

1 **Pathogen susceptibility and fitness costs explain variation in immune priming**
2 **across natural populations of flour beetles**

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36 survival benefit

37 **Abstract**

38

39 In many insects, individuals primed with low doses of pathogens live longer after being
40 exposed to the same pathogen later in life. Yet, our understanding of the evolutionary and
41 ecological history of priming of immune response in natural insect populations is limited.
42 Previous work demonstrated population-, sex- and stage- specific variation in the survival
43 benefit of priming response in flour beetles (*Tribolium castaneum*) infected with their natural
44 pathogen *Bacillus thuringiensis*. However, the evolutionary forces responsible for this natural
45 variation remained unclear. Here, we tested whether the strength of the priming response
46 (measured as the survival benefit after priming and subsequent infection relative to unprimed
47 controls) was associated with multiple fitness parameters across 10 flour beetle populations.
48 Our results suggest two major selective pressures that may explain the observed inter-
49 population variation in priming: (A) Basal pathogen susceptibility – populations that were
50 more susceptible to infection produced a stronger priming response, and (B) Reproductive
51 success – populations where primed females produced more offspring had lower survival
52 benefit, suggesting a trade-off between priming response and reproduction. Our work is the
53 first empirical demonstration of multiple selective pressures that may govern the adaptive
54 evolution of immune priming in the wild. We hope that this motivates further experiments to
55 establish the role of pathogen-imposed selection and fitness costs in the evolution of priming
56 in natural insect populations.

57

58 **Introduction**

59

60 Immune priming is now regarded as an integral feature of insect immunity, where exposure to
61 low doses of a pathogen can prime the immune response and confer increased protection
62 against subsequent challenge by the same pathogen (reviewed in Little and Kraaijeveld 2004;
63 Milutinović et al. 2015; Contreras-Garduño et al. 2016). Mathematical models predict that the
64 strength of the priming response can have major implications for infection prevalence,
65 epidemiology and population stability in the wild (Tidbury et al. 2012; Best et al. 2013).
66 Thus, it is important to understand how priming ability evolves in insects and what selective
67 forces drive its evolution in natural populations. In a previous study, we reported considerable
68 variation in the priming response (13-fold survival benefit to no detectable response) among
69 wild-caught populations of the red flour beetle *Tribolium castaneum* (Khan et al. 2016).
70 However, a major unanswered question is – what evolutionary forces lead to such large
71 population level divergence in immune priming?

72

73 In general, host immune function can vary due to spatially variable strength of pathogen
74 pressure (Linhart and Grant 1996; N. Reznick and K. Ghalambor 2001; Corby-Harris and
75 Promislow 2008; Mayer et al. 2015). Classic examples of immune investment shaped by
76 parasite-mediated selection include migratory shore birds (Mendes et al. 2006) and island
77 populations of Darwin’s finches (Lindström et al. 2004), in which investment in immune
78 defence (e.g. increased production of natural antibodies) is correlated with infection
79 prevalence. In insects, encapsulation ability increases with the virulence of the pathogen
80 (Kraaijeveld and Alphen 1994), and natural populations of *Drosophila melanogaster* exposed
81 to diverse pathogen communities show an increased ability to clear bacterial infection
82 (Corby-Harris and Promislow 2008). Similarly, strong pathogen selection may also play a
83 direct role in the evolution of the insect priming response (Best et al. 2013), as we
84 demonstrated recently: immune priming evolved rapidly in laboratory selected flour beetle
85 populations exposed to a lethal dose of bacterial infection (Khan et al. 2017). Does pathogen
86 exposure select for priming in natural conditions as well? Wild populations inhabiting
87 pathogen-rich environments have an increased likelihood of reinfection by the same
88 pathogen, and should thus invest more in specific protection against those pathogens
89 (discussed in Corby-Harris and Promislow 2008). We thus reasoned that the strength of the
90 priming response in natural populations can also be determined by the severity of the
91 infection, such that populations with increased pathogen susceptibility should evolve under
92 selection for a stronger priming response.

93

94 The maintenance and deployment of immune responses may be costly (Sheldon and Verhulst
95 1996; Norris and Evans 2000), and it is possible that natural populations vary in their
96 investment in the immune system. If mounting a priming response is metabolically and
97 energetically costly, trade-offs with other fitness components may constrain the strength of
98 immune priming. Mathematical models already highlight the importance of the fitness costs
99 of priming (Tate and Rudolf 2012; Tidbury et al. 2012; Best et al. 2013), although there are
100 only a few studies that experimentally demonstrate costs of priming. For instance, primed
101 female mosquitoes (Contreras-Garduño et al. 2014) and offspring of primed tobacco
102 hornworms (Trauer and Hilker 2013) lay fewer eggs, suggesting a trade-off between priming
103 and reproduction. Maternal immune priming also prolonged offspring development time in
104 mealworm beetles (Zanchi et al. 2011), compromising their competitive ability at high density
105 (Koella and Boete 2016). Based on these results, we speculate that variable fitness costs could
106 also determine the occurrence and maintenance of priming ability in natural populations.

107

108 To elucidate the selective parameters underlying variation in priming, we analysed the
109 response of 10 natural populations of the red flour beetle *Tribolium castaneum* primed with
110 the natural pathogen *Bacillus thuringiensis* (previously described in Khan et al 2016). For
111 each of these populations, we measured the within-generation priming response, basal
112 pathogen susceptibility (without priming), and various fitness and immune components. First,
113 we asked whether the benefit of priming increases with susceptibility to infection. Second, we
114 tested whether potential fitness costs of immune priming trade off with its survival benefit.
115 Finally, we also tested whether tradeoffs with other immune responses may explain the
116 observed variation in priming responses. Our experiments were thus explicitly designed to
117 detect relationships between various life-history and ecological parameters that may drive
118 populations divergence in priming ability.

119

120 **METHODS**

121

122 **Generating experimental beetles**

123

124 We collected 10 natural populations of *Tribolium castaneum* from grain warehouses at
125 different geographical locations throughout India (described in Khan et al. 2016). We
126 reasoned that multiple factors such as individual age, mating, migration history, nutrient, and
127 local environmental factors are likely to influence variability in immune responses across
128 populations. Since it was impossible to account for all these factors, we did not measure
129 immune priming responses on individuals that were directly collected from the grain
130 warehouses. Instead, we maintained them in the laboratory on whole-wheat flour at 34 °C for

131 one year before commencing the experiments (Khan et al. 2016). To generate experimental
132 individuals for each population, we allowed 800-1000 individuals to oviposit in 350g of
133 wheat flour for 48h. We then removed the adults and collected female offspring at the pupal
134 stage (after ~3 weeks). We discarded males since handling both sexes simultaneously was
135 logistically challenging. We housed 3 female pupae in 2 ml micro-centrifuge tubes containing
136 1g flour for 12 days. Separately, we also collected larval offspring after 10 days. Since the
137 pupal stage lasts for 3-4 days and eggs usually hatch in 2 days, we obtained 8-day-old adults
138 and 8-day-old larvae were for all our experiments.

139

140 **Immune priming and challenge**

141

142 We used the natural insect pathogen *Bacillus thuringiensis* to measure within-generation
143 priming for all populations as described in Khan et al (2016). Briefly, we pricked adults
144 between the head and thorax, and larvae between the last and last but one segment, using a
145 0.1mm insect pin (FST, CA) dipped in heat-killed bacterial slurry (i.e. priming) or insect
146 Ringer (i.e. sham priming control). We made bacterial slurry from 10 ml freshly grown
147 overnight culture of *B. thuringiensis* at 30°C (optical density of 0.95; adjusted to $\sim 10^{11}$ cells
148 in 1 ml insect Ringer solution). We used heat-killed cells to prime beetles because this would
149 induce an immune response without a direct cost of virulence. After this, we isolated
150 experimental beetles (i.e. adults or larvae) in wells of 96-well microplates (Corning)
151 containing wheat flour. Six days later, we checked their mortality and then again challenged
152 individuals with live bacterial culture adjusted to $\sim 10^{10}$ ml⁻¹ (delivering ~ 900 bacterial cells
153 per beetle). We did not find any mortality after priming and before live pathogen challenge.
154 Control beetles received mock priming followed by a mock challenge with insect Ringer.
155 After the immune challenge, we immediately returned experimental beetles from each
156 population to wells of fresh 96-well micro plates and measured various traits as described
157 below (also see Figure 1). Since we used a low dose of infection (compare with Khan et al.
158 2016), we observed a late onset of post-infection mortality. For instance, while a few infected
159 larvae (<1%) died before pupation in some populations, there were no deaths during the adult
160 stage until 23 days post-eclosion. We also did not observe any mortality in adults until a week
161 after infection.

162

163 **Joint assay of basal pathogen susceptibility, survival benefit of priming and changes in** 164 **reproductive output after priming**

165

166 One day after the immune challenge, we paired a subset of adult females with uninfected, 8-
167 day-old virgin males from the respective population for 48 hours in a 1.5 ml microcentrifuge

168 tube containing 1 g flour (one pair of beetles per tube). We then separated females to measure
169 the total number of offspring produced by each female (48 h of oviposition; eggs allowed to
170 develop in 6 g flour for 3 weeks). Following this, we returned mated females to 96-well
171 microplates with flour, and noted their survival every 3-5 days for another ~117 days (total
172 120 days post-challenge), transferring them to fresh microplates with food every 5 days to
173 minimize the interaction between females and new offspring (n=15-22
174 females/treatment/population). For larvae, we isolated primed and challenged individuals in
175 their respective wells until they pupated. Subsequently, we identified and retained only
176 female pupae. Fifteen days post-eclosion, we paired each adult female with a virgin male as
177 described above. We allowed females to oviposit for 24 hours and recorded their mortality
178 every 3-5 days for another 100 days (total ~130 days post-challenge) as described above
179 (n=22-28 females/treatment/population). This procedure allowed us to obtain a correlated
180 dataset for early reproductive success and survival of each experimental female after priming
181 and challenge. A few replicate plates for reproductive output after adult priming were
182 accidentally lost during the experiment. Hence, the sample size for the individual correlation
183 was lower than expected (See Table S1).

184
185 The residuals for reproductive success were not normally distributed (tested with Shapiro-
186 Wilks test). We thus used nonparametric Wilcoxon Rank Sum tests to test the impact of larval
187 and adult priming on reproductive success. We quantified the impact of priming on
188 reproductive output as: Mean number of offspring produced by primed females / Mean
189 number of offspring produced by unprimed females. We analyzed post-immune challenge
190 survival data for each population and life stage separately using Cox proportional hazard
191 survival analysis with priming as a fixed factor and lifespan in days as the response variable.
192 We considered beetles that were still alive at the end of the experiment as censored values.
193 For each population and life stage, we calculated pathogen susceptibility as the estimated
194 hazard ratio of unprimed vs. control groups (Rate of deaths occurring in unprimed group /
195 Rate of deaths occurring in full control group). A hazard ratio significantly greater than one
196 indicates an enhanced risk of mortality in unprimed groups compared to control individuals.
197 To estimate the strength of the priming response, we calculated the survival benefit to the host
198 after infection, with vs. without previous exposure to the same pathogen (Rate of deaths
199 occurring in unprimed group / Rate of deaths occurring in primed group). A hazard ratio
200 significantly greater than one indicates an enhanced risk of mortality in unprimed groups
201 compared to primed individuals..

202

203 **Separate assays to measure the impact of priming on development rate, lifespan under**
204 **starvation and immune components**

205

206 In these assays, we did not re-measure the immune priming response in terms of survival
207 benefit after infection, since this was already measured for each population as described
208 above. Instead, we directly measured the impact of priming (i.e. compared primed vs.
209 unprimed groups) for the following fitness and immune components.

210

211 (1) Lifespan under starvation: We first tested whether priming affects lifespan under
212 starvation in different populations. To do this, we isolated a subset of virgin females and
213 larvae individually in 96-well microplates without food ($n = 16$ -
214 20/lifestage/treatment/population) immediately after immune challenge. We noted their
215 mortality every 12 hours (10 am & 10pm ± 1 hour) until all of them died. We quantified
216 the impact of priming (primed vs. unprimed) on lifespan under starvation using hazard
217 ratios, as described above. A hazard ratio significantly greater than one would suggest a
218 higher risk of mortality in primed groups compared to unprimed individuals. Due to
219 logistical reasons, we could only measure starvation resistance of larvae from 7
220 populations (except B1, AM and ND; described in Khan et al 2016).

221

222 (2) Developmental rate: To measure the effect of priming on larval development, we placed
223 immune-challenged experimental larvae individually in 96-well microplates ($n = 21$ -22
224 larvae/treatment/population). We observed larvae every 12 hours (11 am & 11pm ± 1
225 hour) and noted the time to pupation for each larva. We analyzed the data (non-normally
226 distributed) using nonparametric Wilcoxon Rank Sum tests and calculated the impact of
227 priming on larval development as: Mean time to pupation of primed larvae / Mean time to
228 pupation of unprimed larvae.

229

230 (3) Immune components: To measure aspects of immune function, we first primed and
231 challenged adult females from each population (described above). Next day, we used a
232 subset of females to quantify antibacterial activity of beetle hemolymph ($n = 9$ -
233 15/treatment/population) (see Khan et al. 2015 for detailed methods). Briefly, we
234 measured the zone of inhibition produced by beetle homogenates on a lawn of *B.*
235 *thuringiensis* growing on nutrient agar medium. Flour beetles also secrete defensive
236 quinone compounds that inhibit the microbial growth in their surroundings, acting as an
237 external immune defense (Joop et al. 2014, Khan et al. 2015). To quantify this immune
238 response, we measured the zone of inhibition produced by cold-shocked females (-86°C
239 for 20 minutes) embedded vertically in a lawn produced by *B. thuringiensis* growth on
240 nutrient agar plates ($n = 9$ -10 females/treatment/population). A cold shock triggers the
241 release of abdominal and thoracic stink gland contents with antimicrobial quinones (Khan

242 et al. 2015). We analyzed the non-normally distributed immune response data using
243 nonparametric Wilcoxon Rank Sum tests and estimated the impact of priming on immune
244 components as: Mean zone of inhibition produced by primed females / Mean zone of
245 inhibition produced by unprimed females.

246

247 **RESULTS**

248

249 The strength of immune priming is usually quantified as the survival benefit to the host after
250 infection, with vs. without previous exposure to the same pathogen (Roth et al. 2010;Khan et
251 al. 2016). Hence, we quantified the strength of the priming response as the proportional
252 hazard ratio estimated from survival data for primed vs. unprimed individuals, all
253 subsequently infected with live bacteria. In most populations, both larval and adult survival
254 increased significantly after priming (adults: 9/10 populations, larvae: 8/10 populations; **Fig.**
255 **2A**; see **Figs. S1 & S2** for survival curves). As reported earlier (Khan et al. 2016), the
256 priming response also varied substantially across populations, ranging from no detectable
257 response to a 10-fold increase in larval post-infection survival relative to the unprimed
258 control. We found that this variation was strongly associated with susceptibility to infection,
259 measured as the hazard ratio for infected vs. uninfected groups (**Fig. 2A**). These results are
260 consistent with the prediction that the severity of infection may determine the strength of
261 immune priming in insect populations (Best et al. 2013) – more susceptible populations may
262 face stronger selection for evolving priming response.

263

264 The costs of mounting a priming response can also vary across natural populations, in turn
265 limiting the expression of priming. To test this hypothesis, we analyzed the impact of priming
266 on multiple fitness-related traits: reproduction, larval development, lifespan under starvation,
267 and other immune components. As predicted, we found a negative correlation between the
268 strength of adult immune priming and subsequent change in reproductive fitness, though there
269 was no such association for larvae (**Fig. 2B**). Contrary to the cost hypothesis, we found that
270 only two populations showed a significant decrease in reproductive fitness after adult priming
271 (i.e. significant reproductive impact <1). In most populations, females either produced more
272 offspring after priming (5/10) or showed no detectable change in reproduction (3/10) (**Fig. 2B**
273 **& S3**). Similarly, for larval priming, none of the populations showed a significant cost of
274 reproduction (**Fig. S3**). Together, these results suggest that although priming generally does
275 not impose a reproductive cost, it may induce a stage-specific trade-off between survival vs.
276 reproductive benefits.

277

278 Overall, priming did not affect starvation lifespan, except for a few populations where it
279 either prolonged (Larvae: 2/7 populations; Adults: 1/10 populations) or reduced lifespan
280 (Larvae: 1/7 populations; Adults: 1/10 populations) (see **Fig. S4 & S5** for survival curves).
281 Although there was no consistent association between larval priming and lifespan under
282 starvation, our data suggest a potential relationship between adult investment in priming vs.
283 starvation lifespan. Individuals from the population that lacked a priming response lived twice
284 as long under starvation, whereas adult beetles from the population showing the strongest
285 priming response (~5-fold survival benefit) died faster under starvation (**Fig. 2C**). These
286 results suggest a possible cost of priming response in terms of depleting energy reserves, in
287 turn reducing lifespan under starvation.

288

289 We found that immune priming had a contrasting effect on two components of adult
290 immunity. While priming consistently reduced external immunity in several populations
291 (5/10), its impact on antibacterial activity was highly variable (**Fig. S6**). Priming had no
292 impact on antibacterial activity in most populations (7/10), except a few of them where
293 primed females produced either larger (2/10) or smaller zones of inhibition (1/10) than
294 unprimed controls. None of the immune components was correlated with the observed
295 variation in priming response across populations (**Fig. 2D**).

296

297 Finally, we tested whether larval immune priming traded off with larval development rate
298 across populations. We found that immune priming did not alter larval development except in
299 two populations, where larvae either developed faster (AG) or showed delayed development
300 (ND) compared to controls (**Fig. S7**). Thus, developmental rate of primed larvae could not
301 explain population level variation in larval priming response (**Fig. 2F**).

302

303 **DISCUSSION**

304

305 We present the first systematic test of multiple factors that may determine the evolution of the
306 strength of immune priming in natural insect populations. Previously, we documented large
307 variation in the priming response among wild-caught flour beetle populations (Khan et al.
308 2016), ranging from no detectable response to a 13-fold survival benefit in some populations.
309 Although this work provided an empirical framework to understand whether and to what
310 degree priming responses vary in natural populations, the selective forces responsible for this
311 variability remained unclear. Previous theoretical work suggests that the strength of the
312 priming response may depend on the strength of selection imposed by pathogens (Tate and
313 Rudolf 2012; Best et al. 2013), as well as constraints imposed by the cost of immune priming
314 via tradeoffs with other fitness components. Our results are consistent with both types of

315 selection. First, we show that the bacterial pathogen *B. thuringiensis* has a variable impact in
316 different populations, suggesting that the same pathogen can impose divergent selection
317 pressure across populations of the same host species. Subsequently, we find that increased
318 susceptibility to *B. thuringiensis* is positively correlated with an increased survival benefit of
319 immune priming, such that priming is most beneficial for populations that are highly
320 susceptible to infection. This indicates that the pathogen-mediated reduction in lifespan may
321 act to increase selection for priming response in natural populations. Second, we found a life
322 stage-specific negative relationship (a possible trade-off) with reproductive success that may
323 constrain the strength of priming. When primed and infected adult females produced more
324 offspring than unprimed controls (i.e. priming increased reproduction), they showed a
325 reduced survival benefit of priming. Thus, the most important implication of our work is that
326 both specific fitness costs and the fitness impact of infection can determine variability in
327 priming, as well as reflect conditions that may favor the evolution of stronger priming.

328

329 A notable strength of our study is the use of multiple natural populations to gain deeper
330 insights into the evolutionary and ecological history of priming in an insect. Our results
331 indicate a general adaptive role of immune priming such that in many populations, priming
332 not only improves long-term lifespan but also leads to an immediate gain in reproductive
333 effort. This seems to contradict a mathematical model that predicts large reproductive costs
334 associated with priming (Best et al. 2013). However, a careful comparison across populations
335 revealed that immune priming does not improve survival and reproduction equally in a
336 population. Instead, greater benefits of reproduction come at the cost of reduced survival,
337 suggesting a broadly distributed hidden trade-off between these traits. A recent report in
338 mosquitoes also showed that primed females that invested more in egg production show
339 reduced pathogen clearance and greater susceptibility to infection (Contreras-Garduño et al.
340 2014). Such parallel results from intra- vs. inter- population studies suggest the existence of
341 trade-offs at multiple levels. Our data also suggest a weak negative association between
342 priming and starvation resistance. Although priming had no impact on starvation resistance in
343 most populations, populations where priming maximized survival benefit after infection (i.e.
344 ND) or showed no priming response (i.e. AG) also had respectively reduced or increased
345 starvation resistance. Previous studies have documented trade-offs between immune
346 investment and lifespan during starvation in fruit flies (Valtonen et al. 2010) and bumble bee
347 workers (Moret and Schmid-Hempel 2000). In fruit flies, trade-off between immunity and
348 starvation resistance may also have a genetic basis: genotypes that invest more in immunity
349 have lower survival under starvation and vice versa (Hoang 2001). However, it is currently
350 unclear whether such phenotypic or genetic trade-offs are also widespread with respect to

351 priming ability and lifespan during starvation. Thus, we suggest that our experiments uncover
352 an exciting possibility that requires further work.

353

354 What are the mechanisms underlying the potential trade-offs we observed? Both endocrine
355 signaling (e.g. via juvenile hormone and ecdysone) and altered lipid metabolism via
356 insulin/insulin-like growth factor-like signalling pathway (IIS) can mediate physiological
357 trade-offs between immunity and major fitness components (Schwenke et al. 2016; Schwenke
358 and Lazzaro 2017). A previous study in fruit flies suggests that immune activation via the
359 Toll signaling pathway in the fat body (the major immune and lipid storage organ in insects)
360 inhibits insulin signaling activity (DiAngelo et al. 2009). Reduced insulin signalling after
361 infection could further lead to decreased lipid storage (DiAngelo et al. 2009), increased
362 haemolymph lipid concentration (Cheon et al. 2006) and finally, reallocation of energy
363 utilisation to immune response from reproduction (Clancy 2001) or starvation resistance
364 (Beenackers et al. 1986). Interestingly, recent studies suggest that activation of *Toll* pathways
365 and lipid mobilisation are equally important for mounting a successful immune priming
366 response in fruit flies (Pham et al. 2007) and mosquitoes (Ramirez et al. 2015). This sets up
367 the possibility that similar signalling mechanisms are also responsible for the observed
368 negative relationship between immune priming and fitness components in flour beetles.
369 Further studies may provide greater mechanistic insight into how early reproductive success
370 or starvation resistance can alter later immune priming response.

371

372 Another interesting finding of our study is that although priming delayed or accelerated larval
373 development in some populations, it did not explain the observed population level variation in
374 priming response. Previous studies using a single population of different insect species found
375 that immune activation in larvae can accelerate development (Roth and Kurtz 2008) and
376 maternal priming can either accelerate (Tate and Graham 2015) or reduce (Zanchi et al. 2011)
377 offspring development rate. However, ours is the first study that documents the differential
378 impact of immune priming on development rate across populations of the same species.
379 Therefore, an additional implication of our work is that studies using a single population are
380 insufficient to generalize the costs or benefits of priming because priming has variable
381 consequences across populations.

382

383 Multiple lines of evidence suggest that priming is beneficial because it induces more efficient
384 immune responses (reviewed in Milutinović et al. 2016). In contrast, our results present a
385 more complicated scenario – priming increased antibacterial activity only in a few
386 populations, and most populations with large survival benefit of priming did not always
387 increase antibacterial activity. Overall, antibacterial activity did not explain the observed

388 variation in priming response, highlighting that the association between innate immune
389 responses and survival after priming may not be straightforward in wild populations. This is
390 surprising since previous studies with *Tribolium* beetles found that priming with *B.*
391 *thuringiensis* increases expression of a large set of immune related genes (Greenwood et al.
392 2017), correlating strongly with survival after reinfection (Milutinović et al. 2013). We note
393 that whereas our hemolymph antibacterial activity assay may reflect the impact of several
394 immune pathways such as antimicrobial peptides, it is possible that other aspects of innate
395 immunity such as cellular defence (e.g. circulating hemocytes) play a more important role in
396 priming in natural populations (Rodrigues et al. 2010).

397

398 Finally, we note a novel result where priming exerts opposite effects on different aspects of
399 beetle immunity, e.g. antibacterial activity (internal immunity) of the hemolymph vs. quinone
400 secretion outside the body (external immunity, see Joop et al. 2014). In contrast to its variable
401 impacts on antibacterial activity, priming consistently reduced external immune function in
402 several populations, suggesting a crosstalk between priming and quinone production
403 pathways. Although these results are broadly similar to our previous experiment where
404 bacterial infection reduced quinone production in virgin females (Khan et al. 2015), it did not
405 explain the variation in priming response across populations. We suggest further
406 manipulative experiments to disentangle the complex interaction between priming, innate
407 immune pathways and quinone production in flour beetles.

408

409 Overall, our data highlight the importance of explicitly testing the impact of pathogen
410 selection and fitness costs on the immune system of wild populations. While our results
411 provide valuable insight into the macro-evolutionary patterns of priming evolution, a
412 significant limitation is the lack of information on local pathogen pressure that our beetle
413 populations experienced before we brought them into the laboratory. Recent evidence
414 suggests that *Drosophila* populations previously exposed to multiple pathogens are more
415 resistant to a novel *Lactobacillus lactis* infection (Corby-Harris and Promislow 2008).
416 Increased resistance to subsequent *L. lactis* infection can also be selectively favored by
417 previous exposure to conspecific *Lactococcus* species. Similarly, it is possible that our beetle
418 populations have already encountered variable selection imposed by widely distributed
419 natural pathogens such as *B. thuringiensis* in their natural habitat. Therefore, the observed
420 responses against experimental manipulations (e.g. priming and/or live pathogen exposure)
421 can also be influenced by previously experienced pathogen selection. Indeed, at the molecular
422 level, many immune-related genes in insects show signs of strong selection, suggesting rapid
423 coevolution with pathogens (Lazzaro et al. 2006; Sackton et al. 2007).

424

425 In conclusion, we suggest that our work serves as an important first step towards
426 understanding whether and why natural populations of insects differ in their immune priming
427 response. Previously, we used experimental evolution to show that strong pathogen selection is
428 necessary to evolve immune priming in laboratory populations of flour beetles (Khan et al.
429 2017). However, it remains unclear whether wild populations also show a similar response.
430 We suggest future experiments where susceptible wild populations may be allowed to evolve
431 under different levels (low to high) of selection imposed by the pathogen. Such experiments
432 will allow us to directly test for a positive correlation between evolved priming response and
433 the level of pathogen selection, as well as associated evolutionary fitness costs.

434

435 **AUTHOR CONTRIBUTIONS**

436

437 IK conceived experiments; IK, DA and AP designed experiments; AP and IK carried
438 out experiments; IK analyzed data; IK and DA wrote the manuscript with input from
439 AP. All authors gave final approval for publication.

440

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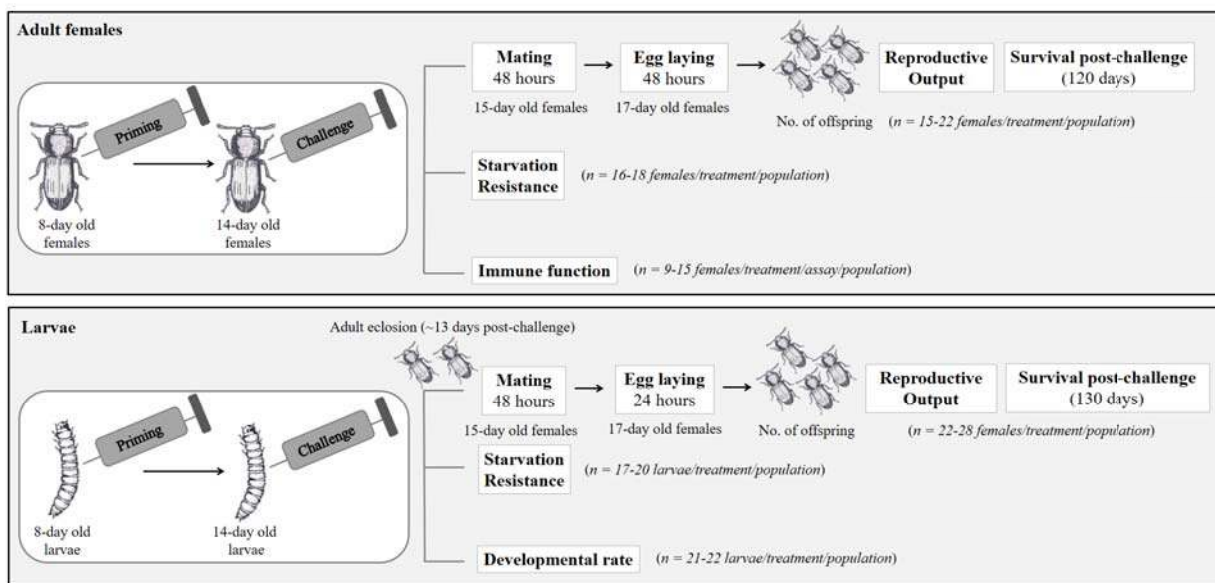
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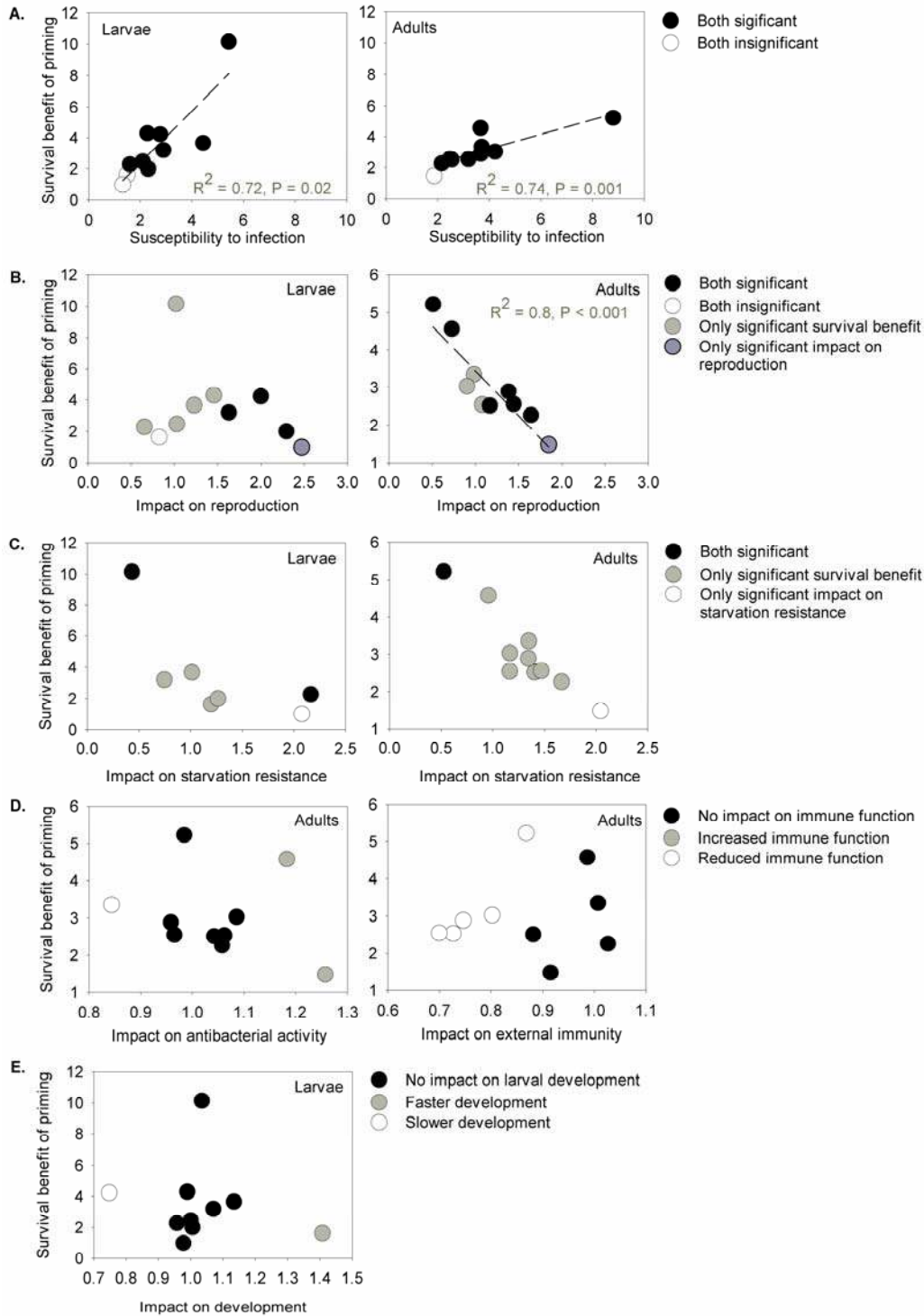
562 **Figure 1. Experimental design**



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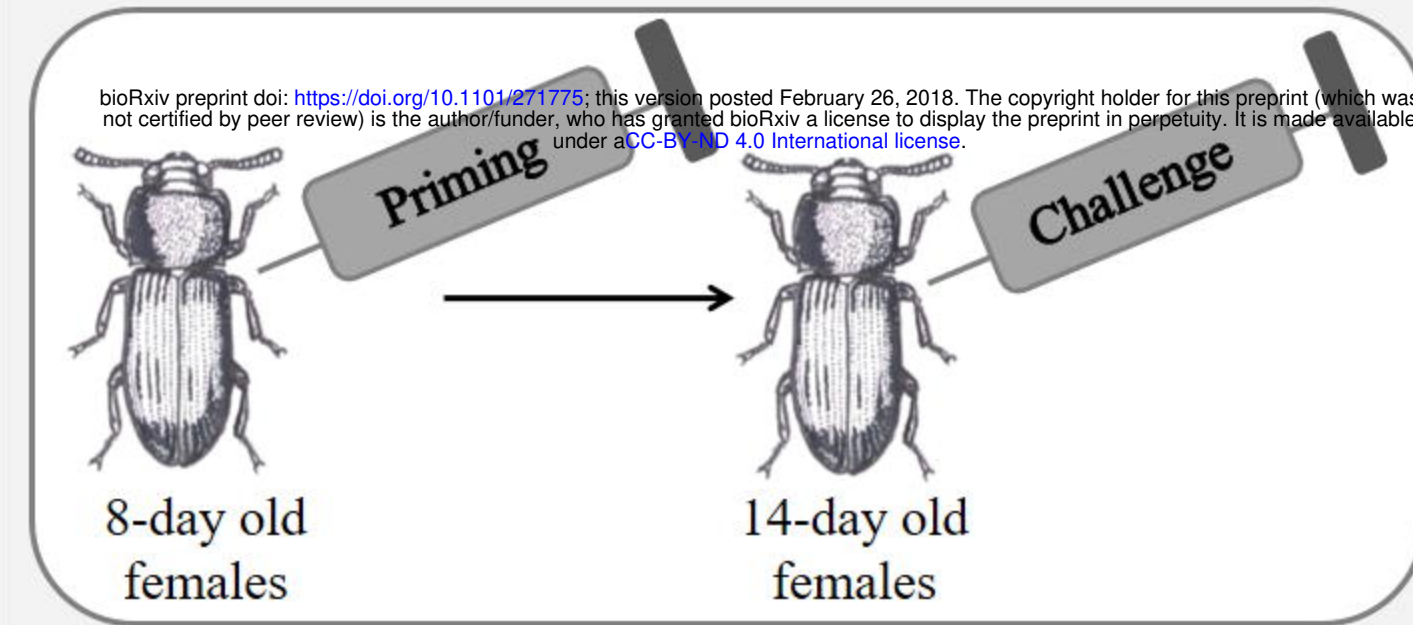
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565 **Figure 2.** Correlation between the survival benefit of immune priming and (A) basal
 566 susceptibility to infection (B) reproductive benefit of immune priming; impact on (C)
 567 starvation resistance (D) antibacterial activity (E) external immunity and (F) larval
 568 developmental rate.
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Adult females



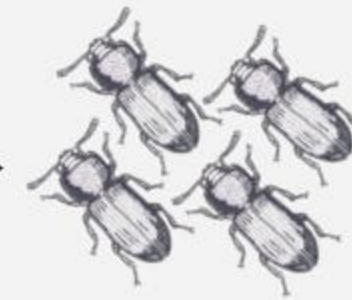
Mating
48 hours

15-day old females



Egg laying
48 hours

17-day old females



No. of offspring ($n = 15-22$ females/treatment/population)

Reproductive Output

Survival post-challenge
(120 days)

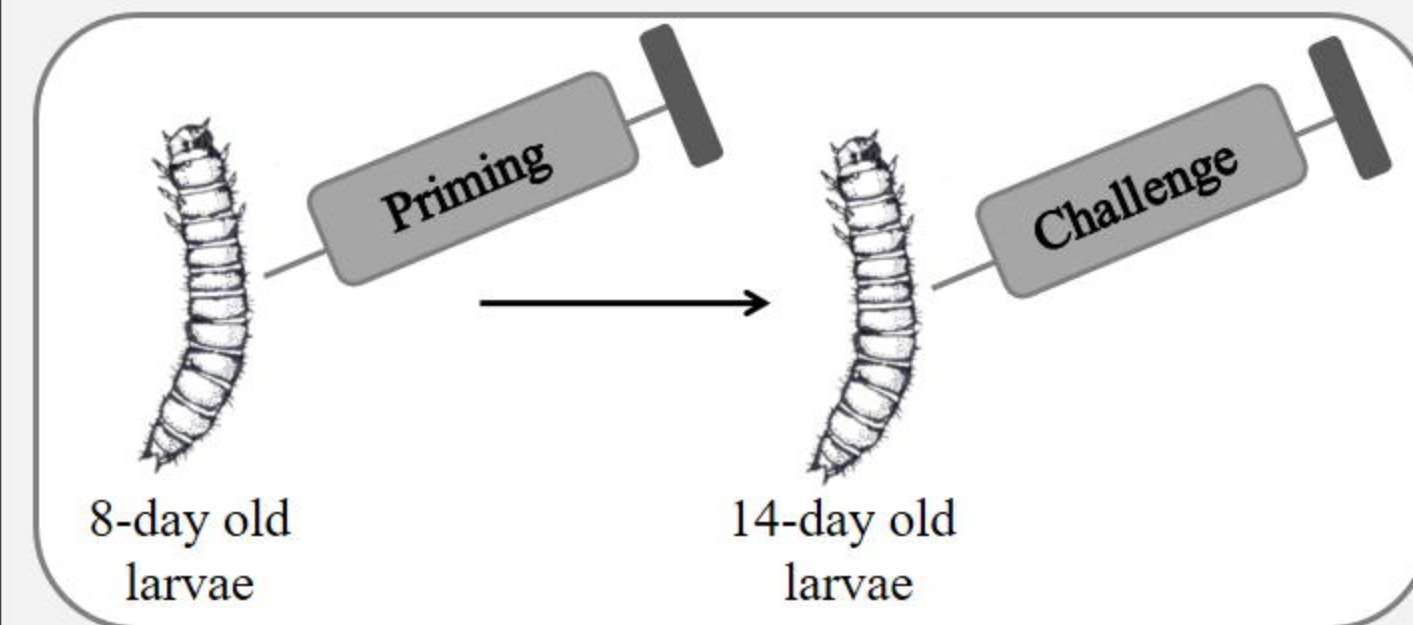
Starvation Resistance

($n = 16-18$ females/treatment/population)

Immune function

($n = 9-15$ females/treatment/assay/population)

Larvae



Adult eclosion (~13 days post-challenge)



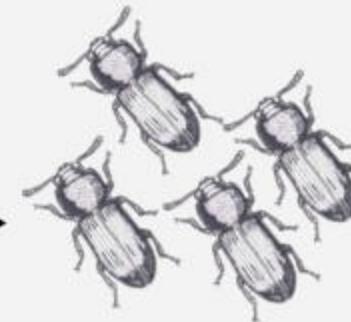
Mating
48 hours

15-day old females



Egg laying
24 hours

17-day old females



No. of offspring

Reproductive Output

Survival post-challenge
(130 days)

($n = 22-28$ females/treatment/population)

Starvation Resistance

($n = 17-20$ larvae/treatment/population)

Developmental rate

($n = 21-22$ larvae/treatment/population)

