (~85% survival).

465 Interestingly, we found that although infection reduced reproduction in all regimes, the effect was less
466 pronounced in evol_resistance_PI beetles; hence, evolved basal resistance was also associated with a
467 relative reproductive advantage. No_evol_P (priming only) beetles also had higher reproduction than

468 control beetles after infection, which is counterintuitive because these beetles never experienced live
469 infection during experimental evolution. Note that this relative reproductive advantage would be
470 important during experimental evolution, since beetles reproduced for 5 days after infection in each
471 generation (see methods). How do we interpret these apparent reproductive fitness benefits in
472 evol_resistance_PI and no_evol_P beetles? First, as described earlier, the reduced cost of infection
473 might also represent evolved tolerance (Ayres and Schneider, 2012), whereby beetles do not invest in
474 directly clearing pathogens via canonical resistance mechanisms and may therefore be able to make a
475 greater reproductive investment during infection. Since we could not compare the relative importance
476 of tolerance vs resistance (e.g., by quantifying the fitness changes with increasing pathogen burden;
477 Ayres and Schneider, 2012) in our beetle lines, future studies are necessary to test these possibilities
478 explicitly. Second, our results could reflect a trade-off between early vs late reproduction. In other
479 words, higher reproduction might represent a terminal investment in no_evol_P and evol_resistance_PI
480 populations, whereas no_evol_C and evol_priming_I populations instead suppress immediate
481 reproduction after infection to maintain survival and somatic maintenance later in life (Luu and Tate,
482 2017). However, long-term reproductive effects are probably not meaningful in the present context,
483 because our beetles were selected to reproduce for only 7 days after infection. Overall, we suggest that
484 divergent immune strategies can have important consequences for reproductive success, which deserve
485 further attention.

486 We explored most of the plausible contexts in which immunity-fitness trade-offs could be expressed in
487 our populations, given the experimental evolution regime wherein beetles evolved the divergent
488 immune functions. It is possible that any underlying fitness trade-offs remained hidden because
489 evol_resistance_PI beetles lived in a relatively stable environment, with *ad libitum* food (McKean et al.,
490 2008). In contrast, suboptimal living conditions such as limited access to food and nutrition (McKean et
491 al., 2008) could reveal fitness costs. To test this possibility, we compared the reproductive output of

492 beetles from evol_resistance_PI lines vs. no_evol_C control lines when reared in a poor-quality corn diet
493 (Agashe et al., 2011). Surprisingly, beetles that had evolved resistance to Bt still produced as many
494 offspring as control beetles, indicating that evolved resistance did not impose reproductive costs even
495 under stressful conditions. However, we want to reiterate that even if fitness costs of evolved resistance
496 are expressed under other externally imposed environmental stresses (which could not be assayed in
497 the present study), these conditions are not relevant for our selected populations that evolved under
498 standard laboratory conditions. As such, any tradeoffs observed in these conditions cannot possibly
499 explain the particular divergence in immune responses that we observed in our present experiment.

500 Our results might also contradict our prior hypothesis that at a low pathogen frequency (experienced by
501 evol_priming_I beetles), priming may be more favourable than resistance due to its low maintenance
502 costs (Khan et al., 2017a). Instead, we found that the overall maintenance of priming responses is costly.
503 Although evolved priming did not affect lifespan or survival under starvation, it directly reduced egg
504 hatchability, offspring viability and development rate in naïve evol_priming_I beetles compared to
505 control beetles. These results broadly corroborate results from another beetle study showing that Bt-
506 driven evolution of priming in larvae delayed their development (Ferro et al., 2019). However, such
507 responses were observed only after mounting a priming response, suggesting a deployment cost;
508 whereas basal maintenance costs in naïve beetles were not analysed. In our study, priming also had
509 variable effects on reproduction across selection regimes. For instance, mounting a within-generation
510 priming response helped no_evol_C beetles to increase their reproduction after infection; whereas
511 infected evol_priming_I beetles, despite evolving survival benefits, could not improve their
512 reproduction. These results mirror our recent observations with wild-caught populations, where primed
513 and infected females with increased post-infection lifespan produced fewer offspring (Khan et al., 2019)
514 and vice versa. We thus speculate that a hidden trade-off with post-infection reproduction might
515 constrain the survival benefits of within-generation priming responses at a much lower level than

516 resistance (see **Fig.** 1B). We also speculate that the evolution of costly priming perhaps requires reactive
517 and self-damaging immune responses (e.g., phenoloxidase; see Ferro et al., 2017) to clear pathogens
518 (Khan et al., 2017b; Khan et al., 2019) and hence physiologically constrains the extent of post-infection
519 fitness benefits. In contrast, evol_resistance_PI beetles either used less toxic immune responses to clear
520 pathogens or evolved tolerance, reducing immunopathological costs and maximising fitness benefit
521 (Khan et al., 2017b). Mounting trans-generational priming responses, on the other hand, did not affect
522 offspring reproduction, suggesting that fitness effects are not uniform across different types of evolved
523 priming responses. Nevertheless, these results broadly corroborate other work showing the negative
524 effects of priming on various fitness parameters (Trauer and Hilker, 2013; Contreras-Garduño et al.,
525 2014). However, these studies primarily used phenotypic manipulations within a single generation,
526 whereas ours is the first detailed study to directly measure the complex fitness costs associated with
527 evolved priming across multiple generations of pathogen exposure.

528 Our experiments also provide the first empirical evidence that insects can evolve multiple priming
529 responses simultaneously. Interestingly, both trans-generational and within-generation priming
530 provided almost equivalent fitness benefits, corroborating our prior work showing similar benefits of
531 WGIP and TGIP across 10 distinct wild-caught beetle populations (Khan et al., 2016). Such parallel results
532 from natural and laboratory-evolved populations might indicate that natural pathogens such as Bt may
533 serve as a potent source of selection favouring the evolution of diverse immune responses in insects. As
534 discussed earlier, Bt reduces the survival of flour beetle larvae and adults equally (Khan et al., 2016),
535 which should favour the simultaneous evolution of WGIP and TGIP (Tate and Rudolf, 2012). However,
536 during experimental evolution, we only infected adult beetles, which should have restricted host-
537 pathogen interaction to adults. It is possible that infected adults directly transmitted Bt to eggs,
538 imposing selection favouring TGIP (see Tate and Rudolf, 2012). Alternatively, infected adults could have
539 transmitted Bt (or antigen) to larvae via the flour, either through infected beetle cadavers (~10-15%

540 mortality during oviposition period in evol_priming_I beetles) or excreta (Argôlo-filho and Loguercio,
541 2014). Another possibility is that ancestral beetle populations may have already coevolved with Bt in
542 their natural habitat before they were brought into the lab. Consequently, despite being infected only as
543 adults during experimental evolution, the beetle immune system could perhaps readily recognise Bt as a
544 risk across life stages. Finally, if WGIP and TGIP involve shared molecular pathways, direct pathogen
545 pressure on adults could result in simultaneous evolution of both types of priming. Although the
546 molecular details responsible for immune priming are still unclear (Cooper and Eleftherianos, 2017) and
547 perhaps confounded by diverse processes such as metabolic changes (Ferro et al., 2019) and epigenetic
548 reprogramming of immune cells (Tate et al., 2017), recent data hint at shared immune pathways
549 between different priming types. For instance, both within- (Pham et al., 2007) and trans-generationally
550 primed bumble bees (Barribeau et al., 2016) show increased expression of Toll signalling pathways (also
551 see Ferro et al., 2019). Further experiments to determine the molecules underlying different immune
552 responses can help distinguish between the above hypotheses.

553 **6. CONCLUSION**

554 In closing, we note that the relative importance of priming vs general resistance has long been debated,
555 primarily because it was unclear whether (a) diverse priming types (within- vs trans-generational)
556 together constitute distinct strategies, separate from basal resistance (b) their costs vs benefits differ
557 substantially, and (c) they involve different or overlapping sets of immune pathways. Our work
558 represents one of the first steps to address the first two problems, demonstrating distinct costs and
559 benefits of multiple priming responses vs resistance evolving simultaneously in response to selection
560 imposed by the same pathogen. While these results highlight the remarkable diversity and flexibility of
561 insect innate immune adaptation against infections, they also suggest that the early survival vs
562 reproductive costs of priming can constrain its evolution, much more so than resistance. We hope that
563 our results will motivate further experiments to understand this intriguing problem. Specifically, we look

forward to detailed mechanistic studies to test how host-pathogen interactions at a low frequency of infection only favour the evolution of priming, mechanistically precluding more beneficial resistance alleles from fixing in host populations.

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AUTHOR CONTRIBUTIONS

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

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COMPETING INTERESTS

We have no competing interests

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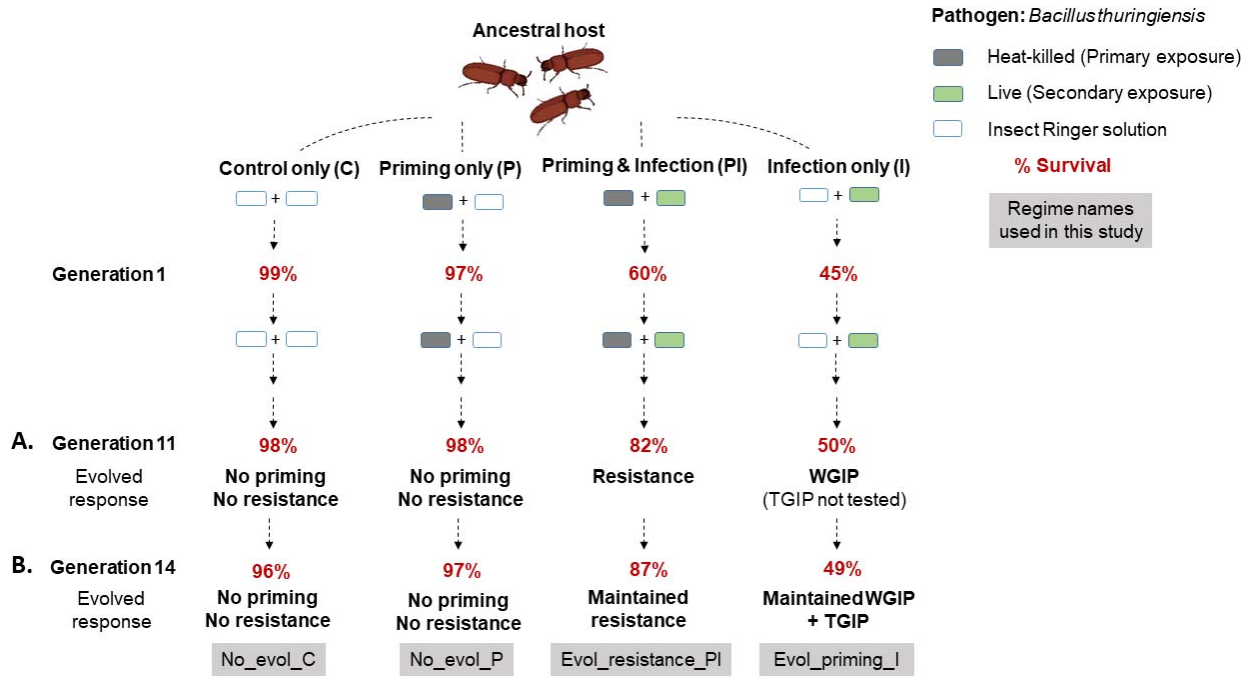
679

680 **FIGURES**

681 **Figure 1.** Summary of the design and outcome of experimental evolution of *Tribolium castaneum* flour
682 beetles against the bacterial pathogen *Bacillus thuringiensis*. The schematic indicates beetle survival as
683 well as evolved immune strategies (resistance or different priming forms) observed in all populations of
684 each regime, after **(A)** 11 and **(B)** 14 generations of experimental evolution, described in Khan et al.,
685 2017a and the present study respectively. C, P, PI and I populations assayed at 11th generation (Khan et
686 al., 2017a) were renamed as no_evol_C, no_evol_P, evol_resistance_PI and evol_priming_I populations
687 respectively in the present study. Every generation, 10-day-old virgin beetles were either injected with
688 heat-killed bacterial slurry (no_evol_P & evol_resistance_PI beetles) or sterile insect Ringer solution
689 (no_evol_C & evol_priming_I beetles) (primary exposure) (See **SI** methods). After six days, individuals
690 from evol_priming_I and evol_resistance_PI regimes were challenged with live Bt, whereas no_evol_C
691 and no_evol_P beetles were pricked with sterile insect ringer solution (secondary exposure) (See **SI**
692 methods). In the current study, we analysed 3 replicate populations from each regime. WGIP = Within-
693 generational immune priming; TGIP = Trans-generational immune priming.



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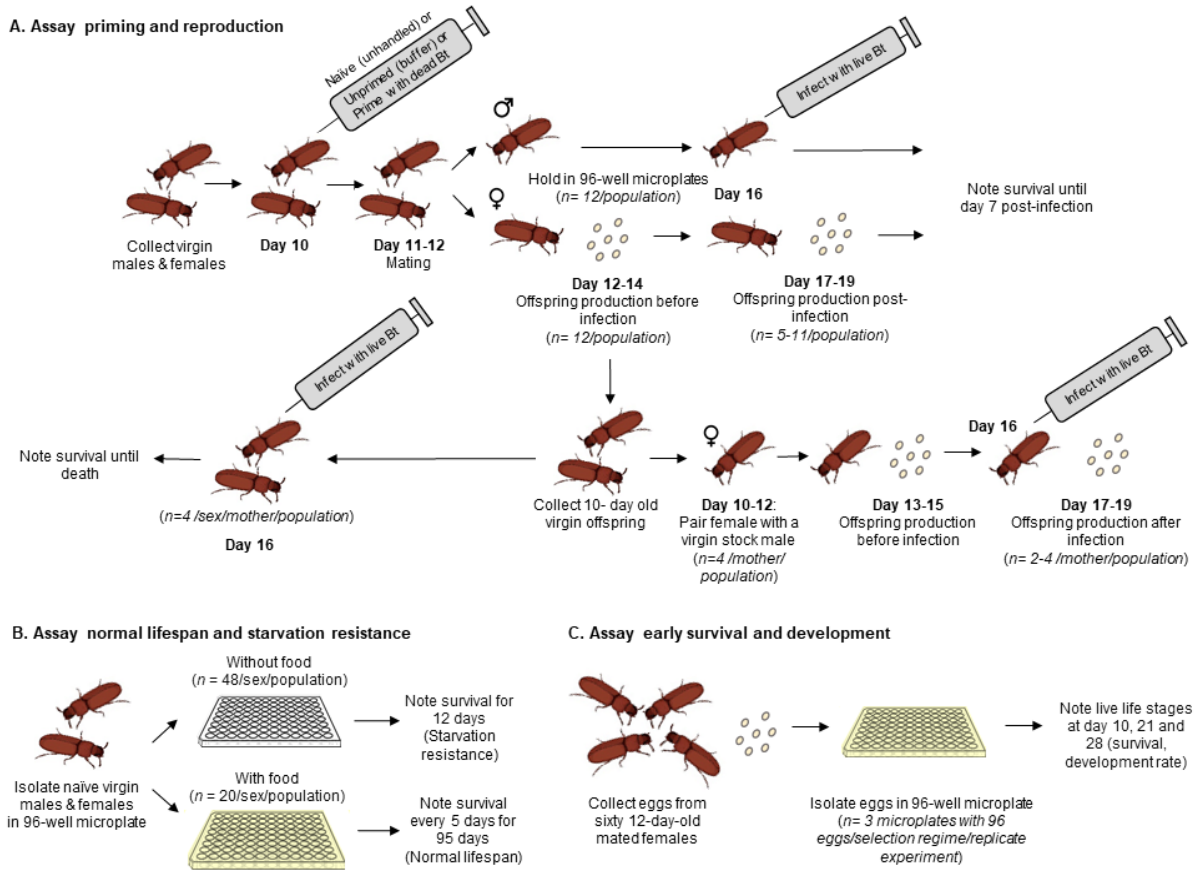
695 **Figure 2.** Design of experiments to assay **(A)** evolved immune responses and their impacts on beetle

696 reproduction **(B)** survival under normal condition and starvation **(C)** early survival and development.



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699 **Figure 3.** Impact of evolved within-generation priming (WGIP) and resistance on female reproductive
700 output, both before (n=12 females/ priming treatment/ replicate population/ selection regime) and
701 after bacterial infection (n=5-11 females/ priming treatment/ replicate population/ selection regime)
702 (partitioned by the vertical line). Different letters denote significant differences in post-infection
703 reproduction of no_evol_C beetles after mounting a within-generation priming response (tested with
704 Tukey's HSD). Error bars denote standard errors.

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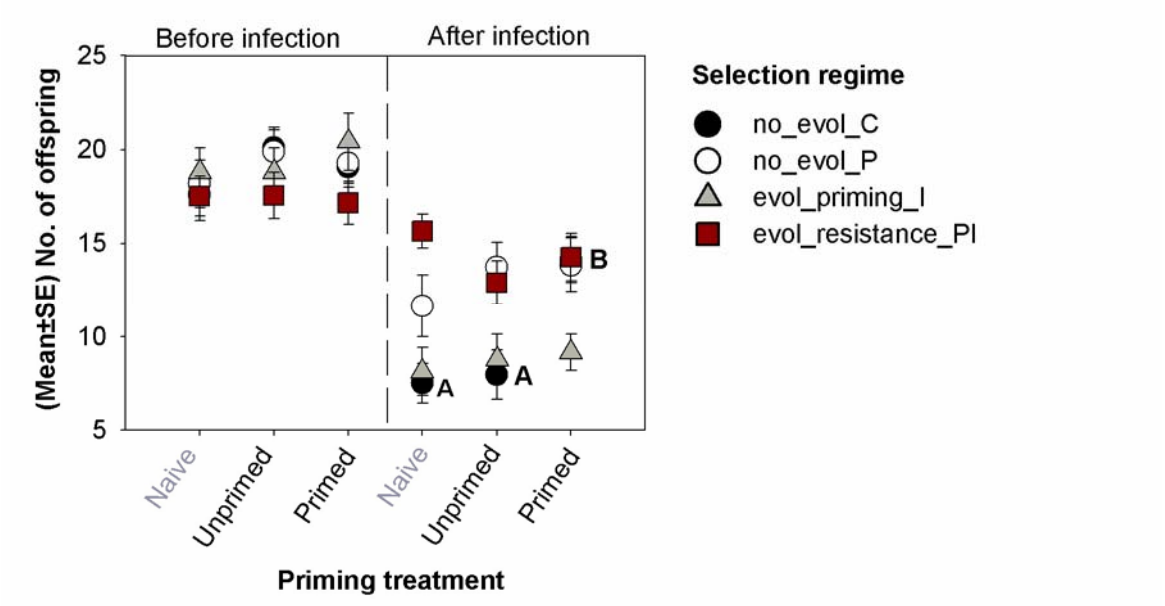

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708 **Figure 4.** Impact of evolved immune responses on **(A)** total number of eggs that hatched into larvae (egg
709 hatchability); proportion of **(B)** pupae at week 3 and **(C)** adults at week 4 as proxies for developmental
710 rate; **(D)** the total number of viable offspring (larvae, pupae and adults) at week 4 (n= 3 × 96 well
711 microplates/ replicate population/ selection regime). P values for the impact of the selection regime are
712 reported in each panel. Significantly different selection regimes are indicated by distinct letters (based
713 on Tukey's HSD). Error bars denote standard errors.

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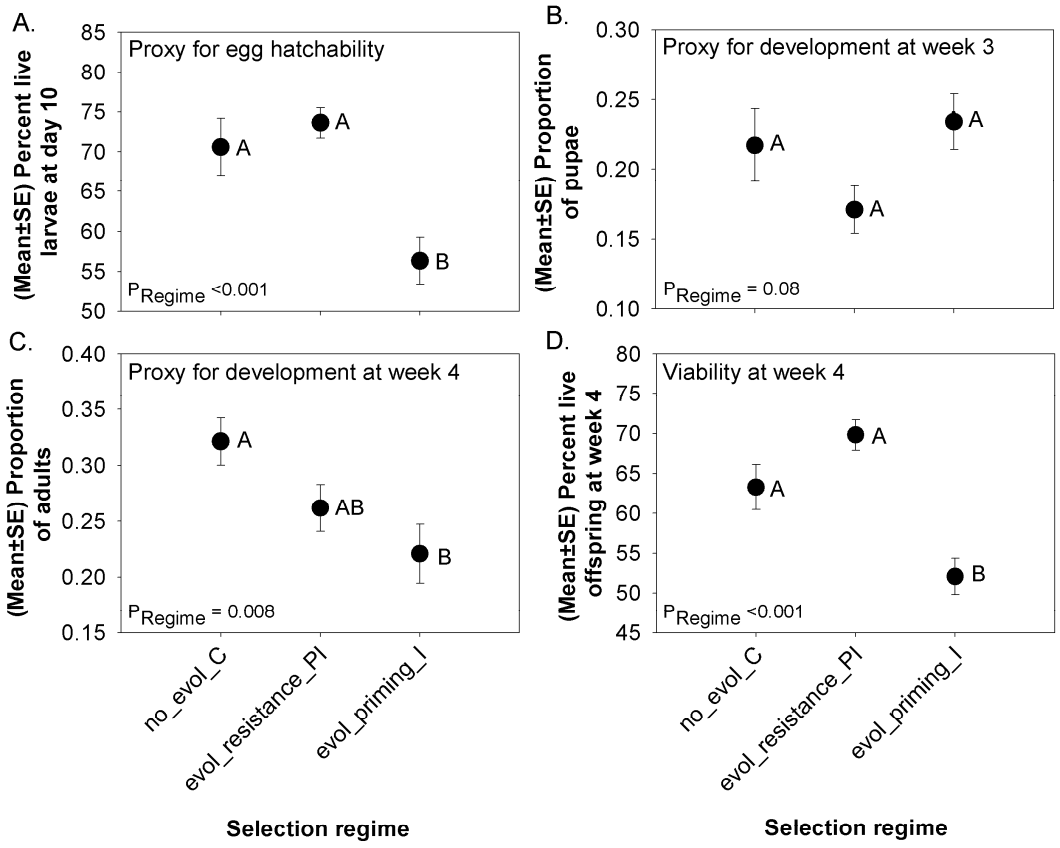

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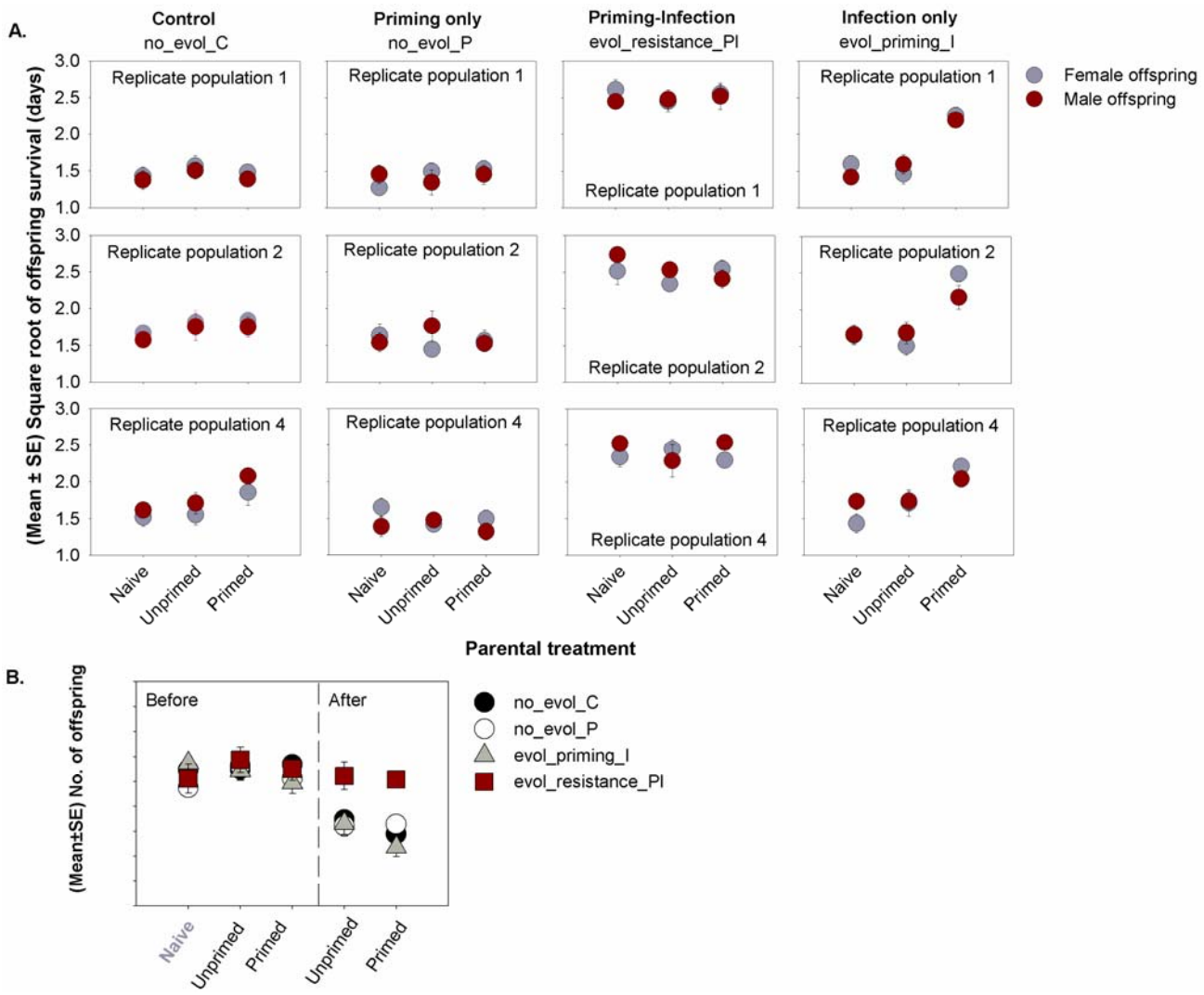
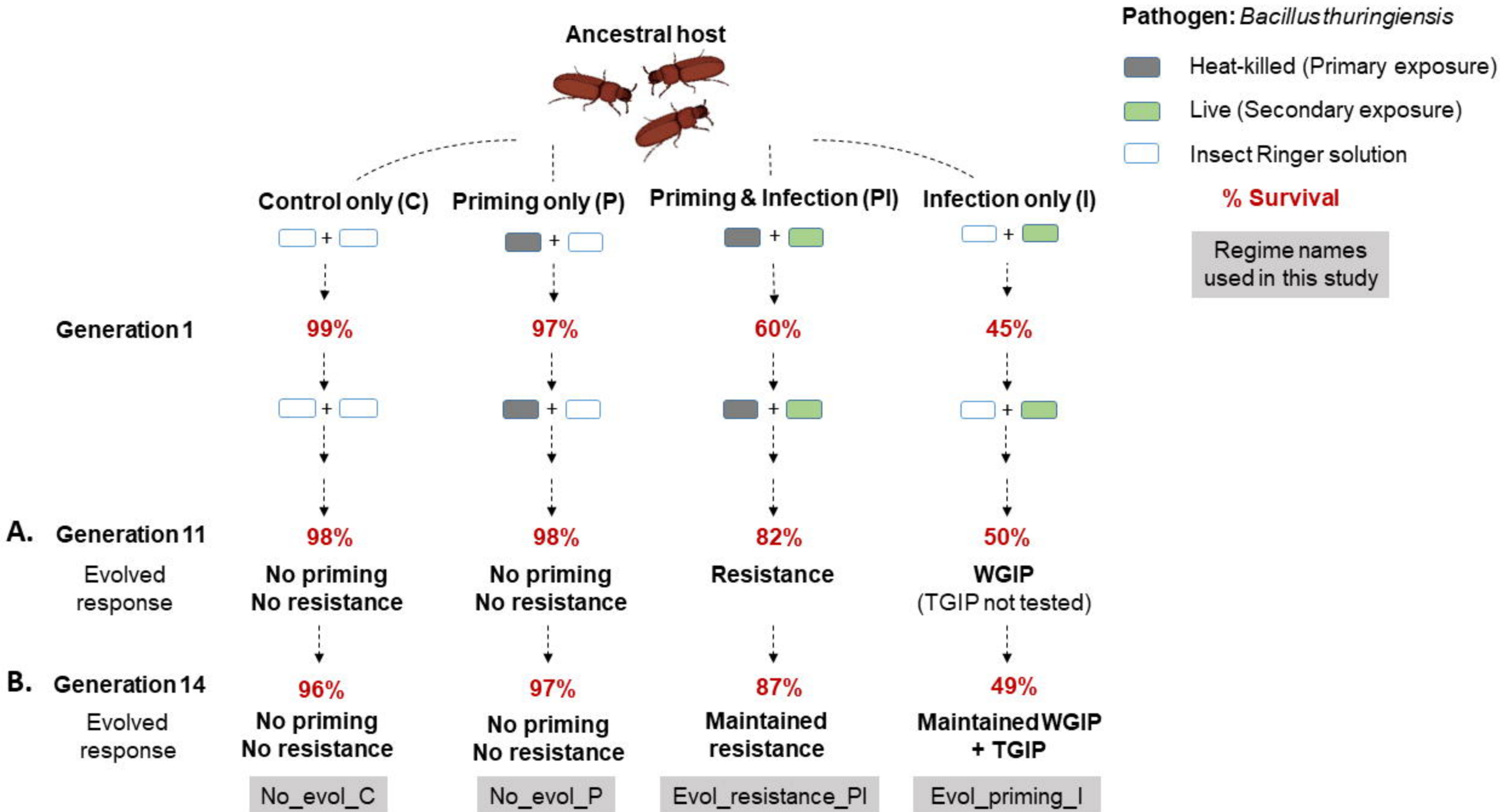
716 **Figure 5. (A)** Offspring survival after trans-generational immune priming (TGIP) and infection (group
717 mean survival of 4 offspring from 8-11 parental pairs/ offspring sex / parental priming status/ replicate
718 population/ selection regime). **(B)** Impact of evolved trans-generational priming on offspring's
719 reproductive output, both with and without infection (partitioned by the vertical line; group mean
720 reproduction of 4 female offspring from 8-11 parental pair/ parental priming status/ replicate
721 population/ selection regime). Error bars denote standard errors.

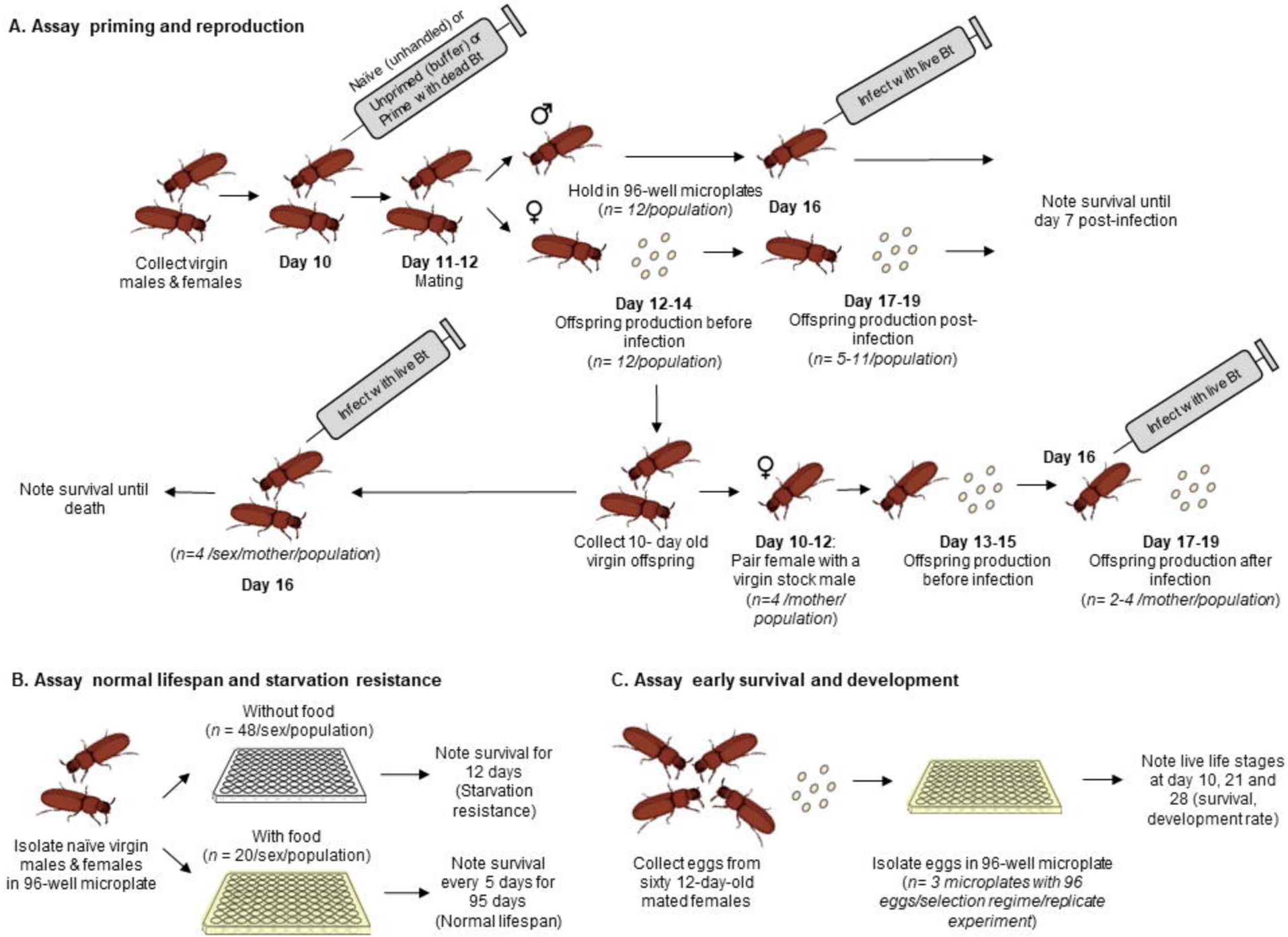
722

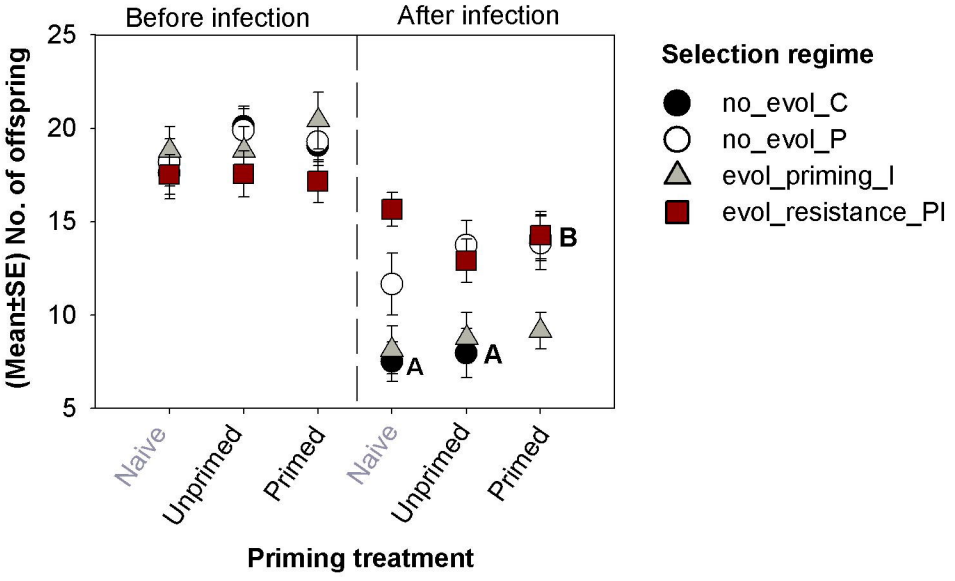


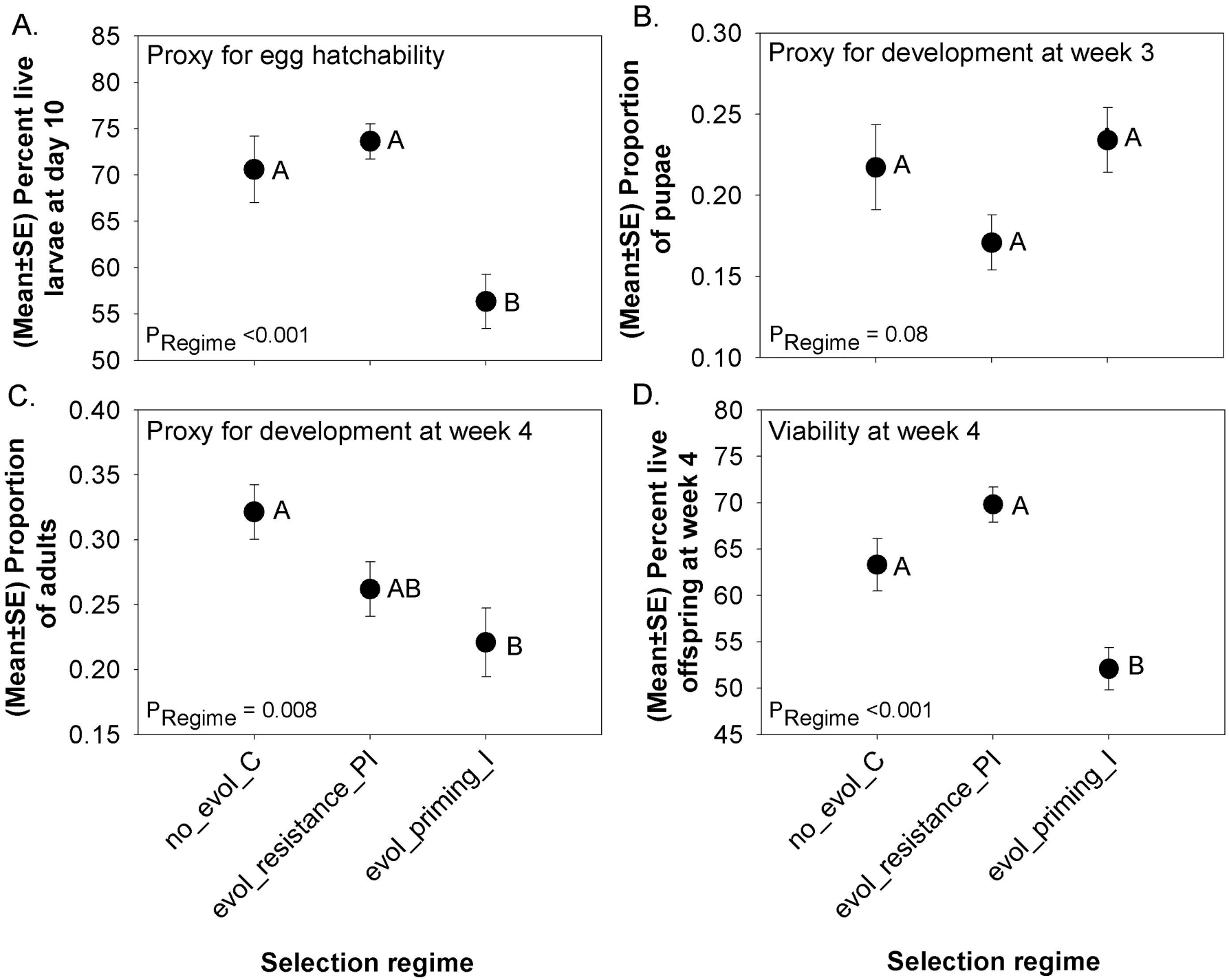
723

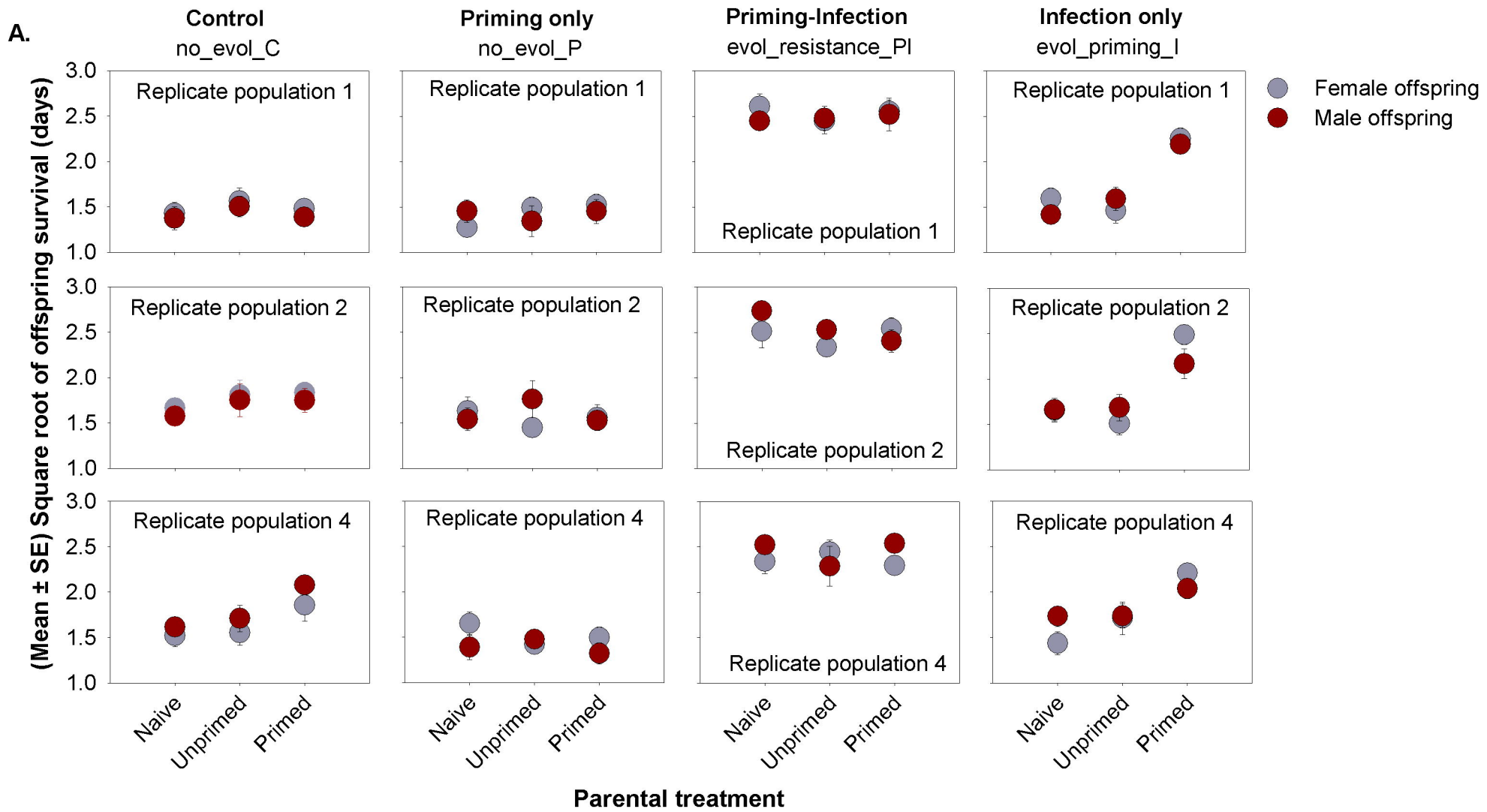
724

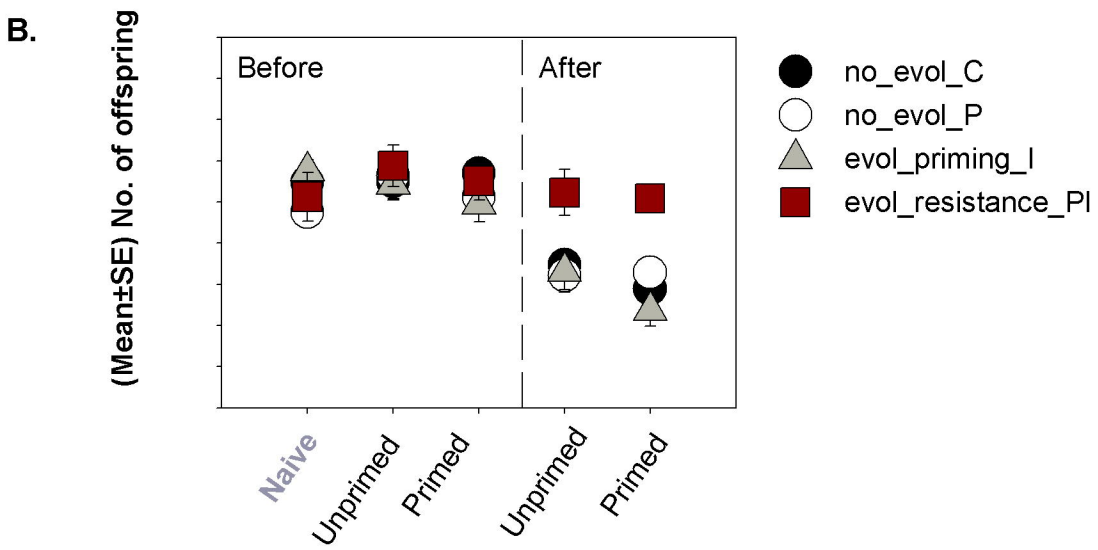

Pathogen: Bacillus thuringiensis
Heat-killed (Primary exposure)
Live (Secondary exposure)
Insect Ringer solution
% Survival
Regime names used in this study
Ancestral host
Control only (C)
Priming only (P)
Priming & Infection (PI)
Infection only (I)
Generation 1
99%
97%
60%
45%
A.
Generation 11
98%
98%
82%
50%
Evolved response
No priming No resistance
No priming No resistance
Resistance
WGIP (TGIP not tested)
B.
Generation 14
96%
97%
87%
49%
Evolved response
No priming No resistance
No priming No resistance
Maintained resistance
Maintained WGIP + TGIP
No_evol_C
No_evol_P
Evol_resistance_PI
Evol_priming_I


A. Assay priming and reproduction
Naïve (unhandled) or
Unprimed (buffer) or
Prime with dead Bt
Infect with live Bt
Collect virgin males & females
Day 10
Day 11-12
Mating
♂
♀
Hold in 96-well microplates
(n= 12/population)
Day 16
Note survival until day 7 post-infection
Day 12-14
Offspring production before infection
(n= 12/population)
Day 17-19
Offspring production post-infection
(n= 5-11/population)
Infect with live Bt
Note survival until death
(n=4 /sex/mother/population)
Day 16
Collect 10- day old virgin offspring
Day 10-12:
Pair female with a virgin stock male
(n=4 /mother/ population)
Day 13-15
Offspring production before infection
Day 16
Infect with live Bt
Day 17-19
Offspring production after infection
(n= 2-4 /mother/population)
B. Assay normal lifespan and starvation resistance
Isolate naïve virgin males & females in 96-well microplate
Without food
(n = 48/sex/population)
Note survival for 12 days (Starvation resistance)
With food
(n = 20/sex/population)
Note survival every 5 days for 95 days (Normal lifespan)
C. Assay early survival and development
Collect eggs from sixty 12-day-old mated females
Isolate eggs in 96-well microplate
(n= 3 microplates with 96 eggs/selection regime/replicate experiment)
Note live life stages at day 10, 21 and 28 (survival, development rate)


Before infection
After infection
(Mean±SE) No. of offspring
25
20
15
10
5
A
A
B
Naive
Unprimed
Primed
Naive
Unprimed
Primed
Priming treatment
Selection regime
no_evol_C
no_evol_P
evol_priming_I
evol_resistance_PI


A.
Proxy for egg hatchability
(Mean±SE) Percent live larvae at day 10
85
80
75
70
65
60
55
50
A
A
B
P Regime <0.001
B.
Proxy for development at week 3
(Mean±SE) Proportion of pupae
0.30
0.25
0.20
0.15
0.10
A
A
A
P Regime = 0.08
C.
Proxy for development at week 4
(Mean±SE) Proportion of adults
0.40
0.35
0.30
0.25
0.20
0.15
A
AB
B
P Regime = 0.008
D.
Viability at week 4
(Mean±SE) Percent live offspring at week 4
80
75
70
65
60
55
50
45
A
A
B
P Regime <0.001
no_evol_C
evol_resistance_PI
evol_priming_I
Selection regime
no_evol_C
evol_resistance_PI
evol_priming_I
Selection regime


A.
Control
no_evol_C
Priming only
no_evol_P
Priming-Infection
evol_resistance_PI
Infection only
evol_priming_I
Female offspring
Male offspring
(Mean ± SE) Square root of offspring survival (days)
3.0
2.5
2.0
1.5
1.0
Replicate population 1
Replicate population 2
Replicate population 4
Naive
Unprimed
Primed
Parental treatment


B.
(Mean±SE) No. of offspring
Before
After
Naive
Unprimed
Primed
Unprimed
Primed
no_evol_C
no_evol_P
evol_priming_I
evol_resistance_PI