

Genotype-specific fitness cost of resistance to Bt toxin Cry1Ac in pink bollworm

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Abstract

BACKGROUND: To improve resistance management strategies for *Bacillus thuringiensis* (Bt) crops, a better understanding of the relative fitness of pest genotypes with resistance alleles in the absence of Bt toxins is needed. Here, we evaluated the impact of costs of resistance to Bt toxin Cry1Ac on the relative fitness of specific pink bollworm (*Pectinophora gossypiella*) genotypes. We created two heterogeneous strains with an intermediate frequency of mutant cadherin alleles linked with resistance to Cry1Ac, reared the strains on diet without Bt and tracked the decline in frequency of resistant genotypes for 15–30 generations using polymerase chain reaction amplification. We used a population genetics model and sensitivity analyses to estimate the relative fitness of resistant genotypes.

RESULTS: Costs were completely recessive in one strain and almost completely recessive in the other. Estimates of the decline in relative fitness of the resistant homozygotes fed on a diet without Bt were 14–22% in one strain and 21–36% in the other.

CONCLUSION: Our genotype-specific cost estimates and the results of studies discussed herein indicate that costs associated with resistance to Bt are often large enough to significantly delay the evolution of resistance to pyramided Bt crops in pests with recessive inheritance of resistance.

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Keywords: *Bacillus thuringiensis*; cadherin; resistance management; transgenic plants

1 INTRODUCTION

Transgenic crops producing insecticidal proteins from the soil bacterium *Bacillus thuringiensis* (Bt) have been used for more than two decades to control some key pests.^{1,2} However, evolution of pest resistance to Bt crops is rapidly increasing.^{3–5} The primary mechanism of resistance involves mutations that disrupt midgut protein receptors and reduce the binding of Bt toxins.^{6–8} Such genetic changes are expected to compromise the normal functions of the affected midgut proteins, thereby imposing fitness costs that select against resistance in habitats where Bt toxins are absent.^{9–16} Knowledge of such costs can enhance the development of proactive resistance management strategies and remedial actions to address resistance evolution.^{10,11,15–17}

Two main approaches have been used to study the costs of Bt resistance.¹³ The first approach compares fitness components between susceptible and resistant strains, sometimes combining data for several components to estimate the overall effect of costs on fitness.^{18–21} For example, Bird and Akhurst¹⁹ measured female fecundity, egg fertility, survival from egg to adult, and sex ratio to estimate the intrinsic rate of population increase (r_m) for a strain of *Helicoverpa armigera* that was resistant to transgenic cotton producing Bt toxin Cry1Ac, a related susceptible strain, and the F₁ progeny from a cross between these strains. A limitation of this approach is that some fitness components affected by resistance can be missed, such as the cost affecting paternity that is associated with resistance to Cry1Ac in pink bollworm (*Pectinophora*

gossypiella) and *H. armigera*.^{22–24} Thus, the overall fitness cost can be underestimated. The second approach evaluates the rate of decline in resistance over multiple generations in heterogeneous laboratory strains not exposed to Bt toxins.¹³ The decrease in resistance is usually measured in terms of the LC₅₀, the concentration of a toxin killing 50% of the insects tested. Analysis of results from 65 experiments applying this approach showed that on average, a 10-fold decrease in LC₅₀ occurred in seven generations, and the rate of decline was positively associated with the initial magnitude of resistance.¹³ Although this approach considers the cumulative impact of all costs of resistance on fitness over many generations, it does not estimate the relative fitness of specific genotypes, which is needed to improve the simulation models used to devise resistance management strategies and remedial action plans.^{4,11,25}

Here, we expand upon the second approach and use it to estimate the overall effect of costs of resistance to Cry1Ac on

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specific genotypes of the pink bollworm. This invasive pest was the primary target of Bt cotton in Arizona^{26–28} before its virtual eradication by a multi-tactic program that included mass releases of sterile moths and planting of nearly 100% Bt cotton.^{29,30} Previous studies have shown that fitness costs associated with pink bollworm resistance to Cry1Ac affect development time, growth rate, larval survival, overwintering survival, paternity, and sperm transfer.^{31–36} The fitness costs identified in these studies were generally recessive, although the cotton defensive phytochemical gossypol induced larger and less-recessive costs affecting larval survival and growth rate.^{33,36} However, these studies did not assess the relative fitness of resistant genotypes.

Here, we estimate the relative fitness of resistant genotypes reared in the absence of Bt in two heterogeneous strains of pink bollworm: MOV97-H2 and SAF97-H2. MOV97-H2 was created by crossing Cry1Ac-resistant and -susceptible strains from the Mohave Valley of western Arizona; SAF97-H2 by crossing Cry1Ac-resistant and -susceptible strains from Safford in eastern Arizona.³⁵ Each of these hybrid strains had two mutant cadherin alleles tightly linked with recessive resistance to Cry1Ac and Bt cotton producing Cry1Ac.^{10,35,37–39} MOV97-H2 had cadherin resistance alleles r_1 and r_3 and SAF97-H2 had cadherin resistance alleles r_1 and r_2 . We reared both strains on a diet without Bt and tracked the decline in the frequency of resistant genotypes for 15–30 generations using allele-specific polymerase chain reaction (PCR).³⁸ We then used a population genetics model and sensitivity analyses to estimate effects of fitness costs on the resistant genotypes.

2 MATERIALS AND METHODS

2.1 Insect strains

We used subsets of the previously described hybrid strains MOV97-H2 and SAF97-H2 to track changes in genotype frequency and estimate fitness costs.³⁵ These strains were created by selecting two hybrid strains (MOV97-H1 and SAF97-H1) with a discriminating concentration of Cry1Ac ($10 \mu\text{g mL}^{-1}$ of diet) and crossing the resulting *rr* insects of each selected strain with individuals of the MOV97-H1 and SAF97-H1 strains (which were heterogeneous for *r* and *s* alleles, where *r* and *s* respectively represent an allele for resistance and susceptibility to Bt). We crossed the selected and unselected hybrid strains to increase the initial frequency of resistance alleles in the experimental strains.

Hybrid strains were fed an artificial diet⁴⁰ without Bt in the laboratory. Before creating MOV97-H2 and SAF97-H2, insects from MOV97-H1 and SAF97-H1 had been reared on a non-Bt diet for eight generations.³⁵ Individuals from the F5 generation of MOV97-H2 and SAF97-H2 were used to initiate the selection experiment. Rearing insects on a non-Bt diet for 12 generations before onset of the selection experiment contributed to reducing potential linkage disequilibrium between cadherin alleles and other alleles that may affect fitness.³⁵

Compared with the wild-type allele (*s*) that confers susceptibility to Cry1Ac, the r_1 allele found in both MOV97-H2 and SAF97-H2 has a single deletion of 24 base pairs (bp), resulting in the elimination of eight amino acids in the cadherin protein.³⁷ The r_2 allele in SAF97-H2 has a deletion of 202 bp introducing a premature stop codon, resulting in the production of a truncated protein without the membrane proximal, transmembrane and cytoplasmic domain regions.^{37,41} The r_3 allele in MOV97-H2 has a deletion of 126 bp

resulting from the insertion of a retrotransposon that causes splicing out of exon 21 from mRNA.^{37,41,42}

2.2 PCR amplification of cadherin genotypes

PCR amplification was used to measure temporal changes in the frequency of cadherin genotypes over up to 30 generations. DNA of randomly selected neonates was purified using the previously described DNAzol (Molecular Research Center, Inc., Cincinnati, OH, USA) method,³⁸ except that the volumes of DNAzol used for homogenization and rinsing were, respectively, 50 and 450 μL , and the volume of both PolyAcryl carrier (Molecular Research Center) and proteinase K (Fermentas Inc., Hanover, MD, USA) was 4 μL . Individuals were genotyped using allele-specific PCR amplifications following the protocol from Morin *et al.*³⁸

We genotyped randomly selected neonates from MOV97-H2 and SAF97-H2: 39 per strain from the F1 generation; and 100–101 per strain from the F5, F8, F15, F20, F25 and F30 generations for MOV97-H2, and F5, F8 and F15 for SAF97-H2. Ten randomly selected neonates from each strain were screened for the three resistance alleles in every generation to assess potential strain contamination. For SAF97-H2, we did not genotype the full sample of neonates after F15, because analysis of the 10 neonates in F20 revealed many r_3 alleles, indicating that this strain had been inadvertently contaminated with individuals from MOV97-H2 after F15.

2.3 Simulation model

We modified a deterministic population genetic model with a single locus and two alleles^{9,10} to a single locus with three alleles. We used the model to simulate changes in the frequency of the three alleles in each strain: *s*, r_1 and r_3 in MOV97-H2, and *s*, r_1 and r_2 in SAF97-H2. As in previous models,^{9,10} we assumed that selection occurs due to differential mortality among genotypes that mate randomly and are in Hardy–Weinberg equilibrium in each generation. Accordingly, the predicted change in frequency of each allele per generation was calculated from basic equations using the frequency and fitness of each genotype and the average fitness of all the genotypes^{9,10} (Supplementary Material File S1). The predicted frequency of genotypes in subsequent generations was calculated from simulated frequency of alleles, assuming Hardy–Weinberg equilibrium. We used the F1 frequency of *s*, r_1 , r_2 and r_3 in MOV97-H2 and SAF97-H2 estimated with PCR analyses as the initial allele frequency in simulations. For each simulation, we calculated the average of the absolute differences between the observed and simulated frequencies of alleles and genotypes over the F5, F8, F15, F20, F25 and F30 generations in MOV97-H2, and F5, F8 and F15 generations in SAF97-H2. We report analyses of observed and simulated genotype frequencies here because analyses of observed and simulated allele frequencies were similar. Simulations were run with Visual Basic in Microsoft Excel (2007; Microsoft, Redmond, WA, USA).

Fitness of *ss* was 1 in all simulations. Previous studies have shown that the frequency of r_1 is consistently lower than that of r_2 or r_3 in hybrid strains.^{35,36} Furthermore, the frequency of r_1 declined more consistently than that of r_2 and r_3 in the current experiment (see Results), which may reflect a greater cost in r_1r_1 than r_2r_2 or r_3r_3 , or a less-recessive cost in r_1s than r_2s or r_3s (see File S1 and Table S1 in File S1). To assess these possibilities, we simulated three scenarios: (A) recessive costs (fitness of $r_1s = r_2s = r_3s = 1$) and costs equal for all *rr* genotypes (fitness of $r_1r_1 = r_2r_2 = r_3r_3 = r_1r_2 = r_1r_3$); (B) recessive costs, fitness of $r_1r_1 < r_2r_2$ or r_3r_3 , and fitness of r_1r_2 and r_1r_3 varied; and (C) small non-recessive cost in r_1s , recessive

costs in r_2s and r_3s , and fitness of rr genotypes as in (B). The range of fitness values used in simulations for each scenario is shown in Table S2 in File S1. To assess the fit of these scenarios, we compared the smallest average of the absolute differences between the observed and simulated frequencies of genotypes for each scenario (see below).

To further consider the possibility of a small cost affecting r_1s , we also compared patterns of change in the average of the absolute differences between the observed and simulated frequencies of genotypes for models with and without costs in r_1s . First, we compared simulations with the fitness of all rr genotypes equal and no cost in the rs genotypes with similar simulations with small costs in r_1s . In four new sets of simulations for each strain, the fitness of the rr genotypes varied between 0.69 and 0.79 (in 0.01 increments) in SAF97-H2, and between 0.76 and 0.86 in MOV97-H2, fitness of r_2s and r_3s was 1, and fitness of r_1s was 0.97, 0.98, 0.99, or 1. Second, we conducted simulations in which fitness of the rr genotypes varied and no costs affected the rs genotypes, and identical simulations in which small costs reduced fitness of r_1s . We used a factorial design for these sets of simulations in which a range of fitness values of 0.65, 0.7, 0.75, 0.80 and 0.85 was used for the rr genotypes in SAF97-H2. Thus, 125 simulations were conducted for the model without a cost in r_1s (five fitness values for $r_1r_1 \times$ five fitness values for $r_2r_2 \times$ five fitness values for r_3r_3) and another 125 simulations for models with a cost in r_1s (fitness of r_1s was either 0.97, 0.98, or 0.99). For simulations of MOV97-H2, fitness values of 0.7, 0.75, 0.8, 0.85 and 0.9 were used for r_1r_1 and r_3r_3 , and values of 0.7, 0.75, 0.8, 0.85, 0.9 and 0.95 for r_1r_3 (thus 150 simulations were conducted for the model without a cost in r_1s and 150 simulations for the models with a cost in r_1s).

2.4 Statistical analyses

We used Fisher's exact test to evaluate the difference between the initial frequency of r_1 and r_3 in MOV97-H2 and r_1 and r_2 in SAF97-H2; and the difference between the initial and final frequency of each resistance allele in each strain. To describe the trajectory of the decline in frequency of resistance alleles across generations, we used simple linear regression for r_1 and multiple regression in which a linear and quadratic term was included for r_2 and r_3 .

For each of seven generations for MOV97-H2 (F1, F5, F8, F15, F20, F25 and F30) and four generations for SAF97-H2 (F1, F5, F8 and F15), we used a separate Pearson χ^2 test to assess whether the observed genotype frequency differed from the genotype frequency expected under Hardy–Weinberg equilibrium. For simulations of scenario A, we used multiple regression models with a linear and quadratic term for the fitness values of the rr genotypes (explanatory variables) to test whether the average of absolute differences between observed and simulated genotype frequencies (response variables) was minimized at one of the fitness values of the rr genotypes. To assess whether scenarios B and C provided a better fit to the observed trajectories compared with scenario A, we used ANOVA followed by contrasts to compare the smallest average of differences for one scenario (e.g., B) and the smallest average of differences for scenario A.

We used multiple regression analyses to compare simulations with equal fitness of the rr genotypes and no costs in r_1s and identical sets of simulations in which a cost in r_1s was added. The response variable was the average of differences between observed and simulated genotype frequencies, and the explanatory variables were fitness of each rr genotype, a quadratic term for fitness of each rr genotype, and the type of model used (i.e., a cost

in r_1s versus no cost in r_1s). We also used similar multiple regression analyses to compare results of simulations with variable fitness of the rr genotypes and no cost in r_1s and sets of identical simulations in which a small cost in r_1s was added. In these analyses, a significant effect of model type corresponding to lowest deviation in models with a cost in r_1s indicates the presence of a significant cost reducing fitness of r_1s , after taking into account the linear and quadratic associations between fitness of each rr genotype and the average of the absolute differences between observed and simulated frequencies of genotypes.

In our analyses, we defined fitness of ss in the absence of Bt as 1.0 and calculated the fitness cost (%) associated with rr genotypes as: (fitness of ss – fitness of rr) \times 100%. For example, a rr fitness of 0.90 relative to the fitness of 1 for ss yields a cost of 10%.

3 RESULTS

3.1 Decreases in the frequency of resistance alleles

In MOV97-H2, the initial frequency was significantly lower for r_1 (0.13) than r_3 (0.40) (Table 1, Fisher's exact test, $P = 0.0002$). In SAF97-H2, the initial frequency of r_1 (0.17) was marginally lower than the initial frequency of r_2 (0.31) ($P = 0.059$). From F1 to F30 in MOV97-H2, the frequency of r_1 decreased by 77% (from 0.13 to 0.03, $P = 0.0032$), while the frequency of r_3 declined by 60% (from 0.40 to 0.16; $P < 0.0001$). From F1 to F15 in SAF97-H2, the frequency of r_1 declined by 59% (from 0.17 in F1 to 0.07, $P = 0.022$), but the 32% decline in the frequency of r_2 was not significant (from 0.31 to 0.21, $P = 0.12$).

The trajectory of the decline in r allele frequency differed qualitatively between r_1 and r_2 or r_3 (Table 1 and Fig. S1 in File S1). The decline in frequency of r_1 was linear across generations in both strains (MOV97-H2: slope = -0.0036 , $t_5 = -9.96$, $P = 0.0002$; SAF97-H2: slope = -0.0068 , $t_2 = -4.82$, $P = 0.040$), while the decline in frequency of r_2 and r_3 slowed in later generations (r_2 : quadratic coefficient = 0.0015, $t_1 = 2.74$, $P = 0.22$; r_3 : quadratic coefficient = 0.00050, $t_4 = 3.48$, $P = 0.025$).

3.2 Estimating genotypic-specific costs by minimizing differences between simulated and observed decreases in resistance

The observed genotype frequencies (Table 1) did not differ significantly from Hardy–Weinberg equilibrium in any of the generations for MOV97-H2 and SAF97-H2 (all P -values ≥ 0.79), which supports the assumption of Hardy–Weinberg equilibrium used in simulations.

For simulations with scenario A (fitness costs recessive and equal for rr genotypes), fitness of 0.81 for the rr genotypes in MOV97-H2 (i.e., cost of 19%) and 0.74 in SAF97-H2 (i.e., cost of 26%) minimized the difference between simulated and observed genotype frequencies (Fig. 1 and Table 2). With these assumptions, simulated trajectories corresponded well with the observed trajectories of genotype frequency (Fig. 2), as the mean absolute difference between the simulated and observed genotype frequency was 0.024 for MOV97-H2 and 0.025 for SAF97-H2 (Fig. 1). For scenarios B (fitness cost recessive and higher in r_1r_1 than r_2r_2 or r_3r_3) and C (small cost affecting r_1s and higher cost in r_1r_1 than r_2r_2 or r_3r_3), fitness values of the rr genotypes minimizing the difference between simulated and observed genotype frequency ranged between 0.78 and 0.86 in MOV97-H2, and between 0.64 and 0.79 in SAF97-H2 (Table 2). For the fitness values yielding the best fit between observed and simulated genotype frequencies, the mean absolute difference between observed and simulated

Table 1. Changes in allele and genotype frequencies in hybrid pink bollworm strains MOV97-H2 and SAF97-H2 reared without exposure to Bt toxins

Generation	Allele frequency					Genotype frequency				
	<i>s</i>	<i>r</i> ₁	<i>r</i> ₃	<i>ss</i>	<i>r</i> ₁ <i>s</i>	<i>r</i> ₃ <i>s</i>	<i>r</i> ₁ <i>r</i> ₁	<i>r</i> ₁ <i>r</i> ₃	<i>r</i> ₃ <i>r</i> ₃	
MOV97-H2										
1 ^a	0.47	0.13	0.40	0.23	0.11	0.38	0.03	0.10	0.15	
5	0.59	0.13	0.28	0.34	0.18	0.33	0.01	0.06	0.08	
8	0.70	0.09	0.21	0.47	0.15	0.31	0	0.04	0.03	
15	0.74	0.08	0.18	0.53	0.13	0.30	0	0.02	0.02	
20	0.77	0.06	0.17	0.57	0.10	0.29	0	0.02	0.02	
25	0.79	0.04	0.17	0.60	0.07	0.31	0	0	0.02	
30	0.81	0.03	0.16	0.62	0.06	0.31	0	0	0.01	
SAF97-H2										
1 ^a	0.52	0.17	0.31	0.26	0.20	0.34	0.03	0.07	0.10	
5	0.68	0.12	0.20	0.45	0.17	0.30	0.01	0.04	0.03	
8	0.70	0.10	0.20	0.49	0.13	0.30	0.01	0.04	0.03	
15	0.72	0.07	0.21	0.50	0.10	0.34	0	0.03	0.03	

^a The number of neonates genotyped per strain was 39 for generation 1 and 100 per subsequent generation, except for 101 in generation 15 of MOV97-H2.

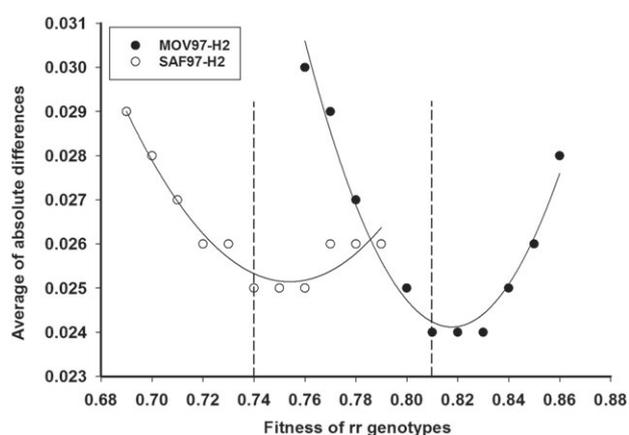


Figure 1. Association between fitness of *rr* genotypes used in simulations with scenario A (no costs affecting *rs* genotypes and equal costs affecting the *rr* genotypes) and the average of the absolute differences between observed and simulated genotype frequencies for MOV97-H2 and SAF97-H2. The quadratic terms of the association between *rr* fitness and the average of the differences between observed and simulated frequencies (continuous lines) are significant (MOV97-H2: $t_8 = 14.53$, $P < 0.0001$; SAF97-H2: $t_8 = 8.29$, $P < 0.0001$), indicating that the fitness values of 0.81 (MOV97-H2) and 0.74 (SAF97-H2) minimized the deviations between observed and simulated frequencies.

genotype frequencies did not differ significantly among scenarios A, B, and C (Fig. 3; MOV97-H2: $F_{2,105} = 0.35$, $P = 0.70$; SAF97-H2: $F_{2,51} = 0.023$, $P = 0.98$). Contrasts between scenario A and B or C also did not indicate that scenarios B or C significantly diminished the mean absolute difference between observed and simulated genotype frequencies relative to scenario A (MOV97-H2: P -values > 0.85 ; SAF97-H2: P -values > 0.45).

3.3 Effects of a small cost in *r*₁*s*

The additional sets of simulations with equal fitness of the *rr* genotypes and with or without costs reducing fitness in *r*₁*s* indicate that a small cost affected *r*₁*s* in SAF97-H2, but not in MOV97-H2. For MOV97-H2, a fitness of 0.99 in *r*₁*s* yielded the best fit with the observed trajectories compared with fitness values of 0.97 and 0.98. However, the average of differences between observed

Table 2. Fitness of genotypes providing best fit of simulation models to observed changes in genotype frequency in the Mohave (MOV97-H2) and Safford (SAF97-H2) strains

Scenario	Strain	Genotype					
		<i>ss</i>	<i>r</i> ₁ <i>s</i>	<i>r</i> ₃ <i>s</i>	<i>r</i> ₁ <i>r</i> ₁	<i>r</i> ₁ <i>r</i> ₃	<i>r</i> ₃ <i>r</i> ₃
A ^a	MOV97-H2	1	1	1	0.81	0.81	0.81
	B	1	1	1	0.78	0.82	0.81
	C	1	0.99	1	0.78	0.86	0.80
A	SAF97-H2	<i>ss</i>	<i>r</i> ₁ <i>s</i>	<i>r</i> ₂ <i>s</i>	<i>r</i> ₁ <i>r</i> ₁	<i>r</i> ₁ <i>r</i> ₂	<i>r</i> ₂ <i>r</i> ₂
	B	1	1	1	0.74	0.74	0.74
	C	1	1	1	0.71	0.72	0.77
C		1	0.98	1	0.64	0.78	0.79

^a Model fit was evaluated by calculating the average of the absolute differences between observed and simulated frequency of genotypes over generations 5, 8, 15, 20, 25 and 30 for MOV97-H2, and generations 5, 8 and 15 for SAF97-H2. Three sets of assumptions were used: (A) recessive costs (fitness of *r*₁*s*, *r*₂*s* and *r*₃*s* = 1) and fitness of all *rr* genotypes equal and < 1 ; (B) recessive costs, fitness of *r*₁*r*₁ $< r$ ₁*r*₂ or *r*₃*r*₃, and fitness of *r*₁*r*₂ or *r*₁*r*₃ varied; and (C) small, non-recessive costs for *r*₁*s* (fitness of *r*₁*s* = 0.97, 0.98 or 0.99), and other conditions as in B.

and simulated genotype frequencies for simulations with fitness of 0.99 in *r*₁*s* were equal or larger than for simulations without a cost reducing fitness of *r*₁*s*. With a fitness of 0.99 in *r*₁*s*, significant linear ($t_{18} = -14.1839$, $P < 0.0001$) and quadratic ($t_{18} = 14.01$, $P < 0.0001$) associations involving the fitness of each *rr* genotype occurred, although the fit of models with and without a cost reducing fitness in *r*₁*s* did not differ significantly ($t_{18} = 1.74$, $P = 0.10$). For SAF97-H2, a fitness of 0.98 in *r*₁*s* best improved the fit with the observed trajectories relative to a fitness of 0.97 or 0.99. Significant linear ($t_{18} = -10.50$, $P < 0.0001$) and quadratic ($t_{18} = 10.27$, $P < 0.0001$) associations occurred for fitness of the *rr* genotypes, and the model with a cost reducing fitness in *r*₁*s* fit significantly better than the model with a completely recessive cost (Fig. 4; $t_{18} = -9.29$, $P < 0.0001$).

Comparisons of simulations with variable fitness of the *rr* genotypes and with and without costs reducing fitness in *r*₁*s* also support the hypothesis that a small cost affected *r*₁*s* in SAF97-H2, but not in MOV97-H2. For MOV97-H2, a fitness of 0.99 in *r*₁*s* yielded

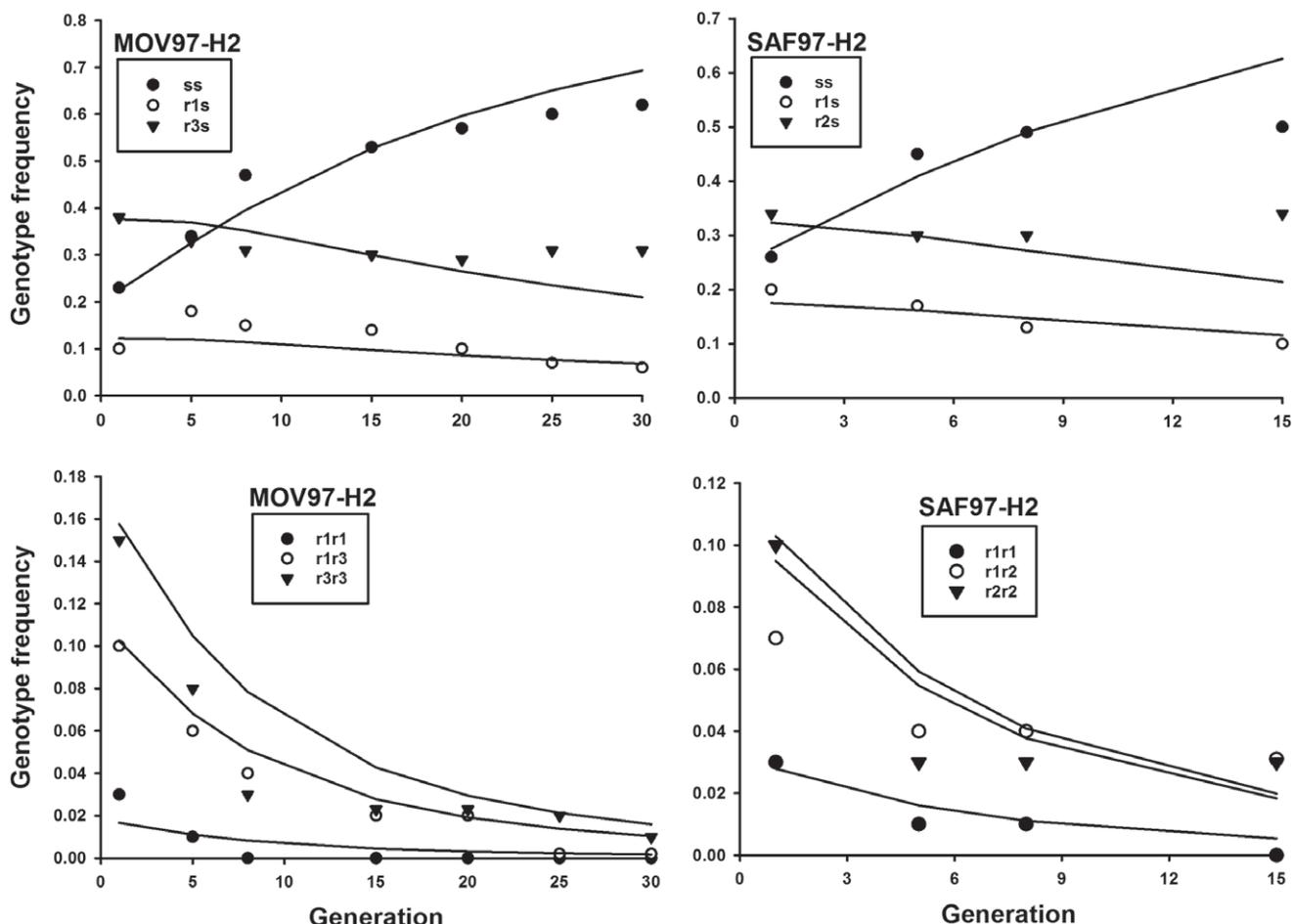


Figure 2. Simulated (lines) and observed (symbols) genotype frequency assuming recessive costs on the *rr* genotypes of 19% in MOV97-H2 and 26% in SAF97-H2. Note that the scale of the x- and y-axes differs between panels. When two genotypes had the same average of differences for a generation, the average of differences was slightly shifted for one genotype to make all points visible.

the best fit with the observed trajectories compared with a fitness of 0.97 and 0.98. With a fitness of 0.99 in r_1s , the linear and quadratic coefficients in the model were significant for r_3r_3 and r_1r_3 (all P -values < 0.0001), but the linear ($t_{292} = -0.77$, $P = 0.44$) and quadratic ($t_{292} = 0.80$, $P = 0.43$) coefficients were not significant for r_1r_1 . No significant difference occurred between models with and without costs reducing fitness of r_1s ($t_{292} = -0.09$, $P = 0.93$). For SAF97-H2 and fitness of 0.98 in r_1s , all linear and quadratic coefficients were significant (P -values < 0.011). Also, for SAF97-H2, the fit to the observed trajectory of genotype frequency was better for the model with a cost affecting r_1s than the model without this cost ($t_{242} = -5.65$, $P < 0.0001$).

4 DISCUSSION

The temporal decline in the frequency of resistance alleles in the hybrid strains (Table 1) confirms previous results identifying fitness costs associated with resistance to Cry1Ac cotton in pink bollworm from Arizona.^{13,31–36} Simulations with equal recessive costs affecting the *rr* genotypes (scenario A with costs of 19% in MOV97-H2 and 26% in SAF97-H2) corresponded well with the observed changes in genotype frequency (Fig. 2). In comparison, simulations with unequal recessive costs (scenario B) or a small cost reducing fitness of r_1s (scenario C) reduced the deviations between observed and simulated genotype frequency slightly,

but not significantly (Fig. 3). Accordingly, our estimates of costs reducing fitness of the *rr* genotypes range between 14 and 22% for MOV97-H2, and between 21 and 36% for SAF97-H2 (Table 2). Further comparisons of simulations with the presence or absence of a cost reducing fitness in r_1s and equal or variable costs in the *rr* genotypes indicate that a small cost (i.e., 2%) reduced fitness of r_1s in SAF97-H2, but not in MOV-H2. Fitness costs of Bt resistance are affected by environmental factors as well as the genetic background.^{13,43,44} Differences in genetic background could have accounted for the less-recessive costs affecting r_1s in SAF97-H2 compared with MOV97-H2.

The decline in frequency of r_1 was linear across generations in both strains but the decline in frequency of r_2 and r_3 slowed in later generations (Table 1 and Fig. S1 in File S1). When the frequency of resistance declines, selection against resistance alleles with recessive costs also declines because homozygous-resistant individuals become relatively rare. By contrast, selection against alleles with non-recessive costs is higher because heterozygotes remain relatively abundant. Accordingly, alleles inducing non-recessive costs should be eliminated at a faster rate than alleles inducing recessive costs⁴⁵ (File S1). The linear and proportionally faster decline in r_1 relative to the decelerating decline in r_2 and r_3 , despite the lower initial frequency of r_1 than r_2 and r_3 , could indicate a greater fitness cost in r_1r_1 relative to r_2r_2 and r_3r_3 , in r_1s relative to r_2s and r_3s ,

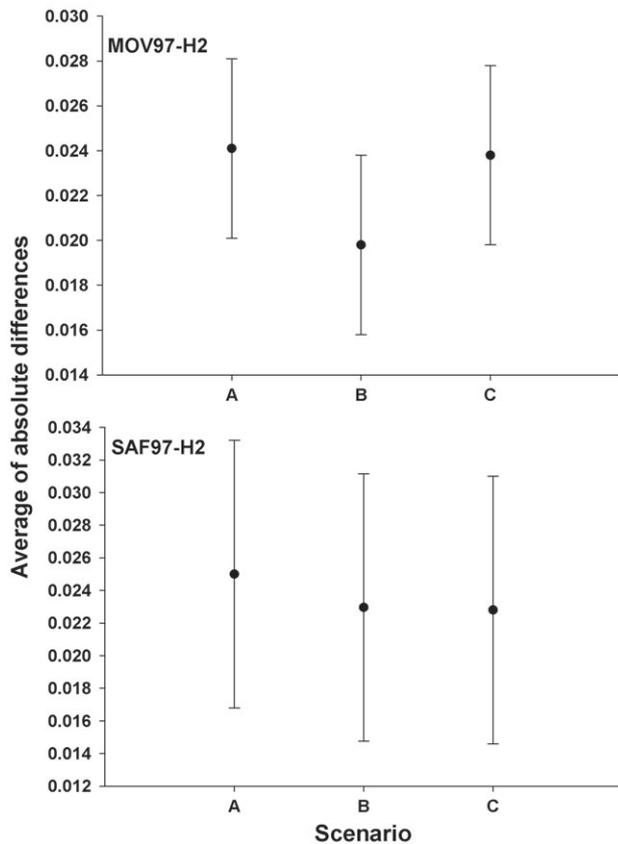


Figure 3. Mean absolute differences (\pm SE) between observed and simulated frequency of genotypes for fitness values yielding the best fit in simulations with scenario A, B, and C (see Table 2).

or both (see File S1). Yet, analyses of results from simulation models revealed a higher cost in r_1s than r_2s in SAF97-H2, but a similar cost in r_1s and r_3s in MOV97-H2, and did not provide conclusive evidence that costs were significantly higher in r_1r_1 than r_2r_2 or r_3r_3 (Table 2 and Fig. 3).

Previous studies of costs of Cry1Ac resistance in pink bollworm strains from Mohave and Safford fed on artificial diet without gossypol have revealed primarily recessive costs affecting development time and growth rate, but no costs affecting pupal weight and survival.^{33,36} Similar studies with non-Bt cotton plants always detected recessive costs affecting development time, pupal weight or survival.^{34,35} However, non-recessive costs of Bt resistance affecting fitness components seem relatively common.¹³ For example, in 16 studies evaluating the dominance of costs, the percentage of non-recessive costs was 43% for development time, 21% for survival and 0% for growth rate.¹³ Here, costs of resistance to Cry1Ac cotton appeared recessive in MOV97-H2, although the relative fitness of r_1s was reduced in SAF97-H2. The dominance of resistance to Bt crops or toxins can be estimated using parameter h , which varies between 0 for recessive resistance and 1 for dominant resistance.⁴⁶ To estimate the dominance of fitness costs, h is calculated as: $h = (W_{ss} - W_{rs}) / (W_{ss} - W_{rr})$, where W_{ss} , W_{rs} and W_{rr} refer to fitness of the ss , rs and rr genotypes, respectively. For example, assuming that fitness of r_1r_1 was 0.74, fitness of r_1s was 0.98, and fitness of ss was 1 in SAF97-H2 (Table 2 and Fig. 4), the estimate of h for the cost affecting r_1s is 0.08.

Meta-analyses of fitness costs of Bt resistance and of fitness costs associated with resistance to Cry1Ac in pink bollworm have

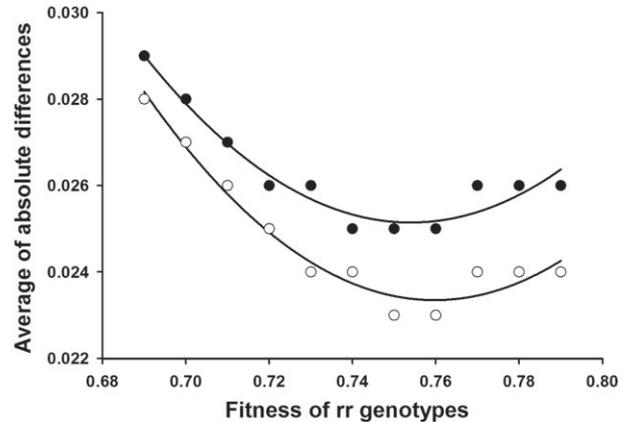


Figure 4. Association between fitness of rr genotypes and the average of the absolute differences between observed and simulated genotype frequencies in SAF97-H2. Simulations with equal fitness of the rr genotypes and no costs affecting the rs genotypes (closed symbols) are compared to identical simulations with a cost of 2% in r_1s (open symbols).

shown that significantly higher costs occurred in strains fed plants rather than artificial diet.¹³ This suggests that the relative fitness of the resistant genotypes estimated here could be overestimated. In previous studies of fitness components in pink bollworm strains from Mohave and Safford with larvae reared on cotton plants, the range of recessive costs associated with resistance to Cry1Ac was 0–11% for development time, 0–8% for pupal weight, and 0–55% for survival on various non-Bt cotton cultivars.^{31,34,35,39} Based on these data, Tabashnik *et al.*¹⁰ calculated a mean recessive cost affecting larval survival on non-Bt cotton of 41%. This cost is higher than the range of fitness costs estimated here from diet experiments (14–36%), supporting the general pattern that fitness costs of resistance to Bt toxins are higher on plants than on artificial diet.¹³

Fitness component studies evaluating r_m in strains fed on plants report similar costs as estimated here in MOV97-H2, but smaller costs than in SAF97-H2. In two separate experiments investigating related Cry1Ac-resistant and -susceptible strains of *H. armigera* on cotton, recessive costs of 22.8% and 27.5% affected r_m .^{18,19} In a similar experiment,²⁰ a Bt-susceptible strain and three related Bt-resistant strains of *Tricoplusia ni* were fed on different host plants (cabbage, cotton, tobacco and tomato). Across host plants, costs affecting r_m varied between 1% and 18% in a Cry1Ac-resistant strain, 2–33% in a Cry2Ab-resistant strain, and 5–26% in a strain resistant to Cry1Ac and Cry2Ab. On cotton, the largest cost affecting r_m in the resistant strains was 26%,²⁰ which was somewhat higher than the largest cost affecting r_m in the same strains on cotton (i.e., 19%) in a separate experiment.²¹ The average cost affecting r_m on plants in these studies^{18–21} was 17.2% ($N = 16$, $SE = 2.4$, 95% confidence interval = 12.0–22.4%).

Cao *et al.*⁴⁷ also estimated costs affecting r_m in 10 Cry1Ac-resistant strains of *H. armigera* fed artificial diet. The Bt-susceptible strain and all Cry1Ac-resistant strains they investigated originated from the Yellow River region of China. The association between costs affecting r_m and levels of resistance to Cry1Ac in these strains was generally positive, although the best-fit relationship was non-linear and reached a plateau indicating that the maximum cost was 24.5%. The strains used by Cao *et al.*⁴⁷ had been selected for resistance to Cry1Ac for a variable number of generations (between 6 and 105), implying that the

non-linear association between levels of resistance and costs could have been due to among-strain variation in the frequency of resistance alleles^{48,49} or in resistance mechanisms associated with different costs.⁴⁷

Resistance to Bt crops cannot always be expected to be associated with significant fitness costs, as illustrated for example by studies of resistance to Cry2Ab in *H. armigera*⁵⁰ and resistance to various Bt toxins in corn rootworm.⁵¹ Furthermore, a high diversity of resistance alleles can occur in some pest populations,^{52,53} implying that selection could favor the spread of resistance alleles associated with low costs and other desirable characteristics in such populations.⁵⁴ Nevertheless, simulation models assuming nearly recessive resistance and efficient redundant killing (which occurs when each toxin in a pyramid kills most susceptible insects)^{55,56} show that relatively small recessive fitness costs (i.e., 5% or 10%) can significantly delay or reverse the evolution of pest resistance to pyramided Bt crops.¹¹ In these simulations, significant delays in resistance occurred even with relatively high frequencies of resistance alleles (e.g., 0.05) and small refuges (e.g., 10%). Our estimates of the overall fitness cost of resistance to Cry1Ac cotton in pink bollworm genotypes and results of studies discussed herein indicate that the cost of Bt resistance could easily be larger than the largest cost (i.e., 10%) simulated by Gould et al.¹¹ This implies that costs could be a major factor for delaying or reversing the evolution of resistance to pyramids in systems where pests show high susceptibility to Bt toxins and recessive inheritance of resistance.^{4,5,7,11} Irrespective of pest susceptibility to Bt toxins, the novel approach outlined here for estimating the relative fitness of resistant genotypes in the absence of Bt could be useful to study impacts of fitness costs, especially if the decline in frequency of resistance was quantified in strains fed on plants.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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