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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

Experimental evolution

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

RESULTS

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

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

Evolved immune responses do not incur reproductive costs

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

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

Evolved priming reduces early survival and extends development time

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

AUTHOR CONTRIBUTIONS

IK conceived experiments; IK, AP and DA designed experiments; AP carried out experiments; IK and AP analyzed 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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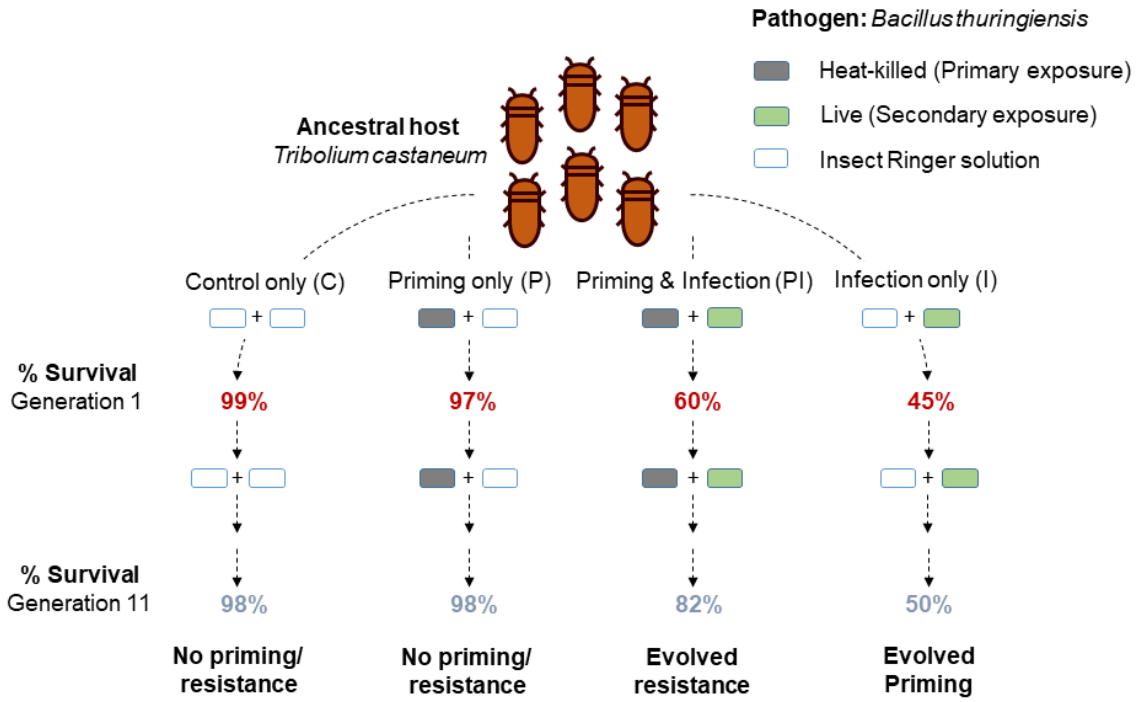
524

FIGURES

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



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

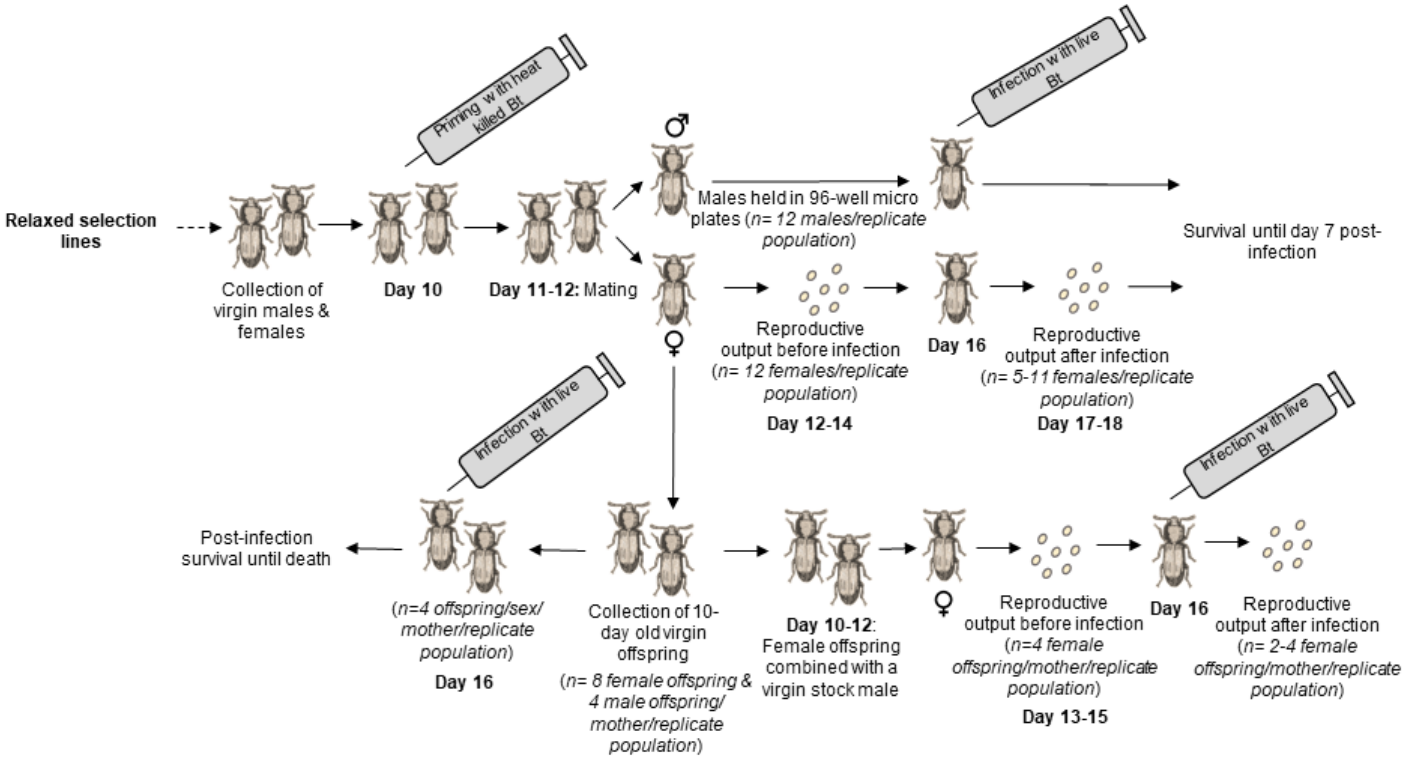


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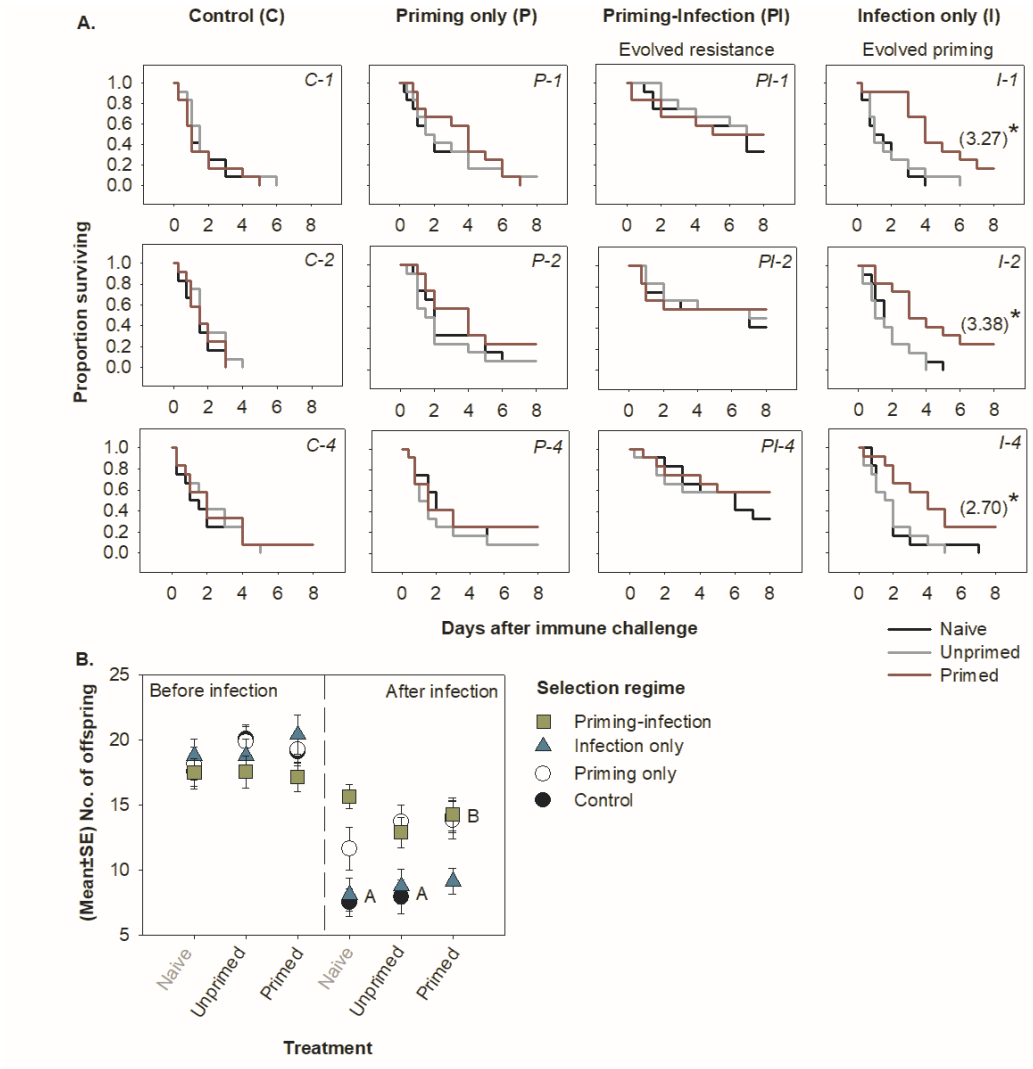
Figure 3. (A) Survival curves for within-generation priming and resistance in females (n= 12 females/treatment/selection regime/replicate population) after 14 generations of selection. Asterisks and the numbers in parentheses for I beetles denote the hazard ratios calculated from survival curves for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



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

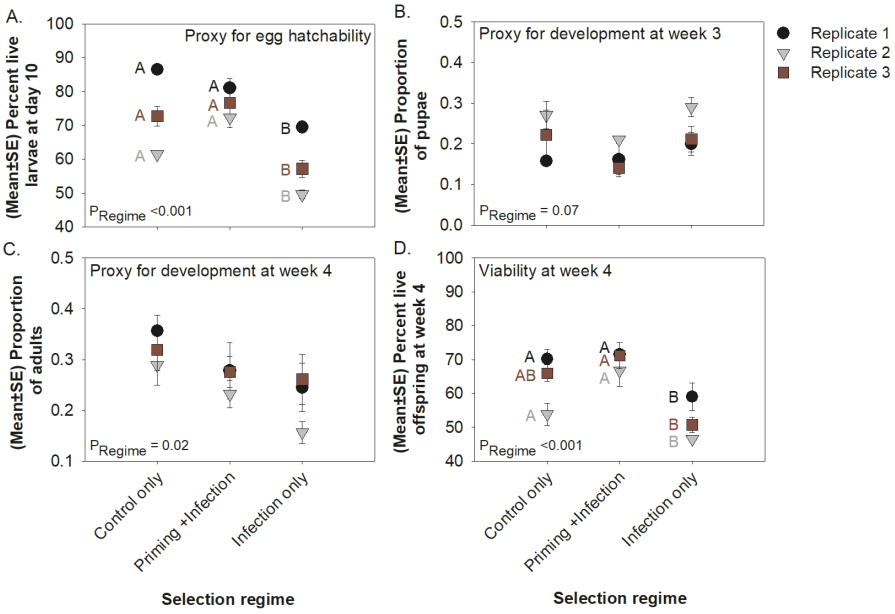


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Figure 5. (A) Offspring survival after trans-generational immune priming (TGIP) and infection (group mean survival of 4 offspring from 8-11 parental pairs/ treatment/ selection regime/ offspring sex). TGIP increased offspring survival only in I regime,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