

1 **DIVERGENT IMMUNE PRIMING RESPONSES ACROSS FLOUR BEETLE**

2 **LIFE STAGES AND POPULATIONS**

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21 **RUNNING TITLE:**

22 Immune priming in natural insect populations

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28 ABSTRACT

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30 Growing evidence shows that low doses of pathogens may prime the immune
 31 response in many insects, conferring subsequent protection against infection in the
 32 same developmental stage (within life stage priming), across life stages (ontogenic
 33 priming), or to offspring (trans-generational priming). Recent work also suggests that
 34 immune priming is a costly response. Thus, depending on host and pathogen ecology
 35 and evolutionary history, tradeoffs with other fitness components may constrain the
 36 evolution of priming. However, the relative impacts of priming at different life stages
 37 and across natural populations remain unknown. We quantified immune priming
 38 responses of 10 natural populations of the red flour beetle *Tribolium castaneum*,
 39 primed and infected with the natural insect pathogen *Bacillus thuringiensis*. We found
 40 that priming responses were highly variable both across life stages and populations,
 41 ranging from no detectable response to a 13-fold survival benefit. Comparing across
 42 stages, we found that ontogenic immune priming at the larval stage conferred
 43 maximum protection against infection. Finally, we found that various forms of
 44 priming showed sex-specific associations that may represent tradeoffs or shared
 45 mechanisms. These results suggest that sex-, life stage-, and pathogen- specific
 46 selective pressures can cause substantial divergence in priming responses even within
 47 a species. Our work highlights the necessity of further work to understand the
 48 mechanistic basis of this variability.

49

50 **Keywords:** Within generation immune priming, ontogenic immune priming, trans-
 51 generational immune priming, wild populations, variability, *Tribolium castaneum*

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53 INTRODUCTION

54

55 Immunologists have long assumed that insects lack immune memory and specificity
 56 because they do not have the lymphocytes and functional antibodies that are
 57 responsible for acquired immunity in vertebrates (Janeway & Medzhitov, 2002).
 58 However, growing evidence suggests that a low dose of a pathogen may prime the
 59 immune response in insects, reducing the risk and severity of infection by the same
 60 pathogen later in life. Evidence for such priming-induced immune protection has been
 61 reported in many insects including mealworm beetles (Daukšte *et al.*, 2012), bumble
 62 bees (Sadd & Schmid-Hempel, 2006; Tidbury *et al.*, 2011), silkworms (Miyashita *et*
 63 *al.*, 2014), fruit flies (Pham *et al.*, 2007), mosquitoes (Contreras-Garduño *et al.*, 2014)
 64 and flour beetles (Roth *et al.*, 2009). Immune priming can also confer sustained
 65 protection via (A) ontogenic priming, where the benefit of priming can persist through
 66 metamorphosis (Thomas & Rudolf, 2010; Moreno-García *et al.*, 2015) and (B) trans-
 67 generational immune priming, where the benefits are manifested in the next
 68 generation (Sadd & Schmid-Hempel, 2006; Sadd & Schmid-hempel, 2009; Moreau *et*
 69 *al.*, 2012; Zanchi *et al.*, 2012; Dubuffet *et al.*, 2015). Theoretical models show that
 70 within- and trans- generational immune priming can significantly alter pathogen
 71 persistence (Tidbury *et al.*, 2012) and reduce infection intensity in populations (Tate
 72 & Rudolf, 2012). Thus, it is clear that immunological memory is widespread in
 73 insects, and immune priming may have large impacts on the outcome of host-
 74 pathogen interactions.

75 Although we have begun to understand immune priming in many insects, it is not
 76 clear how priming evolves. This is partly because the strength, consistency and
 77 relevance of immune priming in natural populations remains largely unexplored and

78 is difficult to gauge from laboratory studies. Other aspects of immune function (post-
79 infection survival and encapsulation ability) vary across fruit fly populations
80 (Kraaijeveld, 1995; Corby-Harris & Promislow, 2008), and parasite burden is strongly
81 correlated with the strength of the innate immune response across damselfly
82 populations (Kaunisto & Suhonen, 2013). Similarly, immune priming responses may
83 also vary across natural populations. In laboratory populations, immune priming is
84 affected by the presence of other pathogens (Sadd & Schmid-hempel, 2009) and food
85 availability (Freitak *et al.*, 2009). However, the impact of these factors on immune
86 priming in natural populations is unknown. Wild populations likely face substantial
87 spatial and temporal variation in pathogen diversity, pathogen abundance, and
88 resource availability, generating variability in the strength of selection on immune
89 priming. Priming also imposes fitness costs in some laboratory populations
90 (Contreras-Garduño *et al.*, 2014), potentially generating tradeoffs with other immune
91 responses, or between different types of immune priming. Finally, these fitness costs
92 may also vary as a function of sex and developmental stage. For instance, life-history
93 theory predicts that females should generally evolve higher immune competence than
94 males (Rolff, 2002; Nunn *et al.*, 2009); hence, males may gain more benefits from
95 priming than females (Moreno-García *et al.*, 2015). Similarly, variable costs of
96 infection across life stages are also predicted to select for stronger priming responses
97 at specific developmental stages (Tate & Rudolf, 2012). A detailed analysis of such
98 variability can indicate factors that influence the evolution of immune priming.
99 Unfortunately, very few studies have quantified priming in wild insect populations
100 (but see (Reber & Chapuisat, 2012) (ants), (Gonzalez-Tokman *et al.*, 2010)
101 (damselflies), and (Tate & Graham, 2015) (closely related flour beetle species)), and

102 none have measured variation in priming responses across multiple natural
103 populations.

104

105 We systematically analyzed immune priming responses of 10 populations of the red
106 flour beetle *Tribolium castaneum* collected from different locations across India (Fig
107 S1). In the laboratory, flour beetles show within life stage (WLS) (Roth *et al.*, 2009),
108 ontogenic (ONT) (Thomas & Rudolf, 2010) and trans-generational (TG) immune
109 priming (Roth *et al.*, 2010), making them an ideal model system to understand the
110 occurrence and abundance of these different types of immune priming responses. We
111 addressed three major questions: (a) Does the immune priming response vary across
112 natural populations and as a function of sex and life stage? (b) Are the different types
113 of priming responses equally beneficial? (c) Are the different types of immune
114 priming responses correlated? Our work is the first report of large within-species
115 variability of priming response across sexes and life stages in natural insect
116 populations. We found that ontogenic immune priming provided greater protection
117 against re-infection, compared to within life stage or trans-generational priming.
118 Finally, our data reveal novel sex-specific links between various forms of immune
119 priming, perhaps representing tradeoffs or even shared mechanistic basis. We hope
120 that our results motivate further investigations to confirm and understand the
121 ecological, evolutionary and mechanistic basis of the observed variability and
122 associations between priming at different stages.

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127 **METHODS**

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129 **Beetle collection and experimental individuals**

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131 Although immune priming responses should be measured on individuals directly
 132 collected from the wild (i.e. grain warehouses), this is difficult to do for the following
 133 reasons. First, natural beetle populations do not always have enough individuals of
 134 different stages to allow sufficient replication. Second, it is impossible to account for
 135 the many factors that may increase within-population variability in immune
 136 responses, such as individual age, migration and diet history, and immediate local
 137 environment. Controlling for within-population variability in immune priming is
 138 essential to quantify variability between populations, which was the major goal of our
 139 study. Hence, we established large laboratory populations using wild-collected beetles
 140 (maintaining most of the initial genetic variability), and then quantified the immune
 141 priming response of individuals of the same age reared under identical conditions. We
 142 collected 50-100 *T. castaneum* adults from a grain warehouse in each of 9 cities
 143 across India. Of the 10 populations analyzed here, 8 were from different cities and 2
 144 were collected from different warehouses in a single city (Fig S1). We allowed all
 145 adults from a site to oviposit for a week on whole-wheat flour at 34°C to start a large
 146 laboratory population (>2000 individuals). We maintained these stock populations on
 147 a 45-day discrete generation cycle for 9-10 generations before starting experiments.

148

149 To generate experimental individuals of equivalent age from all populations, we
 150 allowed ~1000 adults from each population to oviposit in 350 g wheat flour for 48
 151 hours. We removed the adults and allowed offspring to develop for ~3 weeks until

152 pupation, collecting pupae daily after this period. We housed 3-4 pupae of each sex
 153 separately in 2 ml micro-centrifuge tubes containing 1 g flour for 2 weeks. Since
 154 pupae typically eclose in 3-4 days, we obtained ~11-day-old sexually mature virgin
 155 adults for immune priming experiments. For experiments with larvae, we allowed
 156 adults to oviposit in 350 g flour for 24 hours and collected larvae after 10 days (eggs
 157 hatch in 2-3 days; thus, experimental larvae were ~8 days old). In a separate
 158 experiment, we found that eggs from all populations developed at a similar rate (Fig
 159 S2), confirming that we tested all populations at equivalent developmental stages.

160

161 Immune priming and challenge

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163 For each type of immune priming, we tested all populations together to allow a direct
 164 comparison across populations. However, given logistical constraints, we had to test
 165 males and females in separate blocks. Note that we only measured maternal TG
 166 immune priming in our experiments, and did not measure paternal TG priming. The
 167 timeline for each type of immune priming is given in Fig 1 (see supplementary
 168 information for detailed methods). For all infections, we used a strain of *Bacillus*
 169 *thuringiensis* (DSM. No. 2046). Originally isolated from a Mediterranean flour moth,
 170 this is a natural insect pathogen that imposes significant mortality in flour beetles
 171 (Abdel-Razek *et al.*, 1999). On the evening before priming, we inoculated 10 ml
 172 nutrient broth (Difco) with cells from a -80°C stock of *B. thuringiensis*. We incubated
 173 the growing culture overnight in a shaker at 30°C until it reached an optical density of
 174 0.95 (measured at 600 nm in a Metertech UV/Vis Spectrophotometer, SP8001). We
 175 centrifuged the culture at 5000 rpm for 10 minutes, removed the supernatant, and
 176 resuspended the pellet in 100µl insect Ringer solution (7.5g NaCl, 0.35g KCl, 0.21g

177 CaCl₂ per liter) to make bacterial slurry. We killed the bacteria in a heat block at 90°C
178 for 20 minutes as described earlier (Roth *et al.*, 2009; Khan *et al.*, 2015). We used
179 heat-killed bacteria to prime individuals, since this would elicit an immune response
180 without any direct cost of infection.

181

182 To prime individuals, we pricked them with a 0.1 mm minuten pin (Fine Science
183 Tools, Fosters City, CA) dipped either in heat-killed bacteria (primed) or in sterile
184 insect Ringer solution (control). To minimize damage to internal organs we pricked
185 individuals laterally between the head and thorax (adults) or between the last two
186 segments (larvae). After priming (or mock priming), we isolated individuals in wells
187 of 96-well microplates containing flour. When appropriate, we sexed pupae and
188 distributed them individually in wells of 96-well microplates. For subsequent immune
189 challenge, we pricked individuals as described above, but used live bacterial slurry
190 (without heat-killing). After this, we again isolated individuals in fresh microplates
191 and monitored their survival (See Fig 1 for timeline).

192

193 **Data analysis**

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195 We analyzed post-infection survival data for each population, sex and immune
196 priming type separately using Cox Proportional Hazard survival analysis with priming
197 treatment as a fixed factor (see Figs S3-S11 for survival curves). We noted
198 individuals that were still alive at the end of the experiment as censored values. We
199 calculated the strength of a given type of immune priming response within each
200 population (and sex) as the estimated hazard ratio of unprimed vs. primed groups
201 (hazard ratio = rate of deaths occurring in unprimed group/ rate of deaths occurring in

202 primed group). A hazard ratio significantly greater than one indicates a greater risk of
203 death after infection in the unprimed (control) compared to primed individuals.

204

205 To estimate the overall impact of sex on the immune priming response, we analyzed
206 hazard ratios using a two-way ANOVA with sex and type of immune priming as fixed
207 factors. We excluded data from larval within life stage priming (L-WLS) because sex
208 cannot be distinguished in larvae. To test whether the strength of the priming response
209 varies as a function of life stage at priming (larvae vs. adults), we analyzed hazard
210 ratios with a one-way ANOVA. Finally, to compare the strength of priming across
211 different stages (Fig 1), we analyzed data with a one-way ANOVA and used Tukey's
212 honest significant difference (HSD) to estimate pairwise differences after correcting
213 for multiple comparisons.

214

215 We also wanted to test whether the strength of immune priming responses was
216 correlated across types of priming. However, several populations did not show a
217 significant immune priming response; hence, we could not use a linear regression
218 approach. Therefore, we generated a contingency table, categorizing each population
219 according to the presence (proportional hazard test: $p < 0.05$) or absence (proportional
220 hazard test: $p > 0.05$) of each type of priming response (also see Figs S3-S11). We
221 then used a Fisher's exact test to determine whether the presence of the two types of
222 immune priming was qualitatively associated across populations.

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227 **RESULTS**

228

229 **The immune priming response varies across populations**

230

231 We estimated the strength of immune priming as the proportional hazard ratio of
 232 individuals mock-primed with sterile Ringer solution vs. primed with a pathogen
 233 (heat-killed *B. thuringiensis*), followed by a subsequent infection with live *B.*
 234 *thuringiensis*. Surprisingly, we found that only about half the populations showed
 235 significant priming at a given stage, although all populations were capable of
 236 mounting multiple forms of immune priming (Fig 2). The immune priming response
 237 varied substantially in larvae as well as adult males and females across natural
 238 populations (Fig 2; Figs S3-S11). We found that only a few populations showed
 239 significant within life stage immune priming as larvae (L-WLS, 4/10 populations) or
 240 as adults (only females; A-WLS, 4/10 populations) (Fig 2A). In contrast, at least one
 241 sex of many populations showed significant ontogenic (ONT, 9/10 populations; Fig
 242 2B) and trans-generational benefits of adult priming (A-TG, 6/10 populations; Fig
 243 2C). Our data also demonstrate long ranging impact of trans-generational immune
 244 priming in several populations (L-TG, 6/10 populations; Fig 2D), whereby priming
 245 larvae improved post-infection survival of their adult offspring. Finally, we found that
 246 populations B1 and B2 showed very different priming responses (Fig 2), although
 247 they were collected from different warehouses in the same city. Hence, geographical
 248 proximity does not seem to be a good predictor of similarity in immune responses.

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252 **Effect of sex on immune priming**

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254 As explained in the methods, we tested the priming response of each sex separately.
 255 Hence, we could not directly test for an impact of sex in each population. Combining
 256 hazard ratios across populations, we did not find a consistent impact of sex on the
 257 strength of the immune priming response for any type of priming (Table 1A-C).
 258 However, in many populations, only one sex showed a significant priming response.
 259 For instance, the adult WLS response appears to be female-limited, with males
 260 showing no priming in any population (Fig 2A). Similarly, in most populations that
 261 showed ontogenic priming, priming was beneficial for only one sex (7/9 populations;
 262 Fig 2B). However, unlike WLS, we did not find a systematic benefit of ONT priming:
 263 the sex that benefited from ONT priming varied across populations. We also failed to
 264 find clear sex-specific benefits of TG priming for offspring. We observed adult
 265 maternal immune priming (A-TG) in offspring of both sexes (4 populations) or only
 266 one sex (2 populations) (Fig 2C). Intriguingly, all six populations with significant
 267 larval trans-generational (L-TG) priming showed a response in offspring of both sexes
 268 (Fig 2D). Thus, both males and females tend to show parallel benefits of L-TG
 269 priming across populations. Overall, our results show that the impact of sex on
 270 immune priming varies both across populations and type of immune priming.

271

272 **Larval ontogenic priming maximizes protection against subsequent infection**

273

274 Next, we tested the impact of priming life stage on the strength of the priming
 275 response. We found that priming at the larval stage was more beneficial and produced
 276 a greater response than priming adults (Table 1D). However, this result was driven

277 primarily by ontogenic larval priming, which maximized post-infection survival in
278 adults across priming types relative to the respective unprimed controls (Fig 3, Table
279 1E). Larval ONT priming resulted in a ~3 fold survival benefit, compared to the 2-
280 fold benefit observed for other forms of priming, including larval WLS priming (Fig
281 3). We also found that across populations, the strength of ONT priming in females
282 was more variable compared to WLS, L-TG or A-TG priming (Bartlett's test for
283 homogeneity of variance, $p < 0.02$ for each pairwise comparison; compare boxplots in
284 Fig 3). For males, ONT priming was significantly more variable than WLS priming,
285 but not other forms of priming. Together, our results suggest that among different
286 types of immune priming, ONT priming responses are strongest and most variable.

287

288 **Associations between within- and trans- generation immune priming**

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290 We tested whether different types of immune priming responses were associated
291 within populations. We found that most populations either showed significant female
292 WLS priming or significant TG priming in male offspring, but not both (Fig 4A;
293 Fisher's exact test, $p = 0.046$). In contrast, there was no association between female
294 WLS and TG priming in female offspring (Fig S12A; Fisher's exact test, $p = 0.643$).
295 We also found a non-significant trend for an association between ONT priming in
296 males and TG priming in male offspring (Fig 4B; Fisher's exact test, $p = 0.446$), but
297 not for female offspring (Fig S12B; Fisher's exact test, $p = 0.663$). For male
298 offspring, one of the two populations that showed only ONT priming had nearly
299 significant TG priming (population AM, Fig 4B; $p = 0.066$). If this population were
300 counted as showing both types of priming, the association between ONT and male TG
301 priming would be significant (Fisher's exact test, $p = 0.046$). Although the association

is not strong, these results suggest that in populations where male adults benefit from larval ONT priming, they may also benefit from maternal TG immune priming. Overall, our results indicate that trans-generational immune priming responses are associated with within-generation responses, but the association is limited to male offspring.

DISCUSSION

Our work provides the first evidence of substantial variation in both within- and trans-generational immune priming responses among natural populations of an insect. Approximately half the populations did not show a significant response to any given type of priming; on the other hand, all populations showed at least two forms of priming. Relative to unprimed controls, primed individuals showed up to 13-fold higher survival in some cases, whereas others showed no benefits of priming. Note that we reared wild-collected beetles under standard laboratory conditions for 9-10 generations before starting our experiments; hence, we probably underestimated the variation in priming responses across populations. What is the cause of this variability? Potential hypotheses include gain and loss of priming responses via genetic drift; local adaptation to specific pathogen diversity and abundance (Sutton *et al.*, 2011); variable life-history related costs associated with immune investment (Roy & Kirchner, 2000; Miller *et al.*, 2006); and variable susceptibility to pathogens (Best *et al.*, 2013). Currently, we cannot directly test these hypotheses since we do not have information on the local pathogen pressure experienced by our beetle populations, the fitness costs of immune priming, or their relative susceptibility to *B. thuringiensis*. Nonetheless, our work demonstrates the importance of quantifying variability of

immune priming responses in natural populations, and sets up a framework to understand the evolution of immune priming responses.

One of our most interesting findings is that ontogenic priming confers a greater survival benefit than within life stage or trans-generational immune priming response.

A recent theoretical model predicts that if adults incur higher costs of infection than larvae, selection should favor strong ontogenic priming that reduces the proportion of susceptible adults (Tate & Rudolf, 2012). On the other hand, trans-generational priming should be favored when larvae are more susceptible to infection than adults.

Thus, if *B. thuringiensis* imposes stage-specific costs of infection in *T. castaneum*, it may have selected for stronger ontogenic priming in our populations. In a separate experiment, we found that larvae and adults from a laboratory-adapted, outcrossed flour beetle population were equally susceptible to *B. thuringiensis* infection (Fig S13A). These data suggest that beetle life stages are not differentially susceptible to infection, although it is possible that our natural populations do show stage-specific susceptibility. Another interesting result from our analysis is that the strength of larval TG priming is similar to the strength of adult TG priming, but much weaker than larval ontogenic priming. Thus, the high survival benefit of ONT priming (through metamorphosis) is not transmitted to the next generation. Thus, we speculate that during oviposition, priming is “reset”, perhaps because the mechanisms responsible for ontogenic and trans-generational priming are different. Further empirical studies are thus critical to elucidate the complex interplay between immune priming types and their relative impact on the outcome of infection within a population.

351 Our data also revealed novel associations between within- and trans-generational
 352 immune priming responses. In populations where adult females showed significant
 353 within life stage immune priming, male offspring did not show trans-generation
 354 priming. We speculate that this negative relationship may reflect a trade-off between
 355 maternal and offspring immunity (Moreau *et al.*, 2012): transferring immunity to
 356 offspring may be costly for females who also bear the cost of their own immune
 357 priming response. However, this needs to be explicitly tested by quantifying the
 358 difference in the priming response of offspring of individual females that were primed
 359 and challenged as adults, vs. females that were not primed and challenged. Our results
 360 also suggest a weak association between male ONT and male TG priming.
 361 Interestingly, both relationships between trans-generational and within-generation
 362 priming were limited to male offspring. Such male-specific associations may arise
 363 due to sex-specific variation in infection susceptibility, investment in other immune
 364 components, or tradeoffs with other fitness components. We cannot test these
 365 predictions since the relative impact of *B. thuringiensis* infection in both sexes is
 366 unknown in natural beetle populations. However, separate experiments with an
 367 outbred *T. castaneum* population showed that infected males die about twice as fast as
 368 females (Fig S13B). It is possible that the natural populations analysed here also show
 369 similar sex-specific variation in susceptibility to infection, and further work is
 370 necessary to distinguish between these hypotheses.

371

372 We suggest that our results are applicable in many insect-pathogen systems. *B.*
 373 *thuringiensis* infects multiple insect hosts (Bravo *et al.*, 2011), and is commonly
 374 found in diverse habitats such as soil, insect cadavers, water and grain dust (Argôlo-
 375 filho & Loguercio, 2014; Lambert & Peferoen, 2014). Hence, *B. thuringiensis* may

376 impose strong selection on many insects occupying diverse ecological niches,
 377 influencing the evolution of their immune responses in the wild. Although we did not
 378 test whether the immune priming response is specific to the *B. thuringiensis* strain
 379 that we used, an earlier study showed that *T. castaneum* individuals could
 380 differentiate between strains of the same pathogen (Roth *et al.*, 2009). Thus, the
 381 immune priming response that we observed is most likely a specific response against
 382 *B. thuringiensis* and does not represent general protection via an overall upregulation
 383 of immune components. Finally, we assayed immune priming response using septic
 384 injury, whereas many pathogens infect their insect hosts via the oral route. However,
 385 recent studies confirm that both septic injury (Roth *et al.*, 2009) and oral infection
 386 (Milutinović *et al.*, 2014) with *B. thuringiensis* produce comparable immune priming
 387 responses in *Tribolium* beetles, suggesting that our infection protocol is unlikely to
 388 bias our results.

389

390 We would like to end by highlighting several open questions that have emerged from
 391 our work. (A) Do sex- and stage- specific differences in immune function and
 392 pathogen susceptibility explain the observed variation in immune priming response?
 393 (B) Do variable fitness costs of immune priming explain the observed variation in
 394 immune priming response across populations? (C) Finally, do mechanisms underlying
 395 various forms of immune priming differ from each other? We suggest that future
 396 work on insect immune priming should focus on variation in both the mechanistic as
 397 well as ecological and evolutionary aspects of natural variation in immune priming. In
 398 particular, experimental manipulation of specific immune priming types across sexes
 399 and life stages promises to shed light on the complex problem of immune priming
 400 responses and their variable outcomes in natural populations.

401 **COMPETING INTERESTS**

402

403 We have no competing interests.

404

405 **AUTHOR CONTRIBUTIONS**

406

407 IK and DA conceived of and designed experiments; IK and AP carried out
408 experiments; IK and DA analyzed data; DA and IK wrote the manuscript with input
409 from AP. All authors gave final approval for publication.

410

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412

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415

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417

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420 Biological Sciences (NCBS), India.

421

422 TABLES

423

424 **Table 1.** Summary of (A) two way ANOVA for immune priming response with type
 425 of immune priming and sex as fixed factors (B) one way ANOVA for WLS and ONT
 426 priming response with sex as a fixed factor (C) two way ANOVA for TG priming
 427 response with sex and type of parental priming (e.g. larval or adult priming) as fixed
 428 factors (D) one way ANOVA for immune priming response with life stage-specific
 429 (larvae or adults) priming as a fixed factor (E) one way ANOVA for immune priming
 430 response with type of immune priming response as a fixed factor. IP = Immune
 431 priming, S = Sex, PP = Type of parental priming, LS = Life stage.

Experiment	Effect	df	SS	F-ratio	P
A. Impact of type of IP and S	IP	2	4.632	9.24	<0.001
(excluding L-WLS)	S	1	0.126	0.503	0.48
	IP × S	2	0.268	0.536	0.587
B. Impact of S on A-WLS response	S	1	0.044	0.37	0.54
Impact of S on ONT response	S	1	0.249	0.61	0.44
C. Impact of S and PP on TG	S	1	0.148	0.601	0.443
response	PP	1	0.167	0.678	0.415
	S×PP	1	0.000	0.003	0.951
D. Impact of priming at larvae vs. adults	LS	1	1.58	5.99	0.016
E. Impact of type of IP (including L-WLS)	IP	4	5.23	5.67	<0.001

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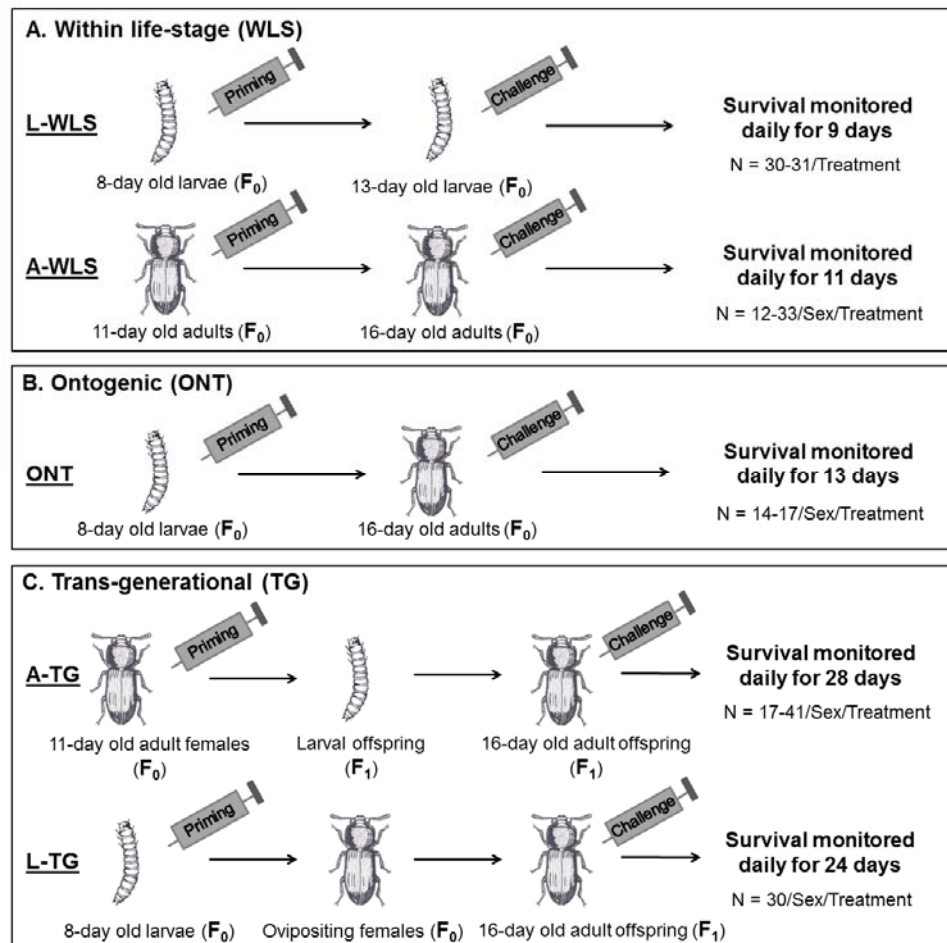
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435

FIGURE LEGENDS

437

438 **Figure 1.** Experimental design to measure the strength of immune priming responses
 439 at different stages: (A) Within life stage priming (individuals primed and challenged
 440 as larvae (L-WLS) or adults (A-WLS)) (B) Ontogenic priming (individuals primed as
 441 larvae and challenged as adults) (C) Trans-generational maternal priming (females
 442 primed as larvae (L-TG) or adults (A-TG) were paired with uninfected virgin males
 443 and their offspring were challenged). Sample sizes are indicated for each treatment
 444 (priming and control) and sex.

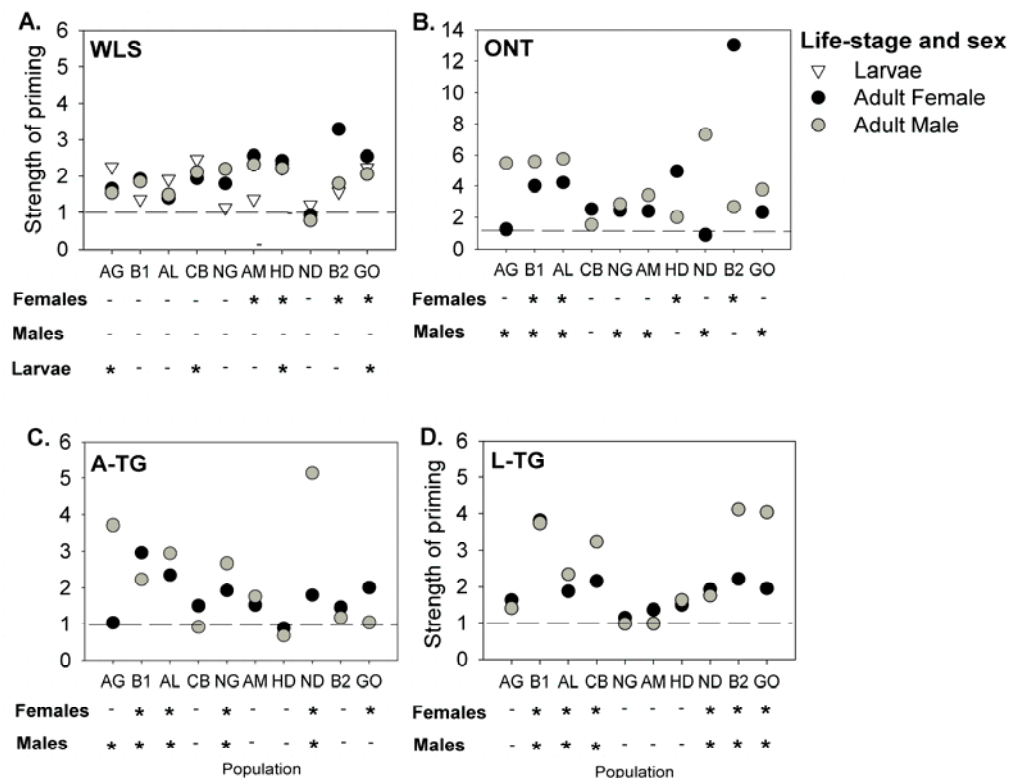


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Figure 2. Variation in priming response across sexes, life stages and populations.

(A) Within life stage immune priming (WLS) benefit in larvae and adults (B) Ontogenic (ONT) immune priming benefit (C) Trans-generational (TG) immune priming benefits from adult females (D) Trans-generation (TG) immune priming benefits from larvae. Strength of immune priming response was calculated as the hazard ratio of the proportion of deaths occurring in the unprimed group compared to the primed group under proportional hazard model. Horizontal dashed lines in each panel indicate a hazard ratio of 1. ‘*’ and ‘-’ denote significant ($p \leq 0.05$) and nonsignificant ($p > 0.05$) impact of immune priming in each stage, sex, and population. Sample sizes for each group are given in Fig. 1.



461 **Figure 3. Strength of each type of immune priming response across different life**
 462 **stages and sexes.** Strength of priming was calculated as described in Fig. 2. Sample
 463 sizes for each assay are shown in Fig. 1. WLS = within life stage immune priming,
 464 ONT = ontogenic priming; A-TG = trans-generation benefits of adult (maternal)
 465 priming; L-TG = trans-generation benefits of larval priming.
 466

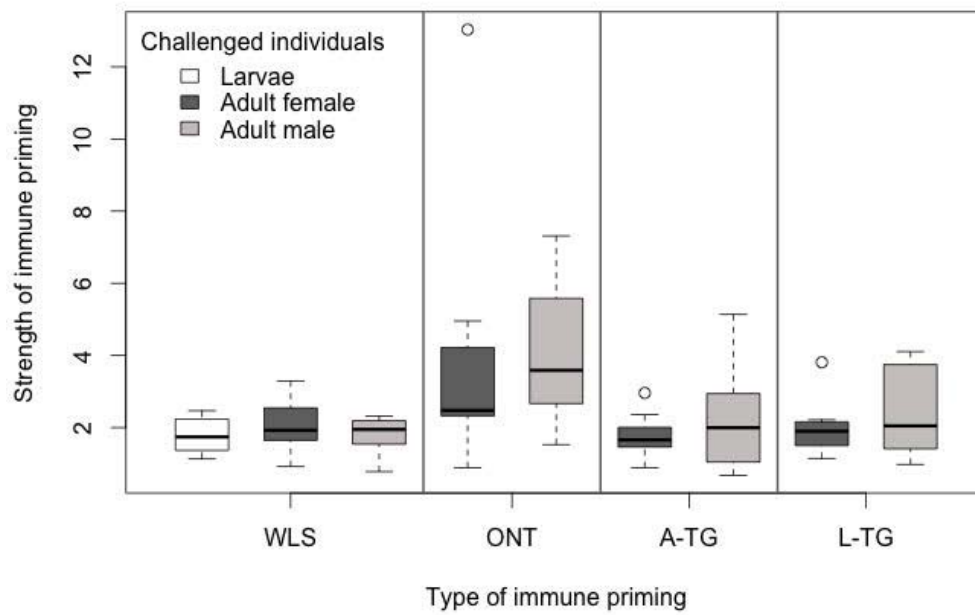
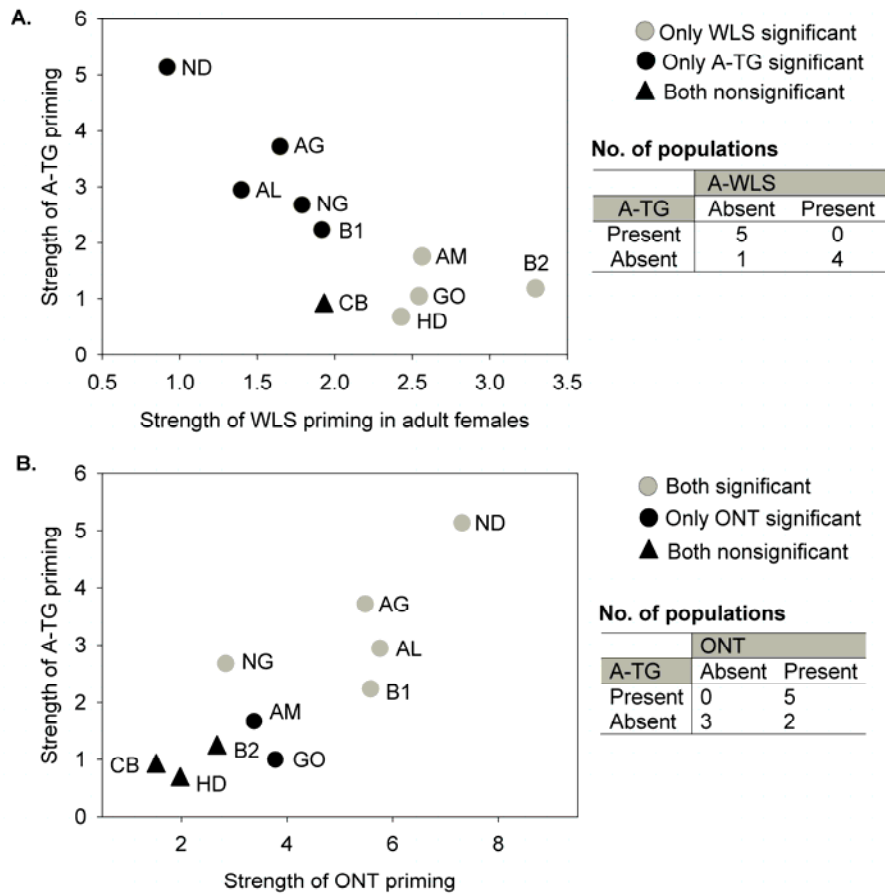


Figure 4. Associations between within- and trans-generation immune priming.

Strength of A-TG response in male offspring as a function of (A) strength of WLS immune priming in female adults (B) ONT priming in males. Strength of priming was estimated as described in Fig 2. Each population (labelled) was categorized based on the presence or absence of each type of priming response (using significant hazard ratios as explained in Fig 2), and contingency tables (shown beside each panel) were used to test the association between two types of immune priming across populations. WLS = Within life stage immune priming, A-TG = Trans-generational benefits of adult (maternal) priming, ONT = Ontogenic immune priming.



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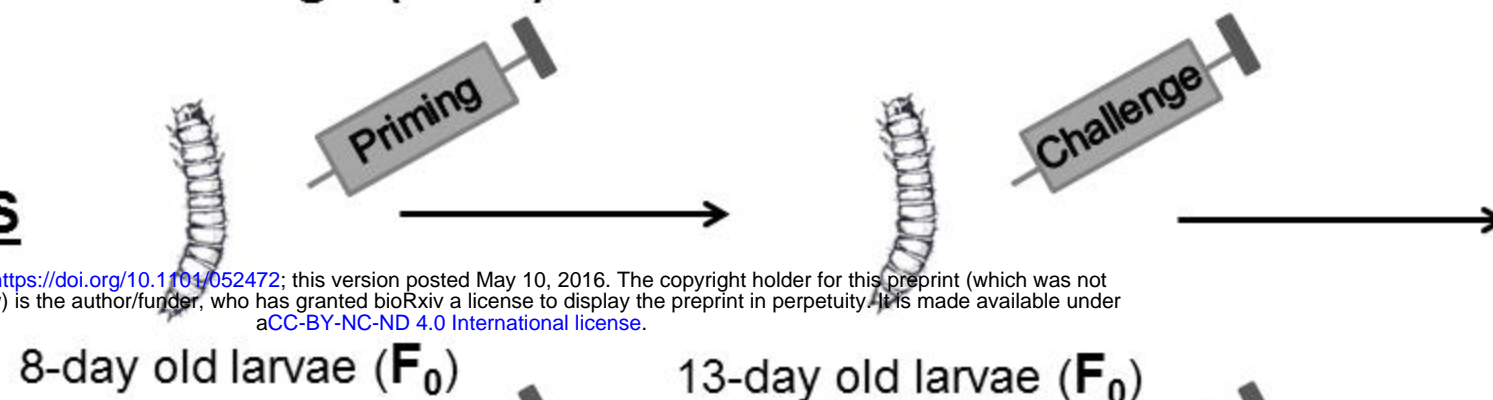
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585

A. Within life-stage (WLS)

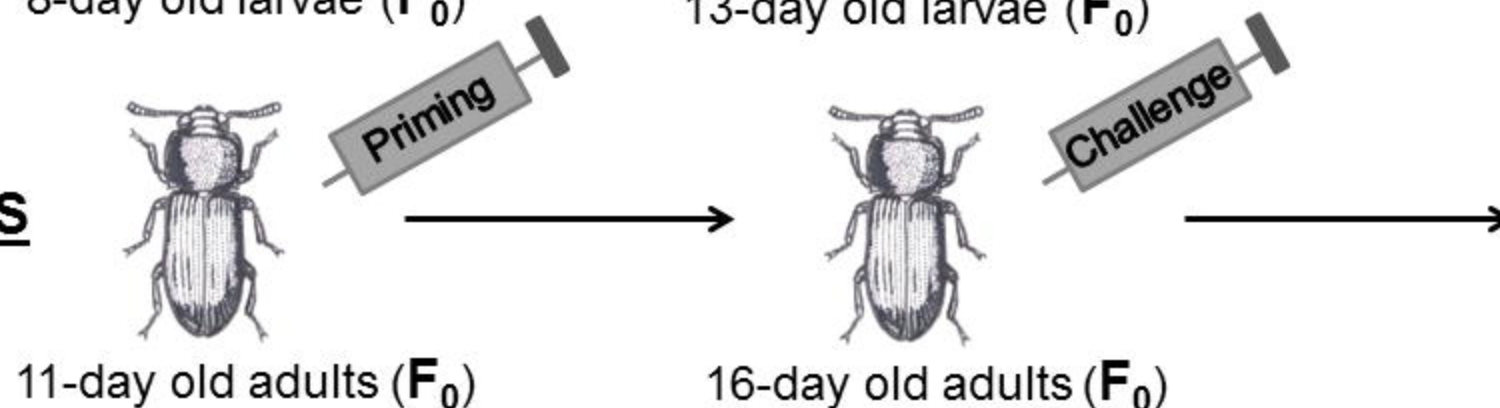
L-WLS



Survival monitored
daily for 9 days

N = 30-31/Treatment

A-WLS

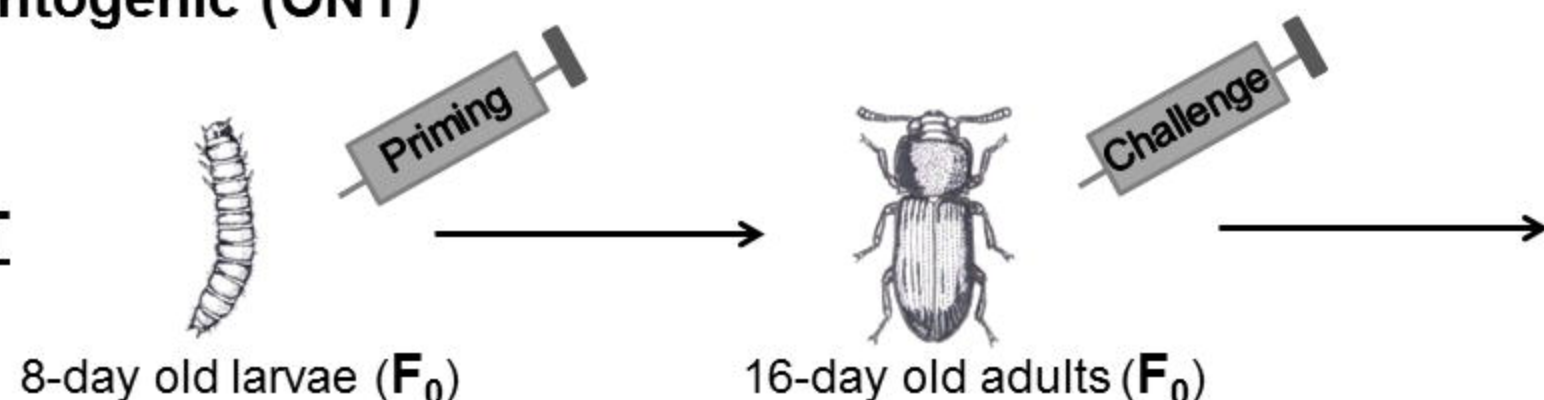


Survival monitored
daily for 11 days

N = 12-33/Sex/Treatment

B. Ontogenic (ONT)

ONT

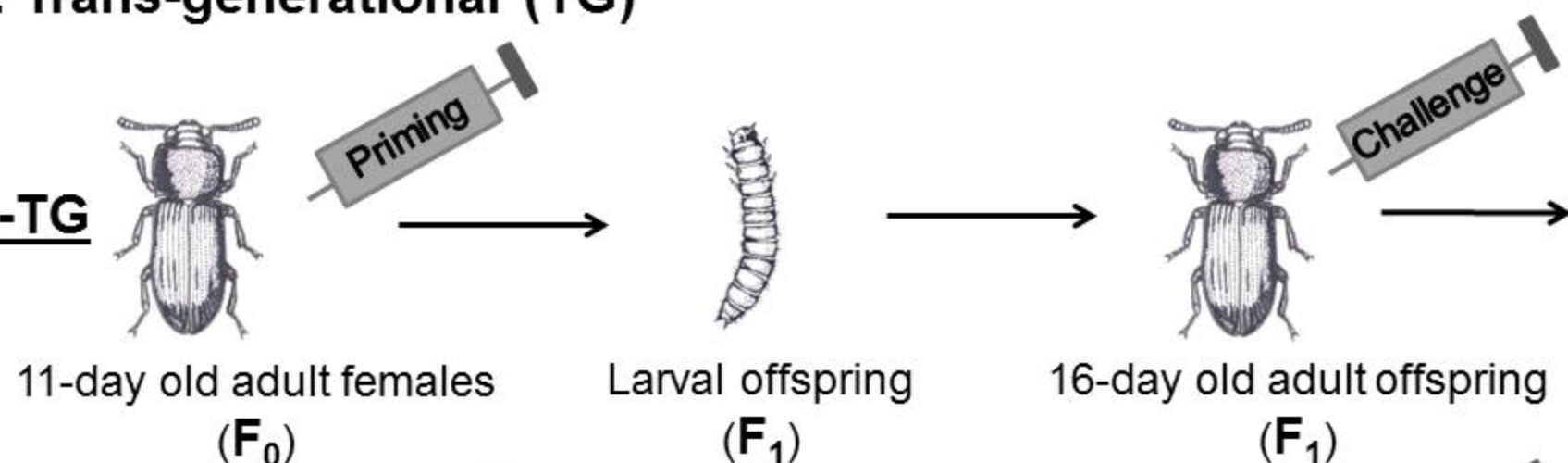


Survival monitored
daily for 13 days

N = 14-17/Sex/Treatment

C. Trans-generational (TG)

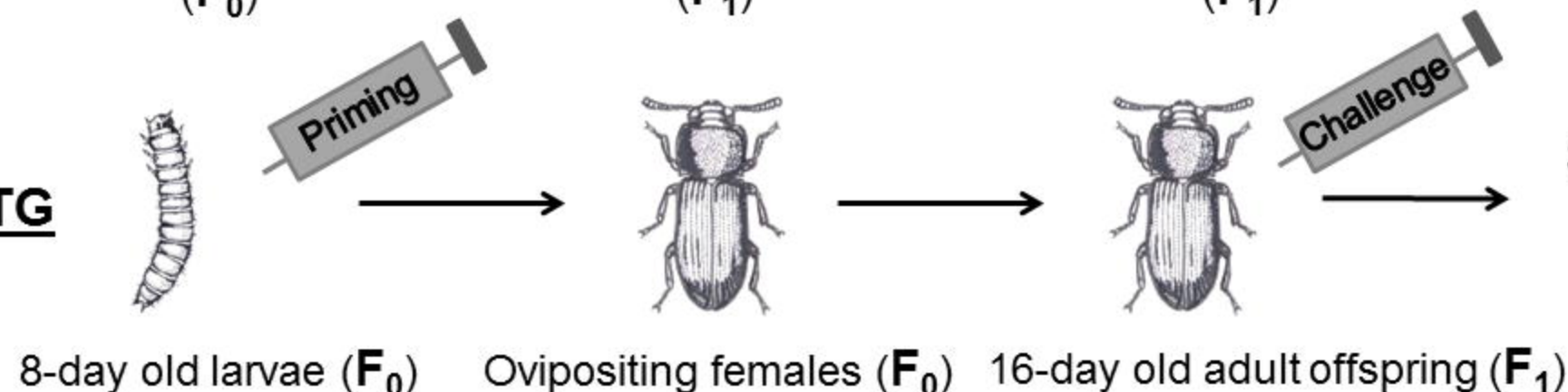
A-TG



Survival monitored
daily for 28 days

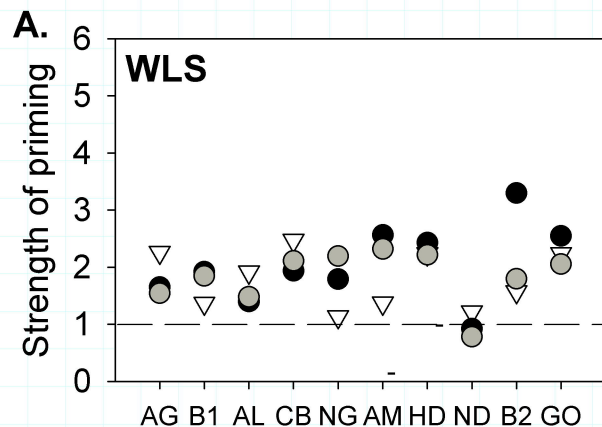
N = 17-41/Sex/Treatment

L-TG



Survival monitored
daily for 24 days

N = 30/Sex/Treatment

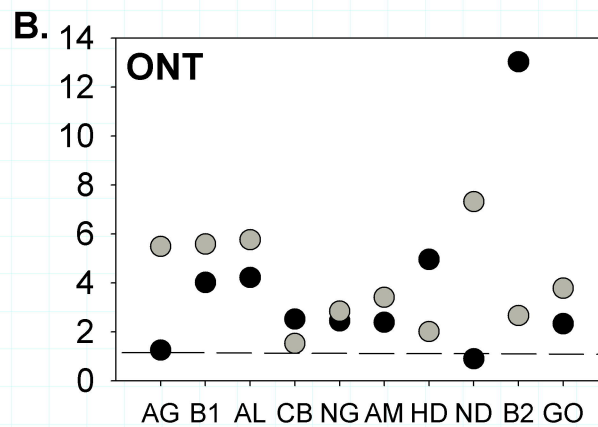


Females

Males

Larvae

-	-	-	-	-	*	*	-	*	*
-	-	-	-	-	-	-	-	-	-
*	-	-	*	-	-	*	-	-	*



Females

Males

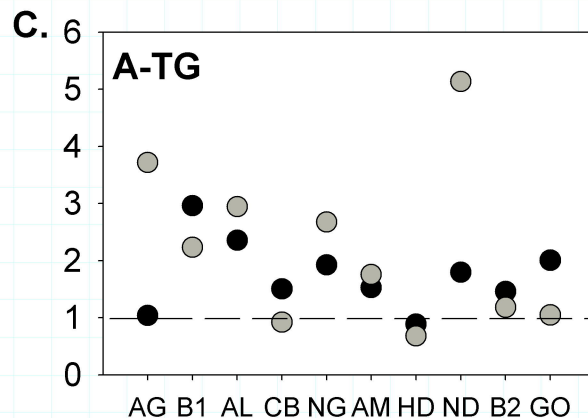
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Life-stage and sex

▽ Larvae

● Adult Female

● Adult Male

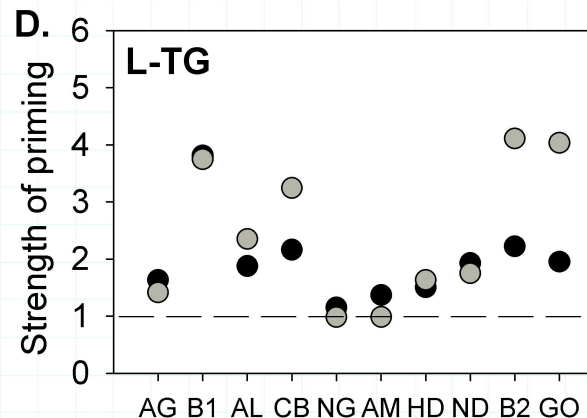


Females

Males

-	*	*	-	*	-	-	*	-	*
*	*	*	-	*	-	-	*	-	-

Population



Females

Males

-	*	*	*	-	-	-	*	*	*
-	*	*	*	-	-	-	*	*	*

Population

