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Effects of entomopathogenic nematodes on evolution of pink bollworm resistance to Bacillus thuringiensis toxin Cry1Ac

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Abstract
The evolution of resistance by pests can reduce the efficacy of transgenic crops that produce insecticidal toxins from *Bacillus thuringiensis* (Bt). However, fitness costs may act to delay pest resistance to Bt toxins. Meta-analysis of results from four previous studies revealed that the entomopathogenic nematode *Steinernema riobrave* (Rhabditida: Steinernematidae) imposed a 20% fitness cost for larvae of pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), that were homozygous for resistance to Bt toxin Cry1Ac, but no significant fitness cost was detected for heterozygotes. We conducted greenhouse and laboratory selection experiments to determine whether *S. riobrave* would delay the evolution of pink bollworm resistance to Cry1Ac. We mimicked the high dose/refuge scenario in the greenhouse with Bt cotton (*Gossypium hirsutum* L.) plants and refuges of non-Bt cotton plants, and in the laboratory with diet containing Cry1Ac and refuges of untreated diet. In both experiments, half of the replicates were exposed to *S. riobrave* and half were not. In the greenhouse, *S. riobrave* did not delay resistance. In the laboratory, *S. riobrave* delayed resistance after two generations but not after four generations. Simulation modeling showed that an initial resistance allele frequency >0.015 and population bottlenecks can diminish or eliminate the resistance-delaying effects of fitness costs. We hypothesize that these factors may have reduced the resistance-delaying effects of *S. riobrave* in the selection experiments. The experimental and modeling results suggest that entomopathogenic nematodes could slow the evolution of pest resistance to Bt crops, but only under some conditions.

Keywords
entomopathogenic nematode, genetically modified crop, meta-analysis, Pectinophora gossypiella, tritrophic interaction

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Effects of Entomopathogenic Nematodes on Evolution of Pink Bollworm Resistance to *Bacillus thuringiensis* Toxin Cry1Ac

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ABSTRACT The evolution of resistance by pests can reduce the efficacy of transgenic crops that produce insecticidal toxins from *Bacillus thuringiensis* (Bt). However, fitness costs may act to delay pest resistance to Bt toxins. Meta-analysis of results from four previous studies revealed that the entomopathogenic nematode *Steinernema riobrave* (Rhabditida: Steinernematidae) imposed a 20% fitness cost for larvae of pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), that were homozygous for resistance to Bt toxin Cry1Ac, but no significant fitness cost was detected for heterozygotes. We conducted greenhouse and laboratory selection experiments to determine whether *S. riobrave* would delay the evolution of pink bollworm resistance to Cry1Ac. We mimicked the high dose/refuge scenario in the greenhouse with Bt cotton (*Gossypium hirsutum* L.) plants and refuges of non-Bt cotton plants, and in the laboratory with diet containing Cry1Ac and refuges of untreated diet. In both experiments, half of the replicates were exposed to *S. riobrave* and half were not. In the greenhouse, *S. riobrave* did not delay resistance. In the laboratory, *S. riobrave* delayed resistance after two generations but not after four generations. Simulation modeling showed that an initial resistance allele frequency >0.015 and population bottlenecks can diminish or eliminate the resistance-delaying effects of fitness costs. We hypothesize that these factors may have reduced the resistance-delaying effects of *S. riobrave* in the selection experiments. The experimental and modeling results suggest that entomopathogenic nematodes could slow the evolution of pest resistance to Bt crops, but only under some conditions.

KEY WORDS entomopathogenic nematode, genetically modified crop, meta-analysis, *Pectinophora gossypiella*, tritrophic interaction

Transgenic corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) that produce insecticidal proteins from *Bacillus thuringiensis* (Bt) were grown on >58 million ha worldwide in 2010 (James 2010). The widespread use of Bt crops not only attests to their success in controlling some key pests but also raises concerns that pests will evolve resistance to Bt crops (Gould 1998, Tabashnik et al. 2009). Field-evolved resistance to a toxin is defined as a genetically based decrease in susceptibility of a population to a toxin caused by exposure to that toxin in the field (Tabashnik et al. 2009). Although Bt crops have remained effective for more than a decade against many pest populations, field-evolved resistance to Bt crops has been reported in several cases (Van Rensburg 2007, Tabashnik et al. 2008, Downes et al. 2010, Storer et al. 2010, Dhurua and Gujar 2011, Gassmann et al. 2011, Zhang et al. 2011).

To delay the evolution of resistance to Bt crops, growers in the United States and elsewhere are required to have refuges of non-Bt host plants near Bt crops to promote the survival of Bt-susceptible individuals (Gould 1998, Tabashnik et al. 2009). Mating between susceptible insects from refuges and resistant pests from Bt fields can delay the evolution of resistance if survival on Bt crops is lower for hybrid progeny from these matings than for homozygous resistant individuals (Carrière et al. 2004, Sisterson et al. 2004, Tabashnik et al. 2004). However, the accumulation of resistance alleles in refuge populations will disrupt this dynamic and eventually lead to resistance (Caprio 2001; Sisterson et al. 2004, 2005).

Fitness costs associated with resistance can reduce the frequency of resistance alleles in refuge populations, thereby increasing the effectiveness of refuges for delaying resistance (Gassmann et al. 2009a,c; Carrière et al. 2010). Fitness costs occur in the absence of Bt toxins when fitness is lower for individuals with alleles for Bt resistance than for individuals without resistance alleles (Carrière and Tabashnik 2001, Gassmann et al. 2008). The dominance and magnitude of fitness costs for Bt resistance may be altered by environmental factors including host plant species

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and cultivar, allelochemicals, intraspecific competition, entomopathogenic viruses, and entomopathogenic nematodes (Gassmann et al. 2009a). These results imply that resistance management can be improved by choosing refuge conditions that magnify fitness costs (Carrière and Tabashnik 2001, Pittendrigh et al. 2004, Gassmann et al. 2009a).

Here, we tested the hypothesis that fitness costs imposed by an entomopathogenic nematode could delay resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), a major pest of cotton throughout much of the world (Henneberry and Naranjo 1998). Although pink bollworm has recently been eradicated from the southwestern United States, pink bollworm was managed for more than a decade largely with transgenic cotton varieties that produce Bt toxins Cry1Ac and Cry2Ab (Tabashnik et al. 2010). By contrast, this insect remains a pest of cotton in India where resistance to Cry1Ac cotton has developed (Bagla 2010, Dhurua and Gujar 2011).

Past research has found that entomopathogenic nematodes can magnify fitness costs of resistance to Bt toxin Cry1Ac by pink bollworm. The entomopathogenic nematode *Steinernema riobrave* Cabanillas, Poinar, and Raulston (Rhabditida: Steinernematidae) has been found to magnify fitness costs in most experiments (Gassmann et al. 2006, 2008, 2009b; Hannon et al. 2010). Some experimental data also indicated that *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae) magnified fitness costs (Gassmann et al. 2009b), but in other cases no effects on fitness costs were detected (Gassmann et al. 2008, Hannon et al. 2010). Two additional species of *Steinernema* and *H. sonorensis* Stock, Rivera-Orduño, and Flores-Lara were evaluated in Hannon et al. (2010), but none affected fitness costs. *Steinernema riobrave* has been found to increase fitness costs for pink bollworm that are homozygous for Bt resistance (i.e., recessive fitness costs) (Gassmann et al. 2006, 2008. 2009b), and in some cases, fitness costs were found for insects that were heterozygous for Bt resistance (i.e., nonrecessive fitness costs) (Hannon et al. 2010). Computer simulation modeling indicated that recessive costs imposed by *S. riobrave* could substantially prolong the efficacy of Cry1Ac cotton against pink bollworm if applied to all refuge fields (Gassmann et al. 2008, 2009b). Cases of nonrecessive costs were found to be especially promising for delaying resistance (Gassmann et al. 2009b, Hannon et al. 2010). This is because when resistance first evolves in a population, the majority of resistance alleles will be present in heterozygous individuals (Gassmann et al. 2009a). Computer simulation modeling indicated that when only a small percentage of refuge field (e.g., 2.5%) were treated with *S. riobrave* that imposed a nonrecessive fitness cost, the durability of Cry1Ac cotton against pink bollworm could be increased by ≈50% (Hannon et al. 2010).

Building on previous research with *S. riobrave* and pink bollworm, we used meta-analysis of data from four previous studies (Gassmann et al. 2006, 2008, 2009b; Hannon et al. 2010) to determine whether *S. riobrave* imposed a significant fitness cost of pink bollworm resistant to Cry1Ac and to measure the magnitude of this cost. Meta-analysis has been used across scientific disciplines (e.g., biomedical science, ecology, and entomology) to combine data from multiple experiments into a single test of significance (Gurevitch and Hedges 1993, Gurevitch et al. 2001). In plant–insect interactions, meta-analysis has been used to test costs of plant defenses against herbivores (Koricheva 2002), the relationship between preference and performance for herbivorous insects (Gripenberg et al. 2010), effects of climate change on plant–insect interactions (Massad and Dyer 2010), and effects of organic agriculture on insect diversity and abundance (Bengtsson et al. 2005). In the research presented here, meta-analysis revealed a significant fitness cost imposed by *S. riobrave* against Cry1Ac-resistant pink bollworm. Thus, we tested the ability of *S. riobrave* to delay pink bollworm resistance to Cry1Ac in laboratory and greenhouse selection experiments. Finally, we used simulation modeling to evaluate the conditions under which nematodes would delay pink bollworm resistance to Cry1Ac.

Materials and Methods

Meta-Analysis. We used MetaWin (Rosenberg et al. 1999) to conduct a meta-analysis of data from all four studies testing fitness costs for Cry1Ac-resistant pink bollworm caused by *S. riobrave* (Table 1). Meta-analysis provided a single test of significance, based on a weighted mean and associated standard error, using data from multiple experiments. This was accomplished by calculating effect sizes and associated variance of the effect size that were based on an experiment's samples size, variance, and mean values for treatment and control. Determining effect sizes placed all experiments on a common metric, allowing data from multiple experiments to be combined into a single test of significance and providing an overall estimate of treatment effects (Rosenberg et al. 1999).

In our meta-analysis, effect size was a response ratio for survival in the presence of nematodes. The response ratio was calculated as the natural log of the quotient for corrected survival of either *rr* and *rs* individuals divided by corrected survival of *ss* individuals: ln (survival of *rr* or *rs*/survival of *ss*). Equations for calculating a response ratio and its associated variance are provided in Rosenberg et al. (1999). Values for corrected survival used in the meta-analysis were survival in the presence of *S. riobrave* for each genotype adjusted for survival of the genotype in experimental controls that were not exposed to nematodes (Gassmann et al. 2006, 2008, 2009b; Hannon et al. 2010). For three of the studies (Gassmann et al. 2006, 2008; Hannon et al. 2010), data were originally reported as mortality in the presence of nematodes adjusted for control mortality with the formula of Abbott (1925). We calculated the complement of these values to obtain corrected survival.

Table 1. Meta-analysis of fitness costs for pink bollworm caused by *S. riobrave*

Reference	Larval host[a]	Genotype	Effect size[b]	Variance of effect size
Gassmann et al. (2006)	Diet	*rr*	−0.763	0.149
Gassmann et al. (2008)[c]	Diet	*rr*	−0.219	0.016
Gassmann et al. (2008)[d]	Diet	*rr*	0.413	0.122
Gassmann et al. (2008)[c]	Diet with gossypol	*rr*	−0.198	0.060
Gassmann et al. (2008)[d]	Diet with gossypol	*rr*	0.003	0.099
Gassmann et al. (2009b)[e]	Cotton bolls	*rr*	−1.418	0.657
Gassmann et al. (2009b)[f]	Cotton bolls	*rr*	0.058	0.326
Hannon et al. (2010)	Diet	*rr*	−0.600	0.086
Weighted mean for effect size = −0.229			95% confidence interval = −0.444 to −0.014	
Gassmann et al. (2008)[c]	Diet	*rs*	0.062	0.012
Gassmann et al. (2008)[d]	Diet	*rs*	0.388	0.113
Gassmann et al. (2008)[c]	Diet with gossypol	*rs*	0.057	0.038
Gassmann et al. (2008)[d]	Diet with gossypol	*rs*	−0.125	0.084
Gassmann et al. (2009b)[e]	Cotton bolls	*rs*	0.235	0.218
Gassmann et al. (2009b)[f]	Cotton bolls	*rs*	−0.708	0.246
Hannon et al. (2010)	Diet	*rs*	−0.406	0.039
Weighted mean for effect size = −0.021			95% confidence interval = −0.214 to 0.171	

[a] Diet refers to standard artificial diet for pink bollworm, which is described in Adkinsson et al (1960). Gossypol is a secondary metabolite of cotton with antibiosis against pink bollworm.

[b] Effect size measures fitness of individuals with *r* alleles relative to homozygous susceptible individuals. Negative values indicate that a fitness cost was present, with larger values indicating greater costs. Positive values indicate higher fitness for individuals with *r* alleles than for homozygous susceptible individuals. Information on calculating effect sizes and their associated variance is given in Materials and Methods.

[c] Measured in the presence of three infective juvenile *S. riobrave* per pink bollworm larva.

[d] Measured in the presence of six infective juvenile *S. riobrave* per pink bollworm larva.

[e] Measured for pink bollworm strain MOV97-H3.

[f] Measured for pink bollworm strain SAF97-H4.

A weighted average for effect sizes and associated 95% confidence intervals (CI) were calculated in MetaWin, with 95% CI that do not include 0 indicating an effect size that differed significantly from 0 (Rosenberg et al. 1999). Effect sizes and their associated 95% CI were back-transformed and used to parameterize a simulation model.

Insect Strains. We used the MOV97-H4 and SAF97-H4 hybrid strains of pink bollworm; each had a mixture of Bt-resistant, susceptible, and heterozygous individuals. We derived MOV97-H4 and SAF97-H4 from the MOV97 and SAF97 strains that originated from individuals collected in 1997 from cotton fields in the Mohave Valley in western Arizona and Safford in eastern Arizona, respectively (Tabashnik et al. 2000, Gassmann et al. 2008, Carrière et al. 2009). In 1997, the Cry1Ac resistance (*r*) allele frequency was >0.15 in MOV97 and SAF97 (Tabashnik et al. 2000). Larvae with two resistance alleles in any combination (e.g., *r1r1* or *r1r3*, referred to as *rr*) can survive on diet with the discriminating concentration of Cry1Ac (10 μg of Cry1Ac per ml diet) and on Bt cotton that produces Cry1Ac, but homozygous susceptible (*ss*) larvae and heterozygous (*rs*) larvae cannot survive (Morin et al. 2003, Tabashnik et al. 2005a). We reared larvae on a wheat germ-diet and maintained each colony at a population size of 1,200 adults (Tabashnik et al. 2000, Carrière et al. 2006, Gassmann et al. 2008).

To produce the *rr* strains MOV97-H4R and SAF97-H4R, we put eggs from MOV97-H4 and SAF97-H4 on diet with the discriminating concentration of Cry1Ac. Only homozygous resistant larvae can survive diet with this concentration of Bt toxin, whereas larvae with zero or one *r* allele die (Tabashnik et al. 2000, 2005b). To create the *ss* strains MOV97-H4S and SAF97-H4S, we used the single-pair mating and screening method of Gassmann et al. (2006). After females laid eggs, we screened the male and female in each pair for *r* alleles by using polymerase chain reaction (PCR) with allele-specific primers (Morin et al. 2004). We used the progeny of pairs from MOV97-H4 and SAF97-H4 that tested negative for *r* alleles to begin MOV97-H4S and SAF97-H4S, respectively.

To begin the greenhouse selection experiments, we pooled moths as follows: 360 adults from MOV97-H4R, 360 adults from SAF97-H4R, 300 adults from MOV97-H4S, and 180 adults from SAF97-H4S. For the laboratory selection experiment, the number of moths pooled was 360 adults from MOV97-H4R, 360 adults from SAF97-H4R, 240 adults from MOV097-H4S, and 240 adults from SAF97-H4S. For each experiment, the expected initial *r* allele frequency of the 1,200 pooled moths was 0.60 (720 *rr* + 480 *ss*), and the pooled strains were reared for two generations without exposure to Bt toxin to increase population size.

Nematodes. For both the greenhouse and laboratory experiments, we used the entomopathogenic nematode *Steinernema riobrave* (ML-29 strain), originally isolated from southeastern Arizona (Stock and Gress 2006). Nematodes were maintained in the laboratory through periodic culturing in larvae of the greater wax moth, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) (Kaya and Stock 1997). Infective juveniles (IJs) were harvested from modified White traps after emergence from greater wax moth and were used within 2 wk, following Kaya and Stock (1997).

Laboratory Selection Experiment. We ran this experiment from December 2008 to May 2009, and a single trial was conducted. As described under Insect

Strains, we produced a pooled strain of pink bollworm by combining moths from MOV97-H4R, MOV97-H4S, SAF97-H4R, and MOV97-H4S. We reared this pooled strain for two generations without exposure to Bt toxin. We then divided the F3 generation of the pooled strain into 10 lines, and randomly assigned five lines as nematode-treated and five as controls not exposed to nematodes. Refuges were incorporated into the experiment by randomly assigning half the eggs in each line to paper diet cups (0.5 liters) containing diet not treated with Bt toxin (refuge). Selection for Bt resistance was incorporated by assigning the other half of the eggs to diet with the discriminating concentration of Cry1Ac. This even distribution of larvae between refuge and Bt environments probably differed from the field where oviposition in refuges may be greater than Bt fields due to the higher population density of pests expected in refuge areas. Consequently, the intensity of selection for Bt resistance in the laboratory experiment may have been greater than would occur in the field. Diet cups with larvae were held in plastic 1-liter containers in a growth chamber (29°C and a photoperiod of 14:10 [L:D] h). Lines were selected for four generations (F3–F6). To determine the initial *r* allele frequency at F3, we randomly sampled 100 larvae from among the 10 lines that had developed on non-Bt diet before exposure to nematodes. These larvae were preserved in ethanol and were screened for *r* alleles by using PCR (Morin et al. 2004).

When larvae reached the wandering stage of the fourth and final instar they chewed through the paper diet cups and were held within 1-liter plastic containers. Larvae exiting Bt-diet cups were allowed to pupate on paper towels (Kimwipes, Kimberly-Clark, Mississauga, ON, Canada). Larvae exiting from non-Bt diet cups (refuge) were collected and placed in sterile petri dishes (100 by 15 mm; Falconer Brand, VWR International, West Chester, PA) containing 15 g of sterile sand. Ten larvae were placed in each petri dish. For the five control lines, the sand was treated with 3.75 ml of deionized water, and for the five nematode treatment lines, the sand was treated with 3.75 ml of nematode suspension at a concentration of 4–8 IJs per ml. We used 3.75 ml of water following Gassmann et al. (2006) because this provided a near saturated sand substrate and thus was conducive to infection of pink bollworm by nematodes. We adjusted nematode concentration between 4 and 8 IJs per ml, in an attempt to achieve ≈50% mortality from nematodes, with concentrations increased or decreased depending on the level of mortality observed during the previous generation. The concentration of each nematode solution was determined to the nearest nematode per ml using a counting grid and microscope.

Petri dishes were held in an incubator in the dark at 25°C for 7 d. We chose these conditions based on the biology of pink bollworm in the field, where fourth instars that exit cotton bolls drop to the ground and pupate in the top layer of the soil (Henneberry and Clayton 1979). However, exposure to nematodes in this experiment was probably more even than would occur in the field because nematodes in a petri dish should have a more homogenous distribution than those within the soil of an agricultural field. Consequently, the overall level of mortality from nematodes was probably greater than could be achieved for a similar density of nematodes in the field.

For each line, pupae from non-Bt diet and Bt diet were combined, eclosing adults were allowed to mate within each of the 10 lines, and eggs were collected for each line. Resistance evolution is affected both by refuge proportion (i.e., the proportion of the population that develops in refuges) and fitness costs. To test the effect of fitness costs on resistance evolution independent of the effect of nematodes on refuge proportion, we used the same refuge proportion for the nematode-treated and control lines. For generation F3, the percentage of pupae from Bt diet was set at 47% of the total population (53% refuge). For generations F4 to F6, we decreased the percentage of pupae from Bt diet to a range of 9–10% (90–91% refuge) because *r* allele frequency was increasing in all lines (see Results) and probably would have reached 100% unless we increased the refuge percentage. The number of pupae per line for each generation were as follows: 280 from non-Bt diet and 248 from Bt diet for all lines in F3; 280 from non-Bt diet and 30 from Bt diet for all lines in F4; 114 ± 11 (mean ± SD) from non-Bt diet and 12 ± 1.3 from Bt diet for F5, and 148 ± 57 from non-Bt diet and 16 ± 6 from Bt diet for F6.

We used a diet bioassay (Patin et al. 1999) to determine the proportion of *rr* larvae in each of our 10 lines in the F5 (after two generations of selection, F3 and F4) and in the F7 (after four generations of selection, F3–F6). In each bioassay, we put 5 g of cubed diet in 30 ml plastic cups with lids (Dart Container Corporation, Mason, MI). We used a fine brush to place one neonate on the diet in each cup. Cups were placed in plastic trays and incubated in darkness at 29°C. The number of neonates tested per line was: 50 on Bt diet and 50 on non-Bt diet for F5 and 80 on Bt diet and 45 on non-Bt diet for F7. After 21 d, individuals that were fourth instars, pupae, or adults were classified as *rr*. For each line, mean mortality on Bt diet was adjusted for control mortality by using Abbott's correction.

Greenhouse Selection Experiment. We conducted this experiment in two quarantine greenhouse bays on the University of Arizona campus in Tucson between March and October 2008 at a mean temperature of 28°C (range, 19–34°C). A single trial of the greenhouse experiment was conducted. Each bay contained four 4.5 by 2 by 2.5 m (length by width by height) cages made with a polyvinyl chloride frame and covered in fine white polyester netting (BioQuip, Rancho Dominguez, CA) to prevent movement of pink bollworm among cages. Each cage contained 20 pots (volume, 20 liters; diameter, 29 cm), 10 with Bt cotton plants (DeltaPine 50B) and 10 with non-Bt cotton plants (DeltaPine 50); plants were randomly distributed in each cage. As in Gassmann et al. (2009b), each pot had three plants, for a total of 60 plants per cage. Plants were watered using drip irrigation. During October, we used supplemental lighting (photoperiod of

16:8 (L:D) h; combination of 1,000-W high-pressure sodium lights and 1,000-W metal halide lights) to prevent diapause in pink bollworm. Plants were treated as necessary for thrips with predatory mites (*Amblyseius cucumeris* Oudemans, Thripex Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) and were sprayed twice for whiteflies with a 0.86% emulsifiable concentrate formulation of the insect growth regulator pyriproxyfen (Knack, Valent, Walnut Creek, CA). Pink bollworm larvae feed within cotton bolls; thus, actively feeding larvae would have been sheltered from the insecticide.

After cotton plants began producing bolls (≈4 mo after planting), we infested each cage with 2,400 adult pink bollworm moths over 9 d beginning on 7 July 2008. To estimate the *r* allele frequency of moths used to infest cages, we preserved a random sample of 100 moths in ethanol for later genotyping with PCR (Morin et al. 2004). A PCR-based genotype was successfully obtained from 95 of 100 individuals. After the initial release of moths into a cage, no additional pink bollworms were placed in any cages for the duration of the experiment. Four cages were designated as nematode cages and the other four as control cages. Cages were designated at random, with two nematode cages and two control cages in each greenhouse bay. To the soil of each pot, we applied 500 ml of nematode suspension in nematode cages and 500 ml of water in control cages. These applications started 21 d after infestation with pink bollworm so that the larval progeny from the first generation of adults in cages would be exposed to nematodes when they emerged from cotton bolls and entered the soil to pupate. A volume of 500 ml was selected so that soil would not become oversaturated when nematodes were added. We made seven nematode applications in total during the experiment at an interval of ≈11 d, with a concentration of 11 ± 2.8 IJs per ml (mean ± SD).

To measure the effectiveness of the nematode applications, we monitored pink bollworm mortality in the soil with small plastic cassettes containing pink bollworm larvae (Gouge et al. 1998). One cassette was placed in three flower pots in each cage (four control cages and four nematode-treated cages), with five larvae per cassette. Cassettes were placed in the soil for 1wk after which larval mortality was recorded; cassettes containing fresh larvae were then placed in the soil. This was done a total of eight times beginning 23 d after pink bollworm were initially released, with cassettes used in cages over a period of 73 d. In total, 960 larvae were used in cassettes to measure insect mortality, with 480 larvae evaluated in control cages and 480 larvae evaluated in nematode-treatment cages. Concentrations of nematodes were adjusted weekly based on mortality observed in monitoring cassettes with the goal of achieve ≈50% mortality from nematodes.

To measure *r* allele frequency, we sampled pink bollworm larvae from three sets of non-Bt cotton, sentinel plants. Each set of sentinel plants consisted of nine additional non-Bt plants (three pots with three plants each) in each cage. Each set of sentinel plants was placed in cages for a brief period of ≈22 d. These non-Bt plants were in the process of producing bolls and thus provided an oviposition substrate for adult pink bollworm. Removal of sentinel plants from cages and subsequent collection of larvae from bolls allowed us to obtain a random sample of the population within each cage. These larvae were used to obtain PCR-based genotypes, from which *r* allele frequency for each cage was calculated.

The first set of sentinel plants was in cages from the day moths were released into cages (day 0) to day 18. The second set was in cages from days 25 to 51 and the third set from days 51 to 66. Pink bollworm generation time was ≈18 d at the average temperature of 28°C in the cages (Beasley and Adams 1996). The day on which sentinel plants were removed from cages corresponded roughly to the completion of one generation for the first set (day 18), three generations for the second set (day 51), and four generations for the third set (day 66).

To obtain larvae from sentinel plants after those plants had spent ≈22 d in cages, sentinel plants were first removed from cages and their bolls harvested (65 ± 6 bolls per cage, mean ± SE). These bolls were put in ventilated containers, and checked every 3 d for emerging larvae following Dennehy et al. (2004). After 2 wk, bolls were opened and all additional insects were collected. Insects were preserved in ethanol for subsequent PCR genotyping (Morin et al. 2004).

Four months (day 121; ≈7 generations) after moths were released into cages, we ended the experiment by collecting all bolls from the 10 non-Bt pots per cage (30 plants per cage) and then collected all pink bollworm from these bolls by using the same methods that were applied to sentinel plants. No Bt bolls were sampled. An average of 93 ± 12 bolls were sampled per cage at the end of the experiment.

Of the pink bollworm collected from non-Bt bolls, we obtained a PCR-based genotype from 155 of 807 insects from the sample on day 18, from 28 of 38 insects for the sample from day 66, and 95 of 98 insects on day 121. No PCR analysis was conducted for the sample from day 51 because only one pink bollworm was recovered. For insects collected on day 18, genotypes were taken from insects selected at random from each cage, because genotyping all 807 insects was not logistically feasible. For insects collected on days 66 and 121, a genotype was obtained for as many insects as possible.

Simulation Modeling. Data on fitness costs obtained from the meta-analysis were used to parameterize a spatially explicit stochastic simulation model based on the biology of pink bollworm (Sisterson et al. 2004, 2005). We simulated a landscape of 400 fields, with 20% of fields planted as non-Bt cotton refuges and 80% planted to Bt cotton. We incorporated nematodes into the model following Hannon et al. (2010) by treating 20% of refuge fields with *S. riobrave* (i.e., 5% of the all fields in the landscape were treated with entomopathogenic nematodes).

Three sets of simulations were completed. In all sets, nematodes were applied to 20% of refuge fields. In the

first and second set of simulations, which mimicked effects of *S. riobrave*, nematodes imposed a fitness cost. Because nematodes can affect the rate of resistance evolution by imposing fitness costs (expected to delay resistance) and by killing refuge individuals (expected to hasten resistance), we included a third set of control simulations, in which all assumptions were the same except that we used hypothetical "control nematodes" that did not impose a fitness cost. In all simulations, nematodes killed pink bollworm larvae, but mortality caused by nematodes was greater for larvae with *r* alleles than for *ss* larvae in only the first and second set of simulations.

Without nematodes, survival from egg to adults was $ss = 0$, $rs = 0$, $rr = 0.207$ in Bt fields and $ss = 0.208$, $rs = 0.207$, $rr = 0.207$ in refuges (Sisterson et al. 2004). The slightly lower survival of *rs* and *rr* relative to *ss* in refuges reflects a small fitness cost not imposed by nematodes that we used in all simulations to balance the effect of mutation introducing *r* alleles into the population (Sisterson et al. 2004). In all simulations, nematodes were assumed to kill 50% of the pink bollworm larvae in fields treated with nematodes. This value was chosen because it was in between the level of mortality observed in the laboratory experiment (63%) and the greenhouse experiment (32%) and because it was similar to the level of mortality achieved for pink bollworm from application of *S. riobrave* to Arizona cotton fields (Gouge et al. 1997).

Pink bollworm mortality from *S. riobrave* was based on the results of the meta-analysis. The meta-analysis revealed 20% higher mortality (95% CI, 4–36%) for *rr* individuals in the presence of *S. riobrave* (see Results). Thus, we parameterized one set of simulations with *rr* individuals having 20% higher mortality in the presence of *S. riobrave* (recessive fitness cost). Meta-analysis also revealed a small cost for *rs* individuals of 2% higher mortality (95% CI, −19 to 21%) from *S. riobrave* (see Results). Thus, we parameterized a second set of simulations with *rr* individuals having 20% higher mortality and *rs* individuals having 4% higher mortality from *S. riobrave* (dominant fitness cost).

For *ss* larvae, survival to adulthood in nematode-treated fields was $0.104 = 0.208 \times 0.5$, which reflects the additional 50% mortality imposed by *S. riobrave* on the remaining 20.8% of the population. Mortality of *ss* was unaffected by assumptions concerning fitness costs imposed by nematodes. For *rr* individuals, survival in refuges with nematodes that imposed a recessive or dominant fitness cost was $0.083 = 0.207 \times 0.5 \times 0.8$, which reflects 20% lower survival caused by *S. riobrave*. In contrast, survival of *rr* individuals in refuges with control nematodes was $0.104 = 0.207 \times 0.5$. For *rs* larvae, survival in refuges with nematodes that imposed a dominant fitness cost was $0.099 = 0.207 \times 0.5 \times 0.96$, which reflects a 4% lower survival imposed by *S. riobrave*. In contrast, survival of *rs* individuals was $0.104 = 0.207 \times 0.5$ in refuge fields with control nematodes or for nematodes that imposed a dominant fitness cost.

Simulations were used to test two hypotheses: 1) increasing *r* allele frequency would diminish the benefit of fitness costs at delaying resistance evolution and 2) increasing population bottlenecks through higher overwintering mortality would diminish the benefit of fitness costs at delaying resistance evolution. We used sensitivity analysis to evaluate how initial *r* allele frequency and population bottlenecks affected the rate of resistance evolution. The values for initial *r* allele frequency were 0.001, 0.005, 0.01, 0.015, and 0.02. To incorporate population bottlenecks, we used the following values for overwintering mortality: 0.80, 0.85, 0.90, 0.925, 0.95, 0.975, and 0.99. These values are relevant to the field because *r* allele frequency for field populations of pink bollworm was found to range from 0.00 to ≈0.15 (Tabashnik et al. 2010). Overwintering mortality of pink bollworm can range from ≈0.05 to 0.35 (Brazzel and Martin 1959); however, growers can vary the planting date of cotton such that host plants are unavailable to adults that develop from overwintering larvae, and this can lead to additional mortality of overwinter insects of ≈0.20–1.00 (Carrière et al. 2001b).

For simulations that varied initial *r* allele frequency, overwintering mortality was set at 0.95 and for simulations that varied overwintering mortality, initial *r* allele frequency was set at 0.01. In each simulation, we recorded years to resistance as the number of years required for the *r* allele frequency to reach 0.50. If this criterion was not met in 100 yr, the simulation was stopped and years to resistance was recorded as >100. For each set of parameters, we ran 20 simulations and determined the median years to resistance.

Statistical Analysis of Data From Selection Experiments and Simulations. For the meta-analysis, mean effect sizes and associated 95% CL for *rr* and *rs* genotypes were calculated with MetaWin 2.0 (Rosenberg et al. 1999). An effect size was statistically different from 0 when the 95% confidence limit did not include 0 (Rosenberg et al. 1999).

Data on the frequency of *rr* individuals in the laboratory selection experiment were analyzed with a one-tailed *t*-test (Sokal and Rohlf 1995). The null hypothesis (Ho) was that nematode and control lines did not differ in frequency of *rr* individuals. The alternative hypothesis was that the frequency of *rr* individuals was lower in nematode lines than control lines. Data on *r* allele frequency in the greenhouse experiment also were compared between nematode and control cages using a one-tailed *t*-test. Ho: nematode and control cages did not differ in *r* allele frequency; and Ha: *r* allele frequency was lower in nematode cages than control cages.

A comparison of initial *r* allele frequency (F1) and the *r* allele frequency at the start of both the greenhouse and laboratory selection experiments (F3) was made using a G test of independence (Sokal and Rohlf 1995). A G test also was applied to test whether populations were at Hardy–Weinberg equilibrium immediately before populations experienced selection from either nematodes or Bt (Hartl and Clark 1997). A *t*-test was used to compare mortality of larvae exposed to nematodes versus experimental controls in both the

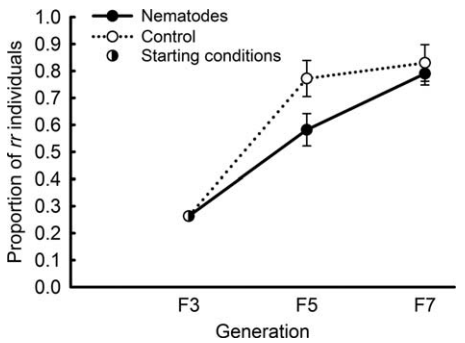

Fig. 1. Frequency of *rr* individuals among lines in the laboratory selection experiment. Points are sample means and error bars are the standard error of the mean. "Nematodes" indicates lines treated with nematodes. "Control" indicates lines that were not treated with nematodes.

greenhouse and laboratory selection experiments (Sokal and Rohlf 1995).

The time for populations to evolve resistance in simulations with *S. riobrave* versus control nematodes was compared for each initial *r* allele frequency and each level of overwintering mortality using a Kruskal–Wallis test. The Kruskal–Wallis test was carried out in SAS (PROC NONPAR1WAY, SAS Institute 2008).

Results

Meta-Analysis. Meta-analysis of data from four previous studies indicated that *S. riobrave* imposed a significant fitness cost for pink bollworm that were homozygous for resistance to Bt toxin Cry1Ac (*rr*), but not for heterozygotes (*rs*) (Table 1). In five of eight comparisons, survival was lower for *rr* than susceptible homozygotes (*ss*), with a mean effect size of −0.23 (95% CI = −0.44 to −0.014), which is significantly less than zero. We obtained the survival of *rr* relative to *ss* by back-transforming the data: $e^{-0.229} = 0.80 =$ (survival of *rr*/survival of *ss*). Therefore, survival of *rr* was 80% of the survival of *ss*. Thus, in the presence of *S. riobrave*, survival was 20% lower for *rr* than *ss* (back-transformed 95% CI = 4–36%). Survival was lower for *rs* than *ss* in three of seven comparisons, with an average effect size of −0.021 (95% CI = −0.214 to 0.171), which does not differ significantly from zero. The back-transformed mean was: $e^{-0.021} = 0.98 =$ (survival of *rs*/survival of *ss*). Therefore, survival of *rs* was 98% the survival of *ss*. Thus, survival was 2% lower for *rs* than *ss* (back-transformed 95% CI = −19–21%).

Laboratory Selection Experiment. In the laboratory selection experiment, bioassay results showed that the frequency of *rr* was significantly lower for nematode-treated lines than for control lines after two generations of selection (F5), but no difference between lines was detected after four generations of selection (F7) (Fig. 1). At the F5, the frequency of *rr* was 0.58 ± 0.06 for nematode-treated lines and 0.77 ± 0.07 for the control lines (mean ± SE). The proportion of resistant (*rr*) individuals was significantly lower in the nematode-treated lines compared with the control lines (one-tailed *t*-test: $t = 2.1$, df = 8, $P = 0.03$) (Fig. 1). At the F7, the frequency of *rr* was 0.79 ± 0.04 in nematode lines and 0.83 ± 0.06 in control lines, and did not differ between these groups (one-tailed *t*-test: $t = 0.38$, df = 8, $P = 0.35$) (Fig. 1).

PCR analysis indicated initial *r* allele frequency for the F3 generation was 0.500, with genotypic frequency of *ss* = 0.263, *rs* = 0.474, and *rr* = 0.263 ($N = 57$ moths), values that did not differ significantly from the expected frequencies based on Hardy–Weinberg equilibrium: $ss_{expected} = 0.250$, $rs_{expected} = 0.50$, $rr_{expected} = 0.250$ (G = 1.55, df = 2, $P = 0.46$). In addition, *r* allele frequency at F3 (for larvae from control diet and not yet exposed to nematodes) did not differ significantly from the initial r allele frequency of 0.6 at F1 (G = 0.02, df = 1, $P = 0.89$).

For lines exposed to nematodes, larval mortality was 63 ± 11% (mean ± SD) per line per generation, and for lines exposed to the experimental control of water only, larval mortality was 5 ± 3%. Larvae exposed to nematodes suffered significantly more mortality than the experimental control ($t = 32$, df = 38, $P < 0.001$). We do not know the cause of death for larvae in the experimental control.

Greenhouse Selection Experiment. In the greenhouse selection experiment, PCR results indicated that the *r* allele frequency did not differ significantly between cages in which pink bollworm larvae were treated with nematodes and control cages without nematodes (Fig. 2a). At day 66 (approximately three generations of selection by nematodes), *r* allele frequency was 0.575 ± 0.101 in treatment cages ($N = 4$ cages) and 0.587 ± 0.140 in control cages ($N = 2$ cages, no insects were recovered from two cages). No significant difference was present between treatment and control cages for mean *r* allele frequency (one-tailed *t*-test: $t = 0.08$, df = 4, $P = 0.47$) (Fig. 2a). After 121 d (approximately six generations of selection by nematodes), *r* allele frequency in the treatment cages was 0.829 ± 0.082 ($N = 3$ cages, no insects were recovered from one cage) and in the control cages 0.835 ± 0.117 ($N = 2$ cages, no insects were recovered from two cages). We did not detect a difference between treatment and control cages (one-tailed *t*-test: $t = 0.05$, df = 3, $P = 0.49$) (Fig. 2a).

When adult pink bollworm were released into cages (day 0), the *r* allele frequency was 0.58, with genotypic frequency *ss* = 0.168, *rs* = 0.495, and *rr* = 0.337 ($N =$ 95 moths), values that did not differ significantly from the expected frequencies based on Hardy–Weinberg equilibrium: $ss_{expected} = 0.172$, $rs_{expected} = 0.486$, $rr_{expected} = 0.342$ (G = 0.54, df = 2, $P = 0.76$). On day 18, before larvae were exposed to nematodes, the mean *r* allele frequency was 0.544 ± 0.025 (mean ± SE) in control cages ($N = 4$ cages) and 0.558 + 0.034 in nematode cages ($N = 4$ cages), and did not differ between these treatment ($t = 0.38$, df = 6, $P = 0.74$). The *r* allele frequency at day 18 represents the frequency before selection by nematodes because nematodes were not placed in cages until 21 d after pink bollworm were released into cages. The overall mean among cages was 0.551 ± 0.02, a value did not differ

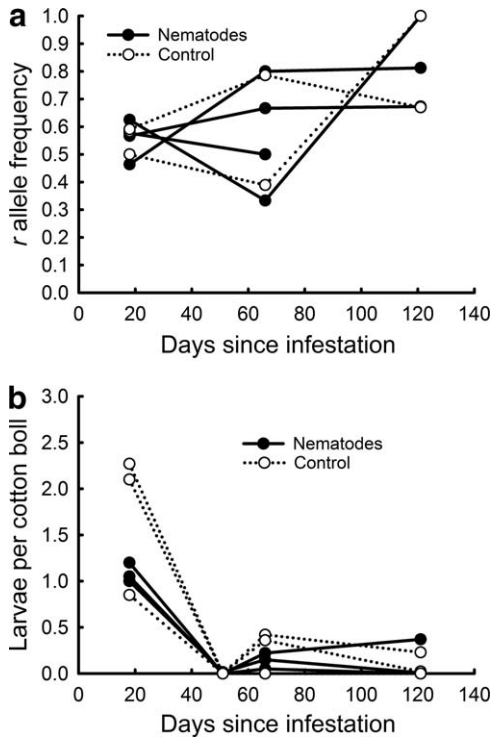

Fig. 2. Pink bollworm *r* allele frequency (a) and population size (b) for the greenhouse selection experiment. Individual points represent values for each cage. "Nematodes" indicates cages treated with nematodes. "Control" indicates cages that were not treated with nematodes. In panel a, data were obtained from four control cages and two treatment cages at day 66 and from three control cages and two treatment cages at day 121.

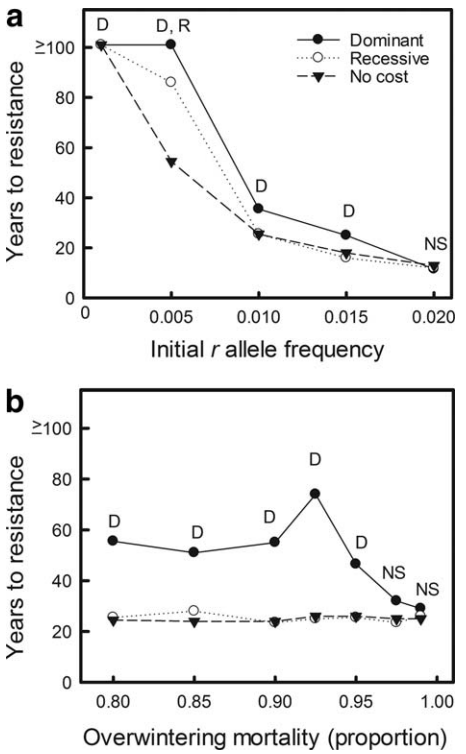

Fig. 3. Simulation modeling for effects of initial *r* allele frequency (a) and population bottlenecks (b) on resistance evolution. Points represent sample medians. D and R indicate that a significant difference ($P < 0.05$ based on a Kruskal–Wallis test) was present between simulations with no costs versus simulations with dominant and recessive costs, respectively. NS indicates that neither of the simulations with costs (dominant and recessive) differed from the simulations with no costs.

significantly from the starting *r* allele frequency of 0.585 (df = 7, $t = 0.58$, $P = 0.48$).

Larval mortality in monitoring cassettes for nematode-treated cages was 32 ± 26% (mean ± SD), and for control cages, larval mortality was 13 ± 14%. Similar to the results of the laboratory selection experiment, mortality of pink bollworm larvae was significantly greater in nematode-treated cages compared with control cages ($t = 5.13$, df = 62, $P < 0.001$). The large standard deviation associated with mortality in control cages and nematode-treated cages probably reflects the high degree of variability in both control mortality and mortality imposed by nematodes.

With the exception of the results from day 18, pink bollworm population density was low during the experiment (Fig. 2b). The number of larvae recovered per boll from sentinel plants was 1.4 on day 18 (N = 564 bolls), 0.0016 on day 51 (n = 590 bolls), 0.23 on day 66 (N = 163 bolls), and 0.13 on day 121 (N = 749 bolls). We do not know why populations were at such low abundance at day 51 and at other times during this experiment.

Simulation Modeling. Application of data on fitness cost to a spatially explicit simulation model demonstrated that both population bottlenecks and increases in initial *r* allele frequency decreased the delays in resistance caused by either dominant or recessive fitness costs (Fig. 3a and b).

When the initial *r* allele frequency was ≤0.015 dominant fitness costs significantly delayed resistance, however, delays in resistance from recessive fitness costs only occurred when *r* allele frequency was 0.005. When initial *r* allele frequency was >0.015, no differences were detected in the number of years until populations evolved resistance in the presence versus absence of fitness costs (Fig. 3a).

Population bottlenecks also decreased the effectiveness of fitness costs at delaying resistance evolution. When overwintering mortality ranged from 0.80 to 0.95, dominant fitness costs approximately doubled the time until population evolved resistance, increasing the time to resistance from ≈25 to ≈50 yr (Fig. 3b). By contrast, under more severe population bottlenecks imposed by increasing overwintering mortality above 0.95, fitness costs did not significantly delay resistance evolution (Fig. 3b).

Delays in resistance were greater for dominant costs than recessive costs. In sensitivity analysis for initial *r* allele frequency, both dominant and recessive costs

delayed resistance evolution but for sensitivity analysis of population bottlenecks, delays were only observed for dominant fitness costs (Fig. 3a and b).

Discussion

Using greenhouse and laboratory selection experiments, we tested the hypothesis that the entomopathogenic nematode *S. riobrave* would enhance the refuge strategy by delaying pink bollworm resistance to Bt toxin Cry1Ac. In one of the two experiments, we found a significant delay in the rate of resistance evolution when nematodes were present in the refuge. This occurred for the F5 generation of the laboratory selection experiment (Fig. 1). However, no significant difference between nematode-treated and control lines occurred in the F7 generation of the laboratory selection experiment, or during any of the three sampling dates for the greenhouse selection experiment (Figs. 1 and 2a). Simulation modeling showed that if the *r* allele frequency was ≥0.015 (Fig. 3a) or if populations experience bottlenecks (Fig. 3b), then the effects of fitness costs in delaying resistance evolution were reduced or absent. Both of those factors were probably present in the greenhouse selection experiment, and the *r* allele frequency was ≥0.54 in the laboratory selection experiment. In the field, population bottlenecks could arise through the excessive or unwarranted use of conventional insecticides in conjunction with Bt crops. In addition, planting of Bt crops in the absence of either sound integrated pest management or insect resistance management could lead to a high frequency of Bt resistance alleles, thus bringing the pest population to a point where enhancing fitness costs will confer little benefit for delaying further resistance evolution.

Refuges delay resistance evolution by reducing the proportion of the population exposed to selection and by providing susceptible insects to mate with resistant pests. Fitness costs can enhance the effectiveness of refuges to delay resistance (Gassmann et al. 2009a, Carrière et al. 2010). Evidence from laboratory experiments suggests that fitness costs of Bt resistance can be increased by several ecological factors including host plant species (Janmaat and Myers 2005, Bird and Akhurst 2007), density (Raymond et al. 2005), entomopathogens (Raymond et al. 2007, Gassmann et al. 2008), and competition for mates (Higginson et al. 2005). In addition, laboratory selection experiments have demonstrated the value of refuges in delaying resistance to Bt (Tang et al. 2001). However, selection experiments have seldom been applied to demonstrate the potential for ecological factors to delay Bt resistance by magnifying fitness costs (Raymond et al. 2007). The application of selection experiments in this study demonstrates that although fitness cost can delay resistance evolution under the high dose/refuge scenario, other population-level factors such as gene frequency and population bottlenecks may diminish the benefits of fitness costs.

In some cases, entomopathogenic nematodes increased the fitness cost of Bt resistance in pink bollworm (Gassmann et al. 2006, 2008, 2009a; Hannon et al. 2010). Although several nematode species were evaluated for their effects on costs, only *S. riobrave* and *H. bacteriophora* magnified costs of resistance, and of these species, costs occurred in a higher percentage of instances for *S. riobrave* (Gassmann et al. 2008, 2009b; Hannon et al. 2010). Application of a meta-analysis revealed that *S. riobrave* increased fitness costs in five of eight cases tested for *rr* individuals, and in three of seven cases tested for *rs* individuals (Table 1), with a 20% fitness cost of survival for *rr* and a 2% cost for *rs* (see Results). Although pink bollworm in India has developed resistance to Bt cotton in the field, the extent to which fitness costs may accompany this trait is unknown (Dhurua and Gujar 2011). However, for strains of pink bollworm from Arizona, which have field-derived resistance alleles and were used in this study, both dominant and recessive fitness costs have been detected (Carrière et al. 2009; Gassmann et al. 2009a,b; Hannon et al. 2010; Williams et al. 2011).

The meta-analysis was performed on data from four studies, which included 15 genotype by treatment combinations (eight for *rr* individuals and seven for *rs* individuals) (Table 1) and 3,934 individual insects. The meta-analysis combined data from multiple studies and has the potential advantage of providing a more statistically powerful test of significance for a treatment effect than can be achieved with a single experiment (Gurevitch and Hedges 1993). A critical assumption of our meta-analysis was that the effect being tested was manifested in a consistent manner across studies (i.e., there is not a treatment by study interaction). Because fitness costs can vary ecology conditions, for example when insects were reared on diet versus cotton bolls, costs may be greater or display nonrecessive effects in some cases but not others (Gassmann et al. 2009a). The meta-analysis preformed here cannot capture this variability and thus cannot provide an assessment of costs under different ecological conditions. Rather our meta-analysis tested the hypothesis that a general effect was present for fitness costs imposed by *S. riobrave*, and quantified the magnitude of this general effect. Conclusions drawn from this analysis do not negate the possibility that larger fitness costs or nonrecessive costs could be elicited under certain ecological conditions, as has been found in some cases (e.g., Hannon et al. 2010).

Resistance was only delayed in one of four cases tested with selection experiments, perhaps suggesting that costs were not present in the other three instances or were not sufficiently large to have a meaningful effect. Among studies testing for fitness costs of Bt resistance across several insect species, the average fitness cost affecting survival was 15.5% (Gassmann et al. 2009a), suggesting that the costs imposed by *S. riobrave* against Cry1Ac-resistant pink bollworm are among the largest costs that can be expected. It also is possible that fitness costs occurred but other factors, such as high initial *r* allele frequency and population bottlenecks, reduced the degree to which fitness costs affected resistance evolution. In the present simulations, only 20% of refuge fields (5% of the entire

landscape) were treated with nematodes. Treating a larger proportion of refuges with nematodes should magnify the effects of fitness costs in delaying resistance, as found in Hannon et al. (2010), and thus may be useful for increasing the effectiveness of costs when populations experience pronounced bottlenecks or when an elevated frequency of resistance allele is present.

Previous simulation modeling has shown that factors including population dynamics and migration will affect the rate of resistance evolution (Comins 1977, Georghiou and Taylor 1977, Caprio 2001, Sisterson et al. 2005). Less modeling work has considered how population-dynamic factors and population-genetic factors will interact with fitness costs to influence resistance evolution in the presence of refuges (Carrière et al. 2001a, Sisterson et al. 2004). Our simulation modeling showed that increases in the initial frequency of resistance alleles and the presence of population bottlenecks reduce the effectiveness of fitness costs at delaying resistance evolution (Fig. 3). A high initial *r* allele frequency probably decreases the effect of fitness costs because the proportion of *rr* individuals increases with *r* allele frequency. Because *rr* individuals can survive on Bt cotton, the fitness advantage of an *rr* individual outweighs the associated costs. Thus, only when *r* allele frequency is low and *rr* individuals are rare may fitness costs have a meaningful effect in delaying resistance evolution. As population bottlenecks become more pronounced, random factors (i.e., genetic drift) will have a greater influence on changes in *r* allele frequency than forces of natural selection such as fitness costs. Thus, when bottlenecks become sufficiently severe, genetic drift affects change in *r* allele frequency more than fitness costs and the effects of fitness costs are probably lost or diminished.

In the greenhouse selection experiment, populations experienced a bottleneck after the initial release of moths into cages (Fig. 2b). Almost no pink bollworms were detected in the sample at day 51, and few were found on days 66 and 121 (Fig. 2b; see Results). These population bottlenecks may have prevented fitness costs from influencing resistance evolution. In the laboratory selection experiment, the proportion of homozygous resistant individuals was significantly lower in the nematode-treated lines for F5 but not F7 (Fig. 1). There was also an increase in the number of resistant individuals from F5 to F7. It may be the case that the *r* allele frequency in the F5 and F6 generations was too great for fitness costs to have an effect on resistance evolution. As indicated by the simulation results (Fig. 3a), after *r* allele frequency becomes sufficiently great (i.e., >0.015), fitness costs no longer delay resistance evolution. This simulation suggests the presence of a threshold (≈0.005–0.01) at which the effectiveness of fitness costs for delaying resistance becomes greatly diminished. This threshold for the effectiveness of fitness costs seems to correspond to the same range of *r* allele frequencies (≈0.005–0.01) at which a threshold is present for the rate of resistance evolution, with resistance evolving at a substantially elevated pace once the threshold is crossed (Fig. 3a).

Results of this study and previous work suggest that fitness costs will be most valuable for delaying resistance evolution when resistance allele frequency is low and pest populations do not exhibit periods of extremely low abundance. Repeating the greenhouse and laboratory experiments described here using pink bollworm strains with a low *r* allele frequency (≈0.01) and with populations that do not suffer bottlenecks would be a worthwhile way to test this hypothesis. Future research on fitness costs may benefit from coupling population dynamics with population genetics to determine useful strategies for incorporating fitness costs into non-Bt refuges under a field setting.

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References Cited

Abbott, W. S. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18: 265–267.

Adkinsson, P. L., D. L. Vaderzant, D. L. Bull, and W. E. Allision. 1960. A wheat germ medium for rearing the pink bollworm. J. Econ. Entomol. 53: 759–762.

Bagla, P. 2010. Hardy cotton-munching pests are latest blow to GM crops. Science 327: 1439.

Beasley, C. A., and D. C. Adams. 1996. Field-based, degree-day model for pink bollworm (Lepidoptera: Gellichiidae). J. Econ. Entomol. 89: 881–890.

Bengtsson, J., J. Ahnstrom, and A. Weibull. 2005. The effects of organic agriculture on biodiversity and abundance: a meta analysis. J. Appl. Ecol. 42: 261–269.

Bird, L. J., and R. J. Akhurst. 2007. Effects of host plant species on fitness costs of Bt resistance in *Helicoverpa armigera* (Lepidoptera: Noctuidae). Biol. Control 40: 196–203.

Brazzel, J. R., and D. F. Martin. 1959. Winter survival and time of emergence of diapausing pink bollworms in central Texas. J. Econ. Entomol. 52: 305–308.

Caprio, M. A. 2001. Source-sink dynamics between transgenic and non-transgenic habitats and their role in the evolution of resistance. J. Econ. Entomol. 94: 698–705.

Carrière, Y., and B. E. Tabashnik. 2001. Reversing insect adaptation to transgenic insecticidal plants. Proc. R. Soc. Biol. Sci. Ser. B 268: 1475–1480.

Carrière, Y., T. J. Dennehy, B. Pedersen, S. Haller, C. Ellers-Kirk, L. Antilla, Y.-B. Liu, E. Willott, and B. E. Tabashnik. 2001a. Large-scale management of insect resistance to transgenic cotton in Arizona: can transgenic insecticidal crops be sustained? J. Econ. Entomol. 94: 315–325.

Carrière, Y., C. Ellers-Kirk, B. Pedersen, S. Haller, and L. Antilla. 2001b. Predicting spring moth emergence in pink bollworm (Lepidoptera: Gelechiidae): implications for managing resistance to transgenic cotton. J. Econ. Entomol. 94: 1012–1021.

Carrière, Y., C. Ellers-Kirk, R. Biggs, D. M. Higginson, T. J. Dennehy, and B. E. Tabashnik. 2004. Effects of gossypol on fitness costs associated with resistance to Bt cotton in pink bollworm. J. Econ. Entomol. 97: 1710–1718.

Carrière, Y., M. E. Nyboer, C. Ellers-Kirk, J. Sollome, N. Colleto, L. Antilla, T. J. Dennehy, R. T. Staten, and B. E. Tabashnik. 2006. Effect of resistance to *Bacillus thuringiensis* cotton on pink bollworm (Lepidoptera: Gelechiidae) response to sex pheromone. J. Econ. Entomol. 99: 946–953.

Carrière, Y., A. M. Showalter, J. A. Fabrick, J. Sollome, C. Ellers-Kirk, and B. E. Tabashnik. 2009. Cadherin gene expression and effects of Bt resistance on sperm transfer in pink bollworm. J. Insect Physiol. 55: 1058–1064.

Carrière, Y., D. W. Crowder, and B. E. Tabashnik. 2010. Evolutionary ecology of insect adaptation to Bt crops. Evol. Appl. 3: 561–573.

Comins, H. N. 1977. The development of insecticide resistance in the presence of migration. J. Theor. Biol. 64: 177–197.

Dennehy, T. J., G. C. Unnithan, S. A. Brink, B. D. Wood, Y. Carrière, B. Tabashnik, L. Antilla, and M. Whitlow. 2004. Update on pink bollworm resistance to Bt cotton in the Southwest. National Cotton Council, Memphis, TN.

Dhurua, S., and G. T. Gujar. 2011. Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India. Pest Manag. Sci. 67: 898–903.

Downes, S., T. Parker, and R. Mahon. 2010. Incipient resistance of *Helicoverpa punctigera* to the Cry2Ab Bt toxin in Bollgard II cotton. PLoS ONE 5: e12567. (doi:10.1371/journal.pone.0012567).

Gassmann, A. J., S. P. Stock, Y. Carrière, and B. E. Tabashnik. 2006. Effect of entomopathogenic nematodes on the fitness cost of resistance to Bt toxin Cry1Ac in pink bollworm (Lepidoptera: Gelechiidae). J. Econ. Entomol. 99: 920–926.

Gassmann, A. J., S. P. Stock, M. S. Sisterson, Y. Carrière, and B. E. Tabashnik. 2008. Synergism between entomopathogenic nematodes and *Bacillus thuringiensis* crops: integrating biological control and resistance management. J. Appl. Ecol. 45: 957–966.

Gassmann, A. J., Y. Carrière, and B. E. Tabashnik. 2009a. Fitness costs of insect resistance to *Bacillus thuringiensis.* Annu. Rev. Entomol. 54: 147–163.

Gassmann, A. J., J. A. Fabrick, M. S. Sisterson, E. R. Hannon, S. P. Stock, Y. Carrière, and B. E. Tabashnik. 2009b. Effects of pink bollworm resistance to *Bacillus thuringiensis* on phenoloxidase activity and susceptibility to entomopathogenic nematodes. J. Econ. Entomol. 102: 1224–1232.

Gassmann, A. J., D. W. Onstad, and B. R. Pittendrigh. 2009c. Evolutionary analysis of herbivorous insects in natural and agricultural environments. Pest Manag. Sci. 65: 1174–1181.

Gassmann, A. J., J. L. Petzold-Maxwell, R. S. Keweshan, and M. W. Dunbar. 2011. Field-evolved resistance to Bt maize by western corn rootworm. PLoS ONE 6: e22629. (doi:10.1371/journal.pone.0022629).

Georghiou, G. P., and C. E. Taylor. 1977. Genetic and biological influences in the evolution of insecticide resistance. J. Econ. Entomol. 70: 653–658.

Gouge, D. H., K. A. Smith, L. L. Lee, and T. J. Henneberry. 1998. Control of pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) with entomopathogenic nematodes (Rhabditida: Steinernematidae) and gossyplure in cotton, pp. 55–85. *In* S. G. Pandalai [ed.], Recent research developments in entomology. Research Signpost, Trivandrum, India.

Gouge, D. H., L. L. Lee, J. R. Van Berkum, T. J. Henneberry, K. A. Smith, C. Payne, and D. Ortega. 1997. Control of pink bollworm *Pectinophora gossypiella,* (Saunders) (Lepidoptera: Gellichiidae) with biocontrol and biorational agents. Proc. Beltwide Cotton Conf. 2: 1066–1072.

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. 43: 701–726.

Gripenberg, S., P. J. Mayhew, M. Parnell, and T. Roslin. 2010. A meta-analysis of preference-performance relationships in phytophagous insects. Ecol. Lett. 13: 383–393.

Gurevitch, J., P. S. Curtis, and M. H. Jones. 2001. Meta-analysis in Ecology, pp. 200–239. *In* H. Caswell [ed.], Advances in ecological research, vol. 32. Academic, London, United Kingdom.

Gurevitch, J., and L. V. Hedges. 1993. Meta-analysis: combining the results of independent experiments, pp. 347–369. *In* S. M. Scheiner and J. Gurevitch [eds.], Design and analysis of ecological experiments. Oxford University Press, New York.

Hannon, E. R., M. S. Sisterson, S. P. Stock, Y. Carrière, B. E. Tabashnik, and A. J. Gassmann. 2010. Effects of four nematode species on fitness costs of pink bollworm resistance to *Bacillus thuringiensis* toxin Cry1Ac. J. Econ. Entomol. 103: 1821–1831.

Hartl, D. L., and A. G. Clark. 1997. Principles of population genetics. Sinauer, Sunderland, MA.

Henneberry, T. J., and T. E. Clayton. 1979. Pink bollworm: movement and distribution of diapause and nondiapause larvae in soil and moth emergence after pupal burial. Environ. Entomol. 8: 782–785.

Henneberry, T. J., and S. E. Naranjo. 1998. Integrated management approaches for pink bollworm in the southwestern United States. Int. Pest Manag. Rev. 3: 31–52.

Higginson, D. M., S. Morin, M. E. Nyboer, R. W. Biggs, B. E. Tabashnik, and Y. Carrière. 2005. Evolutionary tradeoffs of insect resistance to *Bacillus thuringiensis* crops: fitness cost affecting paternity. Evolution 59: 915–920.

James, C. 2010. Global status of commercialized biotech/GM crops: 2010. ISAAA Brief 42. ISAAA, Ithaca, NY.

Janmaat, A. F., and J. Myers. 2005. The cost of resistance to *Bacillus thuringiensis* varies with the host plant of *Tricoplusia ni.* Proc. R. Soc. Biol. Sci. Ser. B 272: 1031–1038.

Kaya, H. K., and S. P. Stock. 1997. Techniques in insect nematology, pp. 281–324. *In* L. Lacey [ed.], Manual of techniques in insect pathology. Academic Ltd., San Diego, CA.

Koricheva, J. 2002. Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. Ecology 83: 176–190.

Massad, T. J., and L. A. Dyer. 2010. A meta-analysis of the effects of global environmental change on plant-herbivore interactions. Arthropod-Plant Interact. 4: 181–188.

Morin, S., S. Henderson, J. A. Fabrick, Y. Carrière, T. J. Dennehy, J. K. Brown, and B. E. Tabashnik. 2004. DNA-based detection of Bt resistance alleles in pink bollworm. Insect Biochem. Mol. Biol. 34: 1225–1233.

Morin, S., R. W. Biggs, M. S. Sisterson, L. Shriver, C. Ellers-Kirk, D. Higginson, D. Holley, L. J. Gahan, D. G. Heckel, Y. Carrière, et al. 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 100: 5004–5009.

Patin, A. L., T. J. Dennehy, M. A. Sims, B. E. Tabashnik, Y.-B. Liu, L. Antilla, D. Gouge, T. J. Henneberry, and R. Staten [eds.]. 1999. Status of pink bollworm susceptibility to Bt in Arizona. National Cotton Council of America, Memphis, TN.

Pittendrigh, B. R., P. J. Gaffney, J. E. Huesing, D. W. Onstad, R. T. Roush, and L. L. Murdock. 2004. "Active" refuges can inhibit the evolution of resistance in insects towards

transgenic insect-resistant plants. J. Theor. Biol. 231: 461–474.

Raymond, B., A. H. Sayyed, and D. J. Wright. 2005. Genes and environment interact to determine the fitness costs of resistance to *Bacillus thuringiensis.* Proc. R. Soc. Biol. Sci. Ser. B 272: 1519–1524.

Raymond, B., A. H. Sayyed, R. S. Hails, and W.D.J. 2007. Exploiting pathogens and their impact on fitness costs to manage the evolution of resistance to *Bacillus thuringiensis.* J. Appl. Ecol. 44: 768–780.

Rosenberg, M. S., D. C. Adams, and J. Gurevitch. 1999. MetaWin Statistical Software for Meta-Analysis. Version 2.0. Sinauer, Sunderland, MA.

SAS Institute. 2008. SAS enterprise guide, version 4.2. SAS Institute, Cary, NC.

Sisterson, M. S., L. Antilla, Y. Carrière, C. Ellers-Kirk, and B. E. Tabashnik. 2004. Effects of insect population size on evolution of resistance to transgenic crops. J. Econ. Entomol. 97: 1413–1424.

Sisterson, M. S., Y. Carrière, T. J. Dennehy, and B. E. Tabashnik. 2005. Evolution of resistance to transgenic Bt crops: interactions between movement and field distribution. J. Econ. Entomol. 98: 1751–1762.

Sokal, R. R., and F. J. Rohlf. 1995. Biometry. W. H. Freeman and Company, New York.

Stock, S. P., and J. C. Gress. 2006. Diversity and phylogenetic relationship of entomopathogenic nematodes (Steinernematidae and Heterorhabditidae) from the Sky Islands of southern Arizona. J. Invertebr. Pathol. 92: 66–72.

Storer, N. P., J. M. Babcock, M. Schlenz, T. Meade, G. D. Thompson, J. W. Bing, and R. M. Huchaba. 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103: 1031–1038.

Tabashnik, B. E., A. L. Patin, T. J. Dennehy, Y. Liu, Y. Carrière, M. A. Sims, and L. Antilla. 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 97: 12980–12984.

Tabashnik, B. E., F. Gould, and Y. Carrière. 2004. Delaying evolution of insect resistance to transgenic crops by decreasing dominance and heritability. J. Evol. Biol. 17: 904–912.

Tabashnik, B. E., R. W. Biggs, D. M. Higginson, S. Henderson, D. C. Unnithan, G. C. Unnithan, C. Ellers-Kirk, M. S. Sisterson, T. J. Dennehy, Y. Carrière, et al. 2005a. Association between resistance to Bt cotton and cadherin genotype in pink bollworm. J. Econ. Entomol. 98: 635–644.

Tabashnik, B. E., T. J. Dennehy, and Y. Carrière. 2005b. Delayed resistance to transgenic cotton in pink bollworm. Proc. Natl. Acad. Sci. U.S.A. 102: 15389–15393.

Tabashnik, B. E., A. J. Gassmann, D. W. Crowder, and Y. Carrière. 2008. Insect resistance to Bt crops: evidence versus theory. Nat. Biotechnol. 26: 199–202.

Tabashnik, B. E., J.B.J. van Rensburg, and Y. Carrière. 2009. Field-evolved insect resistance to Bt crops: definition, theory, and data. J. Econ. Entomol. 102: 2011–2025.

Tabashnik, B. E., M. S. Sisterson, P. C. Ellsworth, T. J. Dennehy, L. Antilla, L. Liesner, M. Whitlow, R. T. Staten, J. A. Fabrick, G. C. Unnithan, et al. 2010. Suppressing resistance to Bt cotton with sterile insect releases. Nat. Biotechnol. 28: 1304–1307.

Tang, J. D., H. L. Collins, T. D. Metz, E. D. Earle, J. Z. Zhao, R. T. Roush, and A. M. Shelton. 2001. Greenhouse tests on resistance management of Bt transgenic plants using refuge strategies. J. Econ. Entomol. 94: 240–247.

Van Rensburg, J.B.J. 2007. First report of field resistance by stem borer, *Busseola fusca* (Fuller) to Bt-transgenic maize. S. Afr. J. Plant Soil 24: 147–151.

Williams, J. L., C. Ellers-Kirk, R. G. Orth, A. J. Gassmann, G. Head, B. E. Tabashnik, and Y. Carrière. 2011. Fitness cost of resistance to Bt cotton linked with increased gossypol content in pink bollworm larvae. PLoS ONE 6: e21863. (doi:10.1371/journal.pone.0021863).

Zhang, H., W. Yin, J. Zhao, L. Jin, Y. Yang, S. Wu, B. E. Tabashnik, and Y. Wu. 2011. Early warning of cotton bollworm resistance associated with intensive planting of Bt cotton in China. PLoS ONE 6: e22874. (doi:10.1371/journal.pone.0022874).

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