Effects of Pink Bollworm Resistance to *Bacillus thuringiensis* on Phenoloxidase Activity and Susceptibility to Entomopathogenic Nematodes

AARON J. GASSMANN,[1,2] JEFFREY A. FABRICK,[3] MARK S. SISTERSON,[4] EUGENE R. HANNON,[1] S. PATRICIA STOCK,[1] YVES CARRIÈRE,[1] AND BRUCE E. TABASHNIK[1]

J. Econ. Entomol. 102(3): 1224–1232 (2009)

ABSTRACT Widespread planting of crops genetically engineered to produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt) imposes selection on many key agricultural pests to evolve resistance to Bt. Fitness costs can slow the evolution of Bt resistance. We examined effects of entomopathogenic nematodes on fitness costs of Bt resistance in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), a major pest of cotton, *Gossypium hirsutum* L., in the southwestern United States that is currently controlled by transgenic cotton that produces Bt toxin Cry1Ac. We tested whether the entomopathogenic nematodes *Steinernema riobrave* Cabanillas, Poinar, and Raulston (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) affected fitness costs of resistance to Cry1Ac in two laboratory-selected hybrid strains of pink bollworm reared on non-Bt cotton bolls. The nematode *S. riobrave* imposed a recessive fitness cost for one strain, and *H. bacteriophora* imposed a fitness cost affecting heterozygous resistant individuals for the other strain. Activity of phenoloxidase, an important component of insects' immune response, did not differ between Bt-resistant and Bt-susceptible families. This suggests phenoloxidase does not affect susceptibility to entomopathogenic nematodes in Bt-resistant pink bollworm. Additionally, phenoloxidase activity does not contribute to Bt resistance, as has been found in some species. We conclude that other mechanisms cause higher nematode-imposed mortality for pink bollworm with Bt resistance genes. Incorporation of nematode-imposed fitness costs into a spatially explicit simulation model suggests that entomopathogenic nematodes in non-Bt refuges could delay resistance by pink bollworm to Bt cotton.

KEY WORDS biological control, fitness cost, immune response, integrated resistance management, simulation modeling

Planting of genetically modified crops that produce insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Bt) continues to increase rapidly. In 2007, Bt crops were planted on >41 million ha worldwide, a 31% increase from 2006 (James 2007). Some insect pests are under intense selection to evolve resistance to Bt, and three cases of field-evolved resistance to Bt crops have been documented (Luttrell et al. 2004, van Rensburg 2007, Matten et al. 2008, Tabashnik et al. 2008).

To counter the threat of pest resistance to Bt crops, the refuge strategy has been adopted in the United States and elsewhere (Gould 1998). This strategy requires non-Bt host plants near Bt crops, with the goal that these non-Bt hosts will serve as refuges and produce large numbers of Bt-susceptible insects. Ideally, the rare resistant individuals that emerge from Bt fields will mate primarily with the abundant susceptible individuals from refuges and produce heterozygous progeny. If transgenic plants produce Bt toxin at a sufficiently high concentration, resistance to Bt will be inherited as a recessive trait (Tabashnik et al. 2004), and the heterozygous progeny produced from matings between Bt-resistant and Bt-susceptible individuals will die on Bt crops. Over time, however, Bt-resistance alleles may accumulate in refuge populations, eventually leading to the evolution of resistance to the Bt crop (Sisterson et al. 2005). Fitness costs of Bt resistance can reduce the frequency of resistance alleles in refuge populations and thereby slow or prevent resistance by pests to Bt crops (Carrière and Tabashnik 2001, Gould et al. 2006). Thus, refuges may have three benefits for delaying resistance: reducing the proportion of the pest population selected for resistance, providing susceptible insects to mate with resistant

[1] Department of Entomology, University of Arizona, Tucson, AZ 85721.
[2] Corresponding author and current address: Department of Entomology, Iowa State University, Ames, IA 50011 (e-mail: aaronjg@iastate.edu).
[3] USDA–ARS, U.S. Arid Land Agricultural Research Center, Maricopa, AZ 85238.
[4] USDA–ARS, San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648.

0022-0493/09/1224–1232$04.00/0

insects, and imposing fitness costs that select against resistance.

Resistance to Bt may result in trade-offs, where the benefit of greater fitness for Bt-resistant versus Bt-susceptible individuals after exposure to Bt is accompanied by fitness costs that reduce the fitness of individuals with resistance alleles in the absence of Bt. Fitness costs are often associated with Bt resistance (Gassmann et al. 2009). Costs may be recessive, occurring only in homozygous resistant genotypes, or costs may be nonrecessive, reducing the fitness of individuals heterozygous for resistance as well as homozygous resistant individuals. When resistance alleles are at low frequency, they will reside predominately in heterozygotes (Hartl and Clark 1997). Thus, fitness costs affecting heterozygotes will be the most effective at delaying pest resistance (Carrière and Tabashnik 2001, Pittendrigh et al. 2004).

Ecological factors can affect both the magnitude and dominance of fitness costs, and consequently, the ability of refuges to delay resistance. This means that some refuges may be better at delaying pest resistance to Bt than other refuges. For example, the host plant on which an insect feeds can alter both the magnitude and dominance of costs (Shirai et al. 1998, Carrière et al. 2005, Janmaat and Myers 2005, Janmaat and Myers 2006, Bird and Akhurst 2007, Raymond et al. 2007b). In addition, entomopathogenic viruses and nematodes can magnify costs in some cases (Gassmann et al. 2006, 2008; Raymond et al. 2007a). Identifying ecological conditions that produce the largest and most dominant costs may lead to the design of refuges that are more effective at delaying pest resistance to Bt crops.

Although a growing body of literature demonstrates that fitness costs of Bt resistance vary with ecological conditions, little is known about the physiological, molecular, or behavioral mechanisms by which some ecological conditions magnify costs (Gassmann et al. 2009). With susceptibility to entomopathogenic nematodes, differences in immune response between Bt-resistant and Bt-susceptible genotypes may contribute to pathogen-mediated fitness costs. Entomopathogenic nematodes in the families Steinernematidae and Heterorhabditidae enter their insect host's hemocoel through natural openings, such as the mouth, and then release symbiotic bacteria that in turn kill the host insect (Burnell and Stock 2000, Dowds and Peters 2002, Park and Stanley 2006). However, insects may fend off infection from nematodes through the encapsulation and/or melanization of nematodes before the nematodes release symbiotic bacteria (Li et al. 2007). Melanization occurs by the action of the prophenoloxidase pathway (Kanost et al. 2004) and differences in the level of phenoloxidase activity could contribute to differences in susceptibility to pathogens between Bt-resistant and Bt-susceptible genotypes. Additionally, resistance to Bt toxin has been associated with increased phenoloxidase activity in some species (Rahman et al. 2004, Ma et al. 2005) but not others (Wang et al. 2007), although it is unknown whether these Bt-resistant genotypes show altered susceptibility to entomopathogenic nematodes. Altogether, it is reasonable to hypothesize that Bt resistance might result in either an increase or decrease in phenoloxidase activity.

In this study, we examined the effects of entomopathogenic nematodes on fitness costs of Bt resistance in pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), a major pest of cotton, *Gossypium hirsutum* L., in the southwestern United States that is currently controlled by Bt cotton that produces Bt toxin Cry1Ac (Henneberry and Jech 2000, Tabashnik et al. 2005b). We tested whether the nematodes *Steinernema riobrave* Cabanillas, Poinar, and Raulston (Rhabditida: Steinernematidae) and *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) affected fitness costs of resistance to Cry1Ac in two laboratory-selected hybrid strains of pink bollworm reared on non-Bt cotton bolls. We also tested whether Bt-resistant and Bt-susceptible pink bollworm differ in phenoloxidase activity. We conclude by applying a simulation model to assess how fitness costs imposed by entomopathogenic nematodes could affect the time until populations evolve resistance to Bt cotton.

Materials and Methods

Greenhouse Experiment. *Insect Strains.* We used pink bollworm from two hybrid strains, each containing a mixture of resistant, susceptible and heterozygous individuals: MOV97-H3 (Carrière et al. 2006) and SAF97-H4. MOV97-H3 originated from MOV97, and SAF97-H4 from SAF97. In 1997 individuals collected from the Mohave Valley and from Safford, AZ, were used to start MOV97 and SAF97, respectively. The initial frequency of Cry1Ac resistance (r) alleles was >15% in MOV97 and SAF97 (Tabashnik et al. 2000).

To maintain an intermediate r allele frequency, hybrid strains derived from MOV97 and SAF97 were periodically split into two substrains, one of which was selected for resistance by rearing on diet containing Cry1Ac for one generation. In reciprocal mass crosses, the survivors of this selection mated with the insects from the unselected substrain (Carrière et al. 2006). At the time of the experiment, 13 generations had passed since MOV97-H3 was produced from its predecessor (MOV97-H2) by using this procedure.

SAF97-H4 was produced by crossing strains SAF97-H1S with SAF97-H1R, which were originally derived from SAF97-H1 (Gassmann et al. 2008). SAF97-H1S was a homozygous susceptible strain (i.e., lacked r alleles) and SAF97-H1R was a homozygous resistant stain (i.e., all individuals were homozygous for r alleles) (Gassmann et al. 2008). Because SAF97-H1 became contaminated by another strain, it was discarded and subsequently reconstituted as SAF97-H4 using insects from SAF97-H1R and SAF97-H1S. This was accomplished by mass mating 100 SAF97-H1S females with 100 SAF97-H1R males, and 100 SAF97-H1S males with 100 SAF97-H1R females. Resulting progeny from these crosses were pooled to begin SAF97-H4, which had been reared on diet without Bt toxin for six generations at the time of the experiment.

Previous work identified cadherin alleles linked with resistance (*r1*, *r2*, and *r3*) or susceptibility (*s*) to Cry1Ac (Morin et al. 2003). Larvae that have two *r* alleles in any combination (e.g., *r1r3* or *r1r1*) can survive on Bt cotton plants, whereas larvae with one (e.g., *r1s*) or no *r* alleles (i.e., *ss*) die on Bt cotton (Tabashnik et al. 2005a). At the time of the experiment, the estimated allele frequencies were $r1 = 0.15$, $r3 = 0.20$, and $s = 0.65$ for MOV97-H3 and $r2 = 0.42$ and $s = 0.58$ for SAF97-H4.

Cotton Plants. We used non-Bt cotton plants (Delta and Pine 5415) started from seed in plastic flats (27 by 90 by 5 cm) and grown in a greenhouse in Tucson, AZ, from April to September 2006. Seedlings were transplanted to 20-liter flower pots after their first true leaves had fully expanded, with three seedling planted per pot. Plants were grown two to three per pot, depending on seedling survival. Plants were watered twice per day with a drip irrigation system and were given supplemental fertilizer (Osmocote, The Scotts Miracle-Gro Company, Marysville, OH) twice per month. Cotton plants were ≈5 mo of age at the start of the experiment.

Nematodes. Two species of nematodes were used: *Steinernema riobrave* (Texas strain) and *Heterorhabditis bacteriophora* (Monterey strain). Nematodes were produced for this experiment through in vivo culturing in *Galleria mellonella* L. (Lepidoptera: Pyralidae) (Kaya and Stock 1997).

Experimental Design. We tested 21 groups of insects: 10 from SAF97-H4 and 11 from MOV97-H3. Each group was generated by putting nine males and nine females from the same strain in a 137-ml paper cup with a vial of honey-water solution and paper toweling as oviposition substrate ("egg sheet") (Chix Masslinn Shop Towel 0930, Chicopee Manufacturing, Benson, NC). We used eggs from the cups to infest bolls on cotton plants in the greenhouse as described by Liu et al. (2001). Briefly, egg sheets were collected from cups every 2 d and cut into pieces containing 20–30 eggs. Bolls were infested by placing a single piece of egg sheet under floral bracts. Bolls were then enclosed in mesh bags (polyester organza, 7.5 by 14 cm; Pouch Depot Inc., Los Angeles, CA). Only egg sheets from a single group were used to infest bolls from cotton plants in the same pot. To prevent movement of neonates between groups, plants from different pots were separated so they did not touch. We used a total of 52 pots, 122 cotton plants, and 312 infested bolls. For each group of insects, the mean ± SD was 2.5 ± 0.6 for pots, 5.8 ± 2 for plants, and 15 ± 10 for bolls.

After 16 d, bolls were cut from plants in the greenhouse, brought to the laboratory, and removed from mesh bags. For each group, bolls were distributed randomly and equally among three treatments: *S. riobrave*, *H. bacteriophora*, and control. We glued two to three bolls with insects from the same group to the side of a 500-ml clear plastic cup at ≈3 cm above the bottom. All cups were lined with 35 g of sterile sand moistened with 9 ml of deionized water. The number of infective juvenile nematodes per milliliter of water was three for the *S. riobrave* treatment, 20 for the *H. bacteriophora* treatment, and zero for the control. This translates to an application rate of 0.4 *S. riobrave* per cm^2 and 2.7 *H. bacteriophora* per cm^2. Cups were held in a growth chamber (29°C; photoperiod of 14:10 [L:D] h). When larvae reached the wandering stage of the fourth (final) instar, some chewed through the cotton bolls and fell to the sand below where they pupated. While on the sand, larvae also came in contact with nematodes, which killed some of them.

Cups were checked every third day for adult emergence. Adults ($n = 276$) found in cups were placed individually in 1.5-ml microfuge tubes containing 95% ethanol and held for later genotyping analysis. Of the 276 moths collected, 220 were randomly selected with a stratified sampling technique and their DNA extracted using the method described in Tabashnik et al. (2005b). The stratified sampling technique consisted of randomly selecting one moth from each combination of group and treatment (i.e., control, *S. riobrave*, and *H. bacteriophora*) during each round of sampling. Seven rounds of sampling were conducted after which sampling was exhaustive for all but seven of the 30 group by treatment combinations for SAF97-H4 and six of the 33 group by treatment combinations for MOV97-H3. Of the 220 moths subjected to DNA extraction, 216 yielded amplifiable DNA at a cadherin locus as determined using intron control primers described in Morin et al. (2004). We used allele-specific polymerase chain reaction (PCR) primers (Morin et al. 2003, 2004) to screen for resistance alleles *r1* and *r2* in SAF97-H4 and *r2* and *r3* in MOV97-H3.

Phenoloxidase Activity. The unit of replication in this experiment was a family. Each family was started from a single mated pair from SAF97-H4, which had been reared on non-Bt diet for 11 generations at the time of this experiment. In total, there were five Bt-susceptible families and eight Bt-resistant families. Bt-resistant pairs were produced by first raising some larvae from SAF97-H4 on artificial diet containing 10 μg Cry1Ac/ml diet. Only homozygous resistant individuals can survive this concentration of toxin (Morin et al. 2003). Mated pairs from survivors on Bt diet were used to initiate the Bt-resistant families. Bt-susceptible families were started by collecting eggs from mated pairs and then screening each pair with cadherin specific primers (Morin et al. 2003, 2004) to test for the absence of *r1* and *r2* alleles. For those pairs lacking *r* alleles, we ran two additional checks to confirm the susceptibility of the families. First, adults from the F1 generation of each family were pooled and screened with cadherin specific primers. Second, ≈100 eggs from the F2 generation of each family were placed on Bt diet with 10 μg Cry1Ac/ml diet and tested for survival to the fourth instar. Families that were negative for these two additional screens were classified as Bt susceptible.

For each family, ≈100 eggs from the F4 generation were placed in 137-ml cups with 75 g of artificial diet (Adkinsson et al. 1960). When wandering larvae emerged, all larvae present in the diet cup were counted, and six wandering larvae were selected at

random to assay for phenoloxidase (PO) activity. Basal PO activity (without the addition of an elicitor) was assayed by using a method adapted from Hall et al. (1995) and Fabrick et al. (2004). Briefly, each larva was chilled on ice and a proleg was removed with a micro-scissors. Approximately 10 μl of hemolymph was drawn into a pipette tip, placed in a 1.5-ml microfuge tube, and centrifuged at 10,000 × *g* for 5 min. Four microliters of supernatant (cell-free plasma) was diluted with 50 μl of sterile deionized water in clean 1.5-ml microfuge tube. Two-microliter samples of dilute cell-free plasma were preincubated with 78 μl of 50 mM sodium phosphate buffer, pH 6.8, for 5 min at 30°C in a 96-well microplate. PO assays were initiated by adding 20 μl of 10 mM dopamine substrate to each plasma sample. Absorbance at 490 nm was measured at 1-min intervals over 15 min at 30°C by using a BioTek Synergy plate reader (BioTek Instruments, Winooske, VT). PO activity was quantified as the slope of change in absorbance at 490 nm milli Optical Density (mOD) per minute, with larger values indicating greater phenoloxidase activity. Slopes were standardized by controls, which contained all reagents but lacked larval hemolymph.

To minimize the time required to process and initiate each PO assay, larvae were assayed in batches of five to 13 randomly selected larvae, with a total of six batches required to assay all larvae. Three larvae were analyzed for each of the 13 families, and PO activity assays for each larva were run in triplicate (total sample sizes were 39 larvae and 117 PO activity assays).

Simulation Modeling. We applied a computer model to consider how nematode-imposed fitness costs in refuges might affect evolution of resistance by pink bollworm to Bt cotton. We used a previously developed spatially explicit, stochastic simulation model based on the biology of pink bollworm in Bt and non-Bt cotton fields (Sisterson et al. 2004, 2005). Each simulation had a landscape of 400 fields that were divided into 16 individual "farms" of 25 fields (five by five). In each simulation run, all farms had the same percentage of refuge (i.e., non-Bt cotton fields). Mortality in Bt and non-Bt cotton fields was estimated from empirical data (Sisterson et al. 2004, 2005). Mortality from egg to pupa in Bt fields was the same in all simulations: *rr* = 0.793, *rs* and *ss* = 1.

We considered four different scenarios in non-Bt fields (i.e., refuges): a) no fitness costs, b) fitness costs affecting only *rs* individuals (see Results), c) recessive fitness costs affecting only *rr* individuals (see Results), and d) dominant fitness costs affecting *rr* and *rs* individuals (Table 1). In simulations without nematodes, we assumed no fitness costs and set mortality in refuges at *rr* = 0.793, *rs* = 0.793, and *ss* = 0.792, with mortality slightly lower for *ss* to balance the effect of mutation introducing *r* alleles into the population (Sisterson et al. 2004). Nematode-imposed fitness costs were incorporated into the model by increasing mortality from egg to pupa by 7.8%. When nematodes were present in refuges, mortality of *rr* and/or *rs* increased to 0.855 ((0.855 − 0.793)/0.793 = 0.078; 0.078 × 100% = 7.8%). We selected this value based on past studies that found a range of nematode-induced costs from 0 to 16% affecting *rr* individuals (Gassmann et al. 2006, 2008). The initial *r* allele frequency was set at 0.01. Simulations were run for 100 yr. The criterion for resistance was a total *r* allele frequency of 0.50.

Table 1. Values used in simulations for mortality of pink bollworm in non-Bt refuges for genotypes *ss*, *rs*, and *rr* under four different assumptions about fitness costs (a–d)

Type of cost	Mortality		
	ss	*rs*	*rr*
a. No fitness cost	0.792	0.793	0.793
b. Cost affecting only *rs*	0.792	0.855	0.793
c. Recessive cost	0.792	0.793	0.855
d. Dominant cost	0.792	0.855	0.855

Data Analysis. Analyses were conducted in SAS and were based on analysis of variance (ANOVA) or covariance (ANCOVA)) with a mixed model (Proc MIXED) (SAS Institute 1999). Random factors in the model were insect group (or family) and its interactions. These random factors were tested with a log-likelihood ratio statistic (−2 RES log likelihood in Proc MIXED), which provides a one-tailed test based on a chi-square distribution with 1 df (Littell et al. 1996). When these random factors were not significant they were excluded from the subsequent model. However, lower order terms were retained in the model if their higher order interactions were significant. Data were transformed as described below to ensure normality of the residuals.

For the greenhouse experiment, the total number of moths surviving per group was analyzed with a mixed model ANOVA that included the fixed factors of strain and nematode treatment, and the random factor of group, which was nested within strain. Data were transformed by the $\log(x + 1)$ function.

For the 216 samples from which we obtained a PCR-based genotype, we tested for the presence of nematode-imposed costs by analyzing the relative fitness of each genotype. Because too few individuals were genotyped per group to accurately estimate the frequency of individual cadherin genotypes, cadherin genotypes *r1r1*, *r2r2*, *r1r3*, *r3r3*, and *r1s*, *r2s*, *r3s* were respectively treated as *rr* or *rs* in the statistical analysis. For each group, relative fitness was calculated as (number of adults for one genotype in the presence of nematodes) / (number of adults for the same genotype in the control). For example, the relative fitness for *rs* genotype from SAF97-H4 group 1 in the presence of *S. riobrave* was obtained by dividing the number of *rs* individuals found for that group in the *S. riobrave* treatment by the number of *rs* individuals for that group in the control. If a genotype was found in a nematode treatment but not in the control, then we assumed emergence of one individual in the control (13 of 117 cases). If no individuals for a genotype were found in both the control and nematode treatment, then the data were excluded from the analysis (20 cases). Relative fitness values were analyzed with a mixed-model ANOVA that included the fixed factors of strain, genotype, and nematode treatment, and the

Table 2. Effects of nematode treatment and pink bollworm strain (MOV97-H3 and SAF97-H4) on survival to the adult stage

Source[a]	df	*F*	*P*
Treatment	2, 38	20.51	<0.0001
Strain	1, 19	0.001	0.97
Treatment × strain	2, 38	0.71	0.50

[a] The random factor of group (strain) ($\chi^2 = 5.7$, df = 1, $P = 0.008$) was included in the analysis.

Table 3. Effects of nematode treatment, cadherin genotype (*ss*, *rs*, and *rr*), and strain on the relative fitness of pink bollworm

Source[a]	df	*F*	*P*
Treatment	1, 85	0.04	0.83
Genotype	2, 85	0.89	0.42
Strain	1, 85	0.06	0.80
Treatment × genotype	2, 85	2.14	0.12
Genotype × strain	2, 85	3.68	0.03
Treatment × strain	1, 85	0.01	0.91
Genotype × treatment × strain	2, 85	0.34	0.71

[a] There were no significant random factors in the analysis.

random factor of group, which was nested within strain. Data were transformed by the square-root function. For each strain by treatment combination, we tested for fitness costs by making a priori linear contrasts of *ss* against *rs* and *rr* (CONTRAST statement in Proc MIXED).

Data on phenoloxidase activity were analyzed with a mixed model ANCOVA. Insect genotype was a fixed factor, family was a random factor, and the density of insects in each diet cup was a covariate. Cadherin genotypes were pooled under the category *rr* for homozygous resistant lines. Any negative values for phenoloxidase activity were set to 0, and data were transformed by the square-root function.

Results

Greenhouse Experiment. Treatment with nematodes significantly reduced the total number of pink bollworm adults emerging for both the MOV97-H3 and SAF97-H4 strains (Table 2). For both strains, survival was decreased by treatment with *S. riobrave* ($F = 21.7$; df = 1, 38; $P < 0.0001$) or *H. bacteriophora* ($F = 37.6$; df = 1, 38; $P < 0.0001$) but did not differ between treatments with these two nematode species ($F = 2.17$; df = 1, 38; $P = 0.15$) (Fig. 1).

We tested for nematode-imposed fitness costs by comparing relative fitness of pink bollworm cadherin genotypes in the presence of nematodes. A fitness cost was present when *rr* or *rs* had lower relative fitness than *ss*. A significant genotype by strain interaction was present (Table 3), indicating that the relative fitness of genotypes in the presence of nematodes differed between the MOV97-H3 and SAF97-H4 strains. Therefore, we analyzed relative fitness of genotypes separately for the two strains.

For MOV97-H3, linear contrasts revealed that the relative fitness of *rr* was significantly lower than *ss* in the *S. riobrave* treatment ($F = 4.08$; df = 1, 85; $P = 0.05$) (Fig. 2a). This indicates that *S. riobrave* imposed a fitness cost in the MOV97-H3 strain. Relative fitness did not differ between *rr* and *ss* in the *H. bacteriophora* treatment ($F = 0.01$; df = 1, 85; $P = 0.93$). Additionally, *ss* and *rs* did not differ in the *S. riobrave* ($F = 0.31$; df = 1, 85; $P = 0.58$) or *H. bacteriophora* ($F = 0.01$; df = 1, 85; $P = 0.94$) treatments.

For SAF97-H4, relative fitness was significantly lower for *rs* than *ss* in the *H. bacteriophora* treatment ($F = 4.15$; df = 1, 85; $P = 0.04$), but *rr* and *ss* individuals did not differ from each other ($F = 0.07$; df = 1, 85; $P = 0.80$) (Fig. 2b). These results indicate that *H. bacte-*

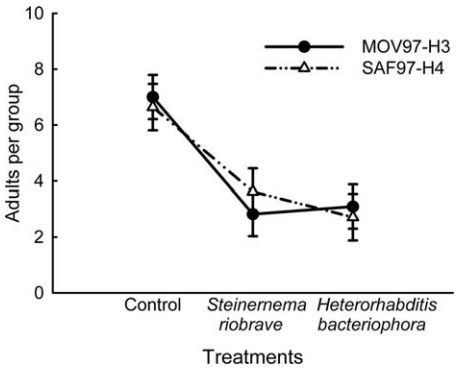

Fig. 1. Effects of nematode treatment on number of pink bollworm surviving to the adult stage (mean ± SE).

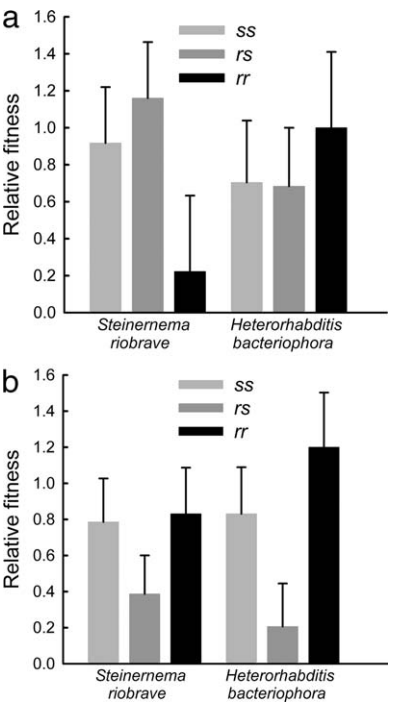

Fig. 2. Relative fitness of genotypes for MOV97-H3 (a) and SAF97-H4 (b). Bar heights represent average fitness of genotypes among groups and error bars are the standard error of the mean.

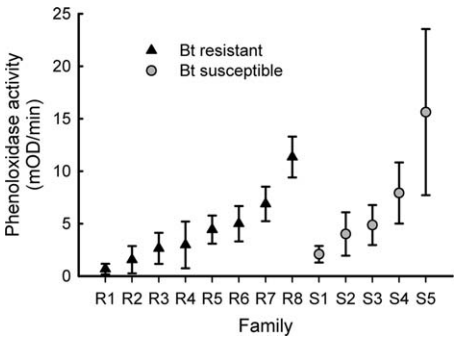

Fig. 3. Phenoloxidase activity for Bt-resistant and Bt-susceptible families. Phenoloxidase activity is quantified as the slope of change in absorbance at 490 nm mOD per minute. Each data point represents the mean value for a family and error bars are the standard error of the mean. Within Bt-resistant and Bt-susceptible genotypes, families are ordered by increasing phenoloxidase activity.

riophora imposed a fitness cost affecting *rs* individuals but not *rr* individuals for the SAF97-H4 strain. No significant differences were found between *ss* and *rs* ($F = 0.67$; df = 1, 85; $P = 0.41$) or *rr* ($F = 0.001$; df = 1, 85; $P = 0.97$) for SAF97-H4 in the *S. riobrave* treatment.

Phenoloxidase Activity. Phenoloxidase activity (mean ± SE) did not differ significantly between resistant (4.48 ± 1.51 mOD/min) and susceptible (6.75 ± 1.94 mOD/min) families of pink bollworm ($F = 0.43$; df = 1, 63, $P = 0.51$), despite significant overall variation among families ($\chi^2 = 5.5$, df = 1, $P = 0.0095$) (Fig. 3). In addition, larval density in rearing containers did not affect phenoloxidase activity ($F = 0.09$; df = 1, 63; $P = 0.76$).

Simulation Modeling. Resistance evolved faster as the percentage of refuge decreased (Fig. 4). Resistance evolved fastest without fitness costs (Fig. 4a). Costs affecting only *rr* individuals (i.e., recessive fitness costs) delayed resistance less than costs affecting only *rs* individuals (Fig. 4b and c). Costs affecting both *rr* and *rs* individuals (i.e., dominant fitness costs) had effects similar to costs affecting only *rs* individuals (Fig. 4b and d).

Discussion

We found that entomopathogenic nematodes increased the fitness cost of Bt resistance in pink bollworm. For the MOV97-H3 strain, *S. riobrave* imposed a recessive fitness cost of higher mortality for the *rr* than *ss* genotype (Fig. 2a). This result, for larvae reared on non-Bt cotton bolls, is consistent with past research, which found that *S. riobrave* imposed a recessive cost in pink bollworm when larvae were reared on diet (Gassmann et al. 2006, 2008). For the SAF97-H4 strain, *H. bacteriophora* imposed a fitness cost affecting *rs* individuals (Fig. 2b). Typically, fitness costs affecting *rs* individuals are accompanied by lower fitness for homozygous resistant (*rr*) individuals (Carrière et al. 2001, 2004; Bird and Akhurst 2004;

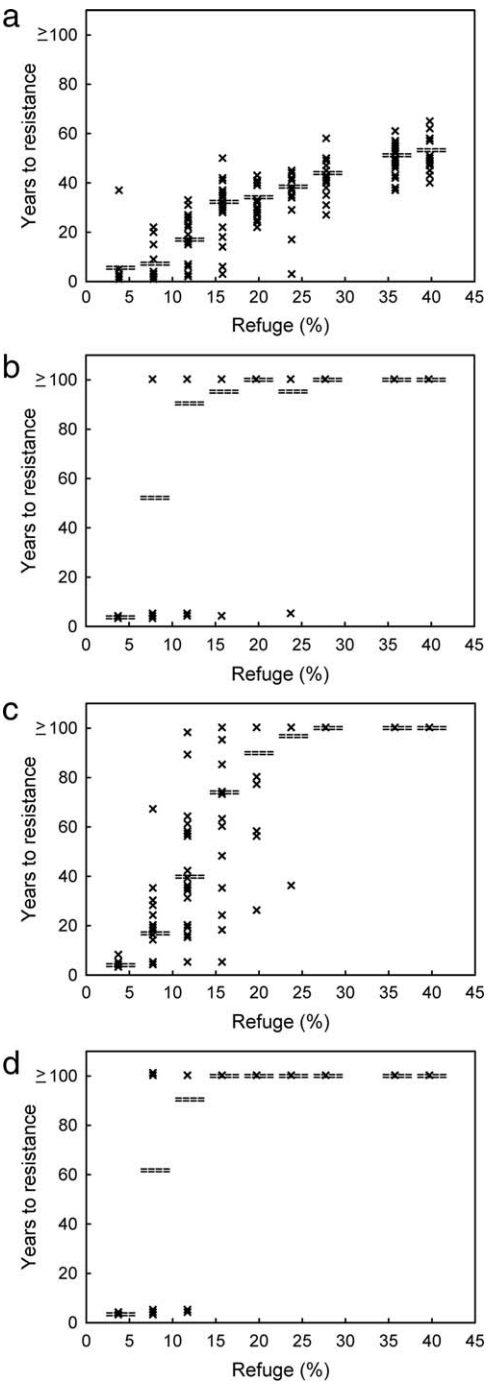

Fig. 4. Simulation results for time until populations evolve Bt resistance with no fitness cost (a), fitness cost affecting only *rs* individuals (b), recessive fitness cost (i.e., cost affecting only *rr* individuals) (c), and dominant fitness cost (i.e., cost affecting *rr* and *rs* individuals) (d). A population was defined as evolving resistance once the *r* allele frequency reached 0.50. For each refuge by fitness cost combination (e.g., dominant cost with a 35% refuge), 20 simulations were run. Broken bars (= = = =) are sample means and x's are results of individual simulations.

Huang et al. 2005; Bird and Akhurst 2007), although some studies have reported fitness costs only affecting *rs* individuals (Sayyed and Wright 2001, Janmaat and Myers 2005). We do not know why a cost affecting only *rs* individuals was found for SAF97-H4. Because the nematode species imposing fitness costs differed between insect strains, it may be that genetic factors in addition to cadherin alleles contribute to nematode-imposed fitness costs in pink bollworm. Similar results have been reported for host plant-mediated fitness costs, with insect strains of the same species differing in the magnitude of fitness costs imposed by host plants (Carrière et al. 2005, Raymond et al. 2007b).

Simulation modeling revealed that fitness costs affecting *rr* or *rs* individuals can greatly delay evolution of resistance by pink bollworm to Bt cotton when refuges of non-Bt cotton are present. Fitness costs affecting only *rs* individuals delayed resistance more than costs affecting *rr* individuals (Fig. 4). This result is consistent with previous modeling work, which found that costs affecting both *rs* and *rr* genotypes (i.e., nonrecessive costs) are more effective at delaying resistance than costs affecting only *rr* individuals (i.e., recessive costs) (Carrière and Tabashnik 2001, Pittendrigh et al. 2004, Crowder et al. 2006). Our finding here, that costs affecting only *rs* individuals can substantially delay resistance, results from almost all *r* alleles residing in heterozygotes when *r* alleles are at low frequency within the population (Hartl and Clark 1997). Consequently, the fitness of *rs* genotypes is a critical factor in determining how quickly populations will evolve resistance to Bt.

The use of entomopathogens in biological control has met with mixed results and it is reasonable to ask whether it would be economically feasible to treat refuges with entomopathogenic nematodes (Gaugler et al. 1997, Lacey et al. 2001). If it were necessary to apply nematodes at currently recommended rates of 25–50 nematodes per cm^2 (Lindegren et al. 1993, Gaugler et al. 1997) and to do so regularly, then it would likely not be economically feasible to use nematodes in integrated resistance management. However, the densities of entomopathogens used in this study were well below the recommended field level with 0.4 and 2.7 nematodes applied per cm^2 for *S. riobrave* and *H. bacteriophora*, respectively. This suggests that naturally occurring abundances of entomopathogenic nematode or those achieved by a few seasonal releases may be sufficient to provide substantial benefits for resistance management. However, it should be noted that far higher nematode concentrations are sometimes needed in the field compared with the laboratory to achieve the same percentage of insect mortality (Lindegren et al. 1993).

We did not find evidence that resistance to Cry1Ac in pink bollworm affects the level of immune defense as mediated by phenoloxidase activity. To date, phenoloxidase activity has been considered in three other species with resistance to Bt: *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae), *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), and *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) (Rahman et al. 2004, Ma et al. 2005, Wang et al. 2007). For both *E. kuehniella* and *H. armigera*, Bt resistance was associated with higher phenoloxidase activity (Rahman et al. 2004, Ma et al. 2005). However, some data suggest that higher phenoloxidase activity does not cause greater Bt resistance (Rahman et al. 2007). By contrast, Bt resistance in *T. ni* did not affect phenoloxidase activity, with resistant insects displaying slightly lower levels than Bt-susceptible insects (Wang et al. 2007). Our data indicate that the cadherin-based resistance to Bt cotton by pink bollworm is not accompanied by changes in phenoloxidase activity.

We hypothesized that lower phenoloxidase activity would contribute to greater susceptibility to entomopathogenic nematodes in Bt-resistant pink bollworm because melanization of infecting nematodes by the action of phenoloxidase may be a defense mechanism by which lepidopteran larvae fend off infection from nematodes (Li et al. 2007). The lack of a difference between genotypes suggests that other mechanisms are responsible for nematode-imposed fitness costs of Bt resistance in pink bollworm, possibly involving cellular immunity, such as a reduction in the number of hemocytes, reduced encapsulation or reduced nodulation. Because phenoloxidase activity is higher in Bt-resistant genotypes of some species, such as *H. armigera* and *E. kuehniella*, it is possible that these Bt-resistant genotypes could have elevated resistance to entomopathogens. If true, entomopathogens may only be effective at magnifying fitness costs of Bt resistance for some species.

Our finding here that entomopathogenic nematodes can increase the fitness cost of Bt resistance complements past work demonstrating ecological factors such as host plants (Janmaat and Myers 2005, Bird and Akhurst 2007), competition (Higginson et al. 2005, Raymond et al. 2005), and viruses (Raymond et al. 2007a) can increase the fitness cost of resistance to Bt. From the perspective of resistance management, these results are important because they point to an opportunity to design refuges to magnify fitness costs. Previous simulation modeling suggests that such refuges would be more effective at delaying pest resistance to Bt crops than refuges that do not impose fitness costs (Carrière and Tabashnik 2001, Pittendrigh et al. 2004). An additional benefit of using nematodes to increase fitness costs is that these organisms also can serve as biological control agents (Gouge et al. 1999, Journey and Ostlie 2000, Koppenhöfer et al. 2000), thereby decreasing the need for conventional insecticides to manage pest populations in refuges. Consequently, entomopathogens may present an opportunity to integrate resistance management with biological control through the use of pest control agents that also delay the evolution of resistance to Bt by magnifying fitness costs.

Acknowledgments

We thank Ana Romo, Alex Yelich, Christa Ellers-Kirk, Ginny Harpold, and Gopalan Unnithan for assistance. Funding was provided by the Center for Insect Science at the

University of Arizona and by the United States Department of Agriculture through grants 2005-00925, 2006-35302-17365, 2007-02227, and 2007-35302-18415.

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Received 16 December 2008; accepted 12 March 2009.