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Synergism between entomopathogenic nematodes and *Bacillus thuringiensis* crops: integrating biological control and resistance management

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Summary

1. The past decade has witnessed a continual increase in the use of crops genetically modified to produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt). This presents the challenge of designing agricultural systems to manage pests and the evolution of resistance to Bt.
2. We tested whether entomopathogenic nematodes might act synergistically with Bt crops by killing pests in non-Bt refuges and by increasing the fitness costs of resistance to Bt. We also tested whether insect mortality and fitness costs were affected by the cotton phytochemical gossypol.
3. The entomopathogenic nematode *Steinernema riobrave* increased the fitness cost of Bt resistance, indicating that its presence in refuges may slow pest adaptation to Bt crops. No effect on fitness costs was detected for the nematode *Heterorhabditis bacteriophora*. Gossypol did not alter nematode-imposed fitness costs.
4. Simulation modelling supported the hypothesis that nematodes in refuges may slow resistance evolution.
5. The effects of gossypol on insect mortality from nematodes and nematode reproduction differed between nematode species. Gossypol increased insect mortality caused by *H. bacteriophora* but did not affect mortality caused by *S. riobrave*. Gossypol enhanced reproduction of *H. bacteriophora* and decreased reproduction of *S. riobrave*.
6. *Synthesis and applications.* Our results point to the value of developing integrated pest management strategies for Bt crops that include non-Bt refuges in which entomopathogenic nematodes are used as a pest-management agent. Because entomopathogenic nematodes can magnify fitness costs of Bt resistance, their presence in refuges may delay resistance by pests to Bt crops. Moreover, entomopathogenic nematodes can serve as biological control agents thereby decreasing dependence on conventional insecticides to manage pest populations in refuges.

Key-words: cotton, Cry1Ac, fitness costs, genetically modified crops, *Heterorhabditis bacteriophora*, *Pectinophora gossypiella*, refuge strategy, simulation modelling, *Steinernema riobrave*

Introduction

Integrated pest management (IPM) applies multiple methods to suppress pest populations, thereby reducing dependence on conventional insecticides, which can have unintended harmful consequences for the environment and human health (Dent 2000). Tactics for IPM have changed considerably in the past decade with the commercialization of genetically modified crops that produce insecticidal toxins from the bacterium *Bacillus thuringiensis* (Bt). Growers have adopted this technology rapidly, with Bt maize *Zea mays* L. and Bt cotton *Gossypium hirsutum* L. covering 32 million hectares world-wide in 2006 (James 2006). Bt crops offer the benefit of decreased dependence on conventional insecticides to manage pest populations (Cattaneo *et al.* 2006; Herdt 2006) but present two new challenges in IPM: (1) how should agricultural systems be managed to slow or prevent pests from evolving resistance to Bt toxins; and (2) what is the optimal strategy for managing pest populations in non-Bt 'refuge' fields?

Currently, the refuge strategy (Gould 1998) is mandated in the United States and elsewhere to delay Bt resistance. This

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strategy requires non-Bt host plants (i.e. a refuge) near Bt crops. If plants produce a sufficiently high concentration of Bt toxin, Bt resistance is effectively a recessive trait (Tabashnik, Gould & Carrière 2004). Resistant insects that emerge from Bt crops will be likely to mate with the more abundant Bt-susceptible individuals from refuges, producing heterozygous progeny that cannot survive on the Bt crop. Consequently, a critical factor in preventing resistance evolution is maintaining Bt-susceptible individuals in refuges (Carrière & Tabashnik 2001; Pittendrigh *et al*. 2004). However, movement of Bt-resistant individuals from Bt fields into refuges will increase the frequency of resistance alleles in refuges, accelerating resistance evolution (Caprio 2001; Sisterson *et al*. 2005). Fitness costs of Bt resistance arise in environments that lack Bt toxin (e.g. refuges) when individuals with Bt-resistance alleles have lower fitness than homozygous susceptible individuals. Fitness costs can reduce the frequency of resistance alleles in refuge populations, thereby slowing the evolution of resistance (Carrière & Tabashnik 2001; Pittendrigh *et al*. 2004; Tabashnik, Dennehy & Carrière 2005).

Surveys of Bt resistance in field populations usually reveal a low frequency of alleles for either resistance to Bt crops or resistance to high levels of Bt toxin in artificial diet (Gould *et al*. 1997; Burd *et al*. 2003; Tabashnik, Dennehy & Carrière 2005; Tabashnik *et al.* 2008), indicating that some natural populations harbour the genetic variation necessary to evolve resistance. Additionally, more than a dozen species have been selected for Bt resistance in the laboratory (Tabashnik 1994; Ferré & van Rie 2002), and resistance to Bt sprays has evolved in field populations of the diamondback moth *Plutella xylostella* (L.) and greenhouse populations of the cabbage looper moth *Trichoplusia ni* (Hübner) (Tabashnik *et al*. 1990; Janmaat & Myers 2003). However, in most cases, pests have not evolved resistance to Bt crops in the field (Tabashnik *et al.* 2008), which raises the possibility that fitness costs act as an evolutionary constraint on Bt resistance (Carrière & Tabashnik 2001; Tabashnik, Dennehy & Carrière 2005).

The hypothesis that fitness costs constrain adaptation by pests to Bt crops is supported by studies that have compared fitness of Bt-resistant and Bt-susceptible genotypes. These studies demonstrate that fitness costs arise for a range of life-history characters (Carrière *et al*. 2001, 2005b; Janmaat & Myers 2003, 2005; Bird & Akhurst 2004, 2007). However, as documented for other traits, fitness costs can vary with ecological conditions (Gassmann & Futuyma 2005 and references therein). Moreover, recent work shows that the magnitude of fitness costs for Bt resistance are variable, for example becoming larger or smaller as a function of the host plant on which an herbivore feeds (Janmaat & Myers 2005; Bird & Akhurst 2007). These studies point to a pattern of ecological plasticity in fitness costs and suggest that non-Bt refuges could be optimized to magnify fitness costs (Carrière *et al*. 2001, 2004; Pittendrigh *et al*. 2004).

Here we test the effects of entomopathogenic nematodes on insect mortality and fitness costs of resistance to Bt toxin Cry1Ac in pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), a major pest of cotton in the southwestern United States controlled currently with Bt cotton (Carrière *et al*. 2003). Recent work shows that entomopathogenic nematodes can increase fitness costs of resistance to Bt toxin (Gassmann *et al*. 2006), an effect that was suggested elsewhere (Baur *et al*. 1998). Additionally, entomopathogenic viruses can increase fitness costs of Bt resistance (Raymond *et al*. 2007). Thus, incorporating entomopathogenic organisms into refuges may enhance resistance management for Bt crops. Moreover, entomopathogenic nematodes also can act as biological control agents, suppressing soil-borne pink bollworm (Gouge *et al*. 1998), which pupates underground, and other pests (Journey & Ostlie 2000; Koppenhöfer *et al*. 2000).

Host plants can influence susceptibility of insects to pathogens (Cory & Hoover 2006) including entomopathogenic nematodes (e.g. Barbercheck, Wang & Hirsh 1995). As such, a tritrophic perspective is needed to understand fully how entomopathogenic nematodes might influence herbivore mortality and fitness costs. We introduced variation in the pink bollworm's diet through the presence or absence of gossypol, a prevalent and ecologically important phytochemical of cotton that affects growth, survival and fitness costs of Bt resistance for pink bollworm (Carrière *et al*. 2004 and references therein). Tritrophic interactions may also have multigenerational effects on biological control, as host plants can alter the reproductive success of entomopathogenic nematodes (Barbercheck, Wang & Hirsh 1995). Therefore, we coupled our measurements of fitness costs and herbivore mortality with measurements of reproduction by nematodes.

Materials and methods

PINK BOLLWORM STRAINS

We used four strains of pink bollworm, two Bt-resistant strains (MOV97-H1R and SAF97-H1R) and their Bt-susceptible counterparts (MOV97-H1S and SAF97-H1S). MOV97-H1R and MOV97-H1S originated from MOV97, and SAF97-H1R and SAF97-H1S from SAF97. Individuals collected from the Mohave Valley and from Safford, Arizona, USA, were used to start MOV97 and SAF97, respectively, and were selected with Bt toxin Cry1Ac to produce SAF97-R and MOV97-R (Tabashnik *et al*. 2005). Resistance to Cry1Ac is linked tightly with a cadherin locus, with cadherin resistance (*r*) alleles *r1* and *r3* present in MOV97-R and *r1* and *r1* in SAF97-R (Morin *et al*. 2003; Tabashnik *et al*. 2005). Only individuals with two *r* alleles survive exposure to a diagnostic concentration of Cry1Ac (10 μg Cry1Ac per ml diet) and survive on Cry1Ac-producing cotton (Morin *et al*. 2003; Tabashnik *et al*. 2005).

SAF97-R (F55) and SAF97 (F60 and F61) were crossed to produce SAF97-H1. To start SAF97-H1S, we screened 80 mated pairs from SAF97-H1 (F21) for *r1* and *r2* using allele-specific polymerase chain reactions (PCR) (Morin *et al*. 2004), selected 18 pairs that lacked *r* alleles and pooled their progeny. We started SAF97-H1R by rearing some SAF97-H1 (F_{21}) on a diet with 10 μg Cry1Ac per ml diet. From the survivors, which were homozygous for resistance to Cry1Ac, we caged 18 pairs and pooled their progeny. Strains were maintained at 1200 individuals per generation. MOV97-H1R and MOV97-H1S were started in a similar manner (Gassmann *et al*. 2006).

During the F_2 generation of SAF97-H1R and SAF97-H1S, we discovered that contamination of SAF97-H1 by MOV97-R occurred at generation F_{16} of SAF97-H1. From the frequency of *r3* (an allele unique to MOV97-R), we estimate the level of contamination at 16%. Paired matings coupled with PCR screening for *r3* alleles were used to remove *r3* alleles from SAF97-H1R and SAF97-H1S. Ten pairs per strain were identified that lacked *r3* alleles and were used to propagate strains. This contamination made SAF97-H1R and SAF97-H1S more similar genetically to MOV97-H1R and MOV97-H1S; however, it should not introduce experimental artifacts because the experimental design, as described below, consisted of making crosses between the Mohave and Safford strains.

INSECT CROSSES

Insects were from generations F_{13}–F_{15} for MOV97-H1R and MOV97-H1S, and F_9–F_{11} for SAF97-H1R and SAF97-H1S. All possible interstrain crosses were made between these Mohave and Safford strains, which generated four classes of genotypes: RR, SS, ♀RS and ♂RS (Table 1). Crosses were made by placing 15 pupae from each strain × sex combination (e.g. 15 SAF97-H1R♂ with 15 MOV97-H1S♀) in a 237 mL paper container with a vial of honey–water solution and oviposition substrate. Eggs were placed on either artificial diet that contained 0·1% gossypol (Sigma-Aldridge Inc., St Louis, MO, USA) or a control diet lacking gossypol (Carrière *et al*. 2004). For each genotype (e.g. SS), an equal number of eggs from both crosses (e.g. SAF97-H1S♀ × MOV97-H1S♂ and SAF97-H1S♂ × MOV97-H1S♀) was added to the diet. Larvae were raised to the wandering stage of the final (i.e. fourth) instar in an incubator (25 °C, 16/8 L/D), and exposed to nematodes when wandering larvae emerged from diet cups (15–18 days after hatching). In the field, wandering larvae leave cotton bolls to pupate in the soil where they could be exposed to entomopathogenic nematodes.

NEMATODE ASSAYS AND EXPERIMENTAL DESIGN

In two experiments, we exposed pink bollworm larvae to two entomopathogenic nematodes: *Steinernema riobrave* (Rhabditida: Steinernematidae) (first experiment) and *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae) (second experiment). *S. riobrave* (Biovector®, Becker Underwood, Ames, IA, USA) and *H. bacteriophora*, collected in Monterey, CA, USA, were maintained in the laboratory through culturing on *Galleria mellonella* (L.) larvae following Kaya & Stock (1997). Only nematodes in the infective juvenile stage were used in these experiments.

For both experiments, we used a fully crossed design with two diet types (control and gossypol), four classes of insect genotype (RR, SS, ♀RS and ♂RS) and three nematode concentrations: control (no nematodes), low treatment (three *S. riobrave* or 10 *H. bacteriophora*) and high treatment (six *S. riobrave* or 20 *H. bacteriophora*). Nine larvae were assigned to the control and 11 to each nematode treatment. This design was replicated over nine experimental blocks. An insufficient number of wandering larvae matured for some blocks. Sample sizes were 1536 treated and 645 control larvae for the experiment with *S. riobrave* and 1458 treated and 605 control larvae for the experiment with *H. bacteriophora*.

Wandering larvae were placed singly into Petri dishes (diameter = 3·5 cm) lined with 3 g of sterile sand, treated with 0·75 mL of deionized water, sealed inside plastic bags and held in an incubator (25 °C, 0/24 L/D). Assays were run in darkness because pink bollworm larvae typically pupate underground. The proportion of larvae failing to eclose as adults (mortality) was recorded.

We measured the number of dead pink bollworm (larvae and pupae) in which nematodes reproduced successfully, and the number of infective juvenile nematodes that emerged. Two, 3 and 4 weeks after larvae were exposed to nematodes, Petri dishes were inspected under a microscope for emergence of juvenile nematodes. For each combination of genotype × diet × nematode concentration × block (i.e. experimental replicate) we recorded the number of dead pink bollworms that yielded nematodes, and for two randomly selected insects we counted the total number of nematodes produced.

We collected nematodes using a modification of the Baermann funnel (Kaya & Stock 1997). Briefly, the dead insect and sand were wrapped in a KimWipe (Kimberly Clark, Dallas, TX, USA) and placed inside a basket made of plastic pipe (diameter 4·25 cm; height 2·0 cm) covered at one end with a fibreglass screen. Baskets, which had small rubber washers (diameter 1·25 cm; thickness 0·3 cm) placed on the bottom, were set inside 5·5 cm Petri dishes filled with deionized water. Nematodes migrated from the sand into the water and were collected over a 4-day period. Nematode density was counted using a haemocytometer and microscope. Sample size for counts in each of the 16 combinations of genotype × diet × nematode concentration was 10·2 ± 2·6 (mean ± standard deviation) for *S. riobrave* (163 in total) and 15·1 ± 2·1 for *H. bacteriophora* (242 in total). Data on nematode reproduction were collected for all experimental blocks except for the first block (run with *S. riobrave*) because our original method for collecting nematodes failed.

Table 1. Interstrain crossing design for producing larvae for nematode assays. Row and column headings list the strain and sex of insects used in crosses, and headings within each cell (e.g. RR) give the class of genotype generated from a cross. Italicized text (e.g. *r1r1*) lists the possible cadherin genotypes present in each parental strain and generated from crosses

Parental lines	MOV97-H1R♀ *r1r1*, *r1r3*, *r3r3*	MOV97-H1R♂ *r1r1*, *r1r3*, *r3r3*	MOV97-H1S♀ *ss*	MOV97-H1S♂ *ss*
SAF97-H1R♀ *r1r1*, *r1r2*, *r2r2*	Not applicable	RR *r1r1, r1r3* *r1r2*, *r2r3*	Not applicable	♀RS *r1s*, *r2s*
SAF97-H1R♂ *r1r1*, *r1r2*, *r2r2*	RR *r1r1*, *r1r3* *r1r2*, *r2r3*	Not applicable	♂RS *r1s*, *r2s*	Not applicable
SAF97-H1S♀ *ss*	Not applicable	♂RS *r1s*, *r3s*	Not applicable	SS *ss*
SAF97-H1S♂ *ss*	♀RS *r1s*, *r3s*	Not applicable	SS *ss*	Not applicable

DATA ANALYSIS

For each experimental replicate, we calculated proportional mortality for a total of 216 mortality scores for *S. riobrave* and 213 for *H. bacteriophora* (no larvae were obtained from one diet cup in one experimental block). Within each combination of genotype × diet × block, the formula of Abbott (1925) was used to adjust mortality in nematode treatments for control mortality (Lacey 1997). Control mortality was compared among genotype × diet combinations using a *G*-test of independence (Sokal & Rohlf 1995).

Each nematode species was analysed separately. Unless stated otherwise, data were analysed with a mixed-model analysis of variance (ANOVA) or analysis of covariance (ANCOVA) (PROC MIXED; SAS Institute Inc. 1999), with the factors of block (nine per nematode species), nematode concentration (low and high), diet (control and gossypol) and insect genotype (RR, SS, ♀RS and ♂RS). When analysing mortality, the control group was not included as a treatment in the ANOVA because it was already incorporated into the data set through the use of Abbott's formula. All means are reported as least squares means (LSMEANS option in SAS). Data were transformed as described below to ensure normality of the residuals and homogeneity of variance. Nematode concentration, genotype and diet and their interactions were fixed factors; block and its interactions were random effects. Random effects were tested with a log-likelihood ratio statistic (–2 RES log likelihood in PROC MIXED) (Littell *et al*. 1996). When block or its interactions were not significant at a level of $\alpha < 0{\cdot}25$, these factors were excluded from the model to increase statistical power (Quinn & Keough 2002). However, lower-order terms were retained if their higher-order interactions were significant. To determine whether maternal effects were present, data were first analysed by considering only the ♂RS and ♀RS classes of genotype and all interactions with block, diet and nematode concentration. No significant differences were detected between the two classes of RS genotypes. Consequently, ♂RS and ♀RS were treated as a single genotype (RS) in the analyses described below.

Mortality was analysed with a four-way ANOVA. Because a significant interaction occurred between genotype and nematode concentration for *S. riobrave* (see Results), pairwise comparisons between genotypes were made at the high and low nematode concentrations. We compared RS against SS to test for a dominant fitness cost of Bt resistance, and RS against RR to test for a recessive cost of Bt resistance. The significance level was set at 0·025 based on the Dunn–Šidák correction with two pairwise comparisons (Sokal & Rohlf 1995).

For each experimental replicate, we calculated nematode reproduction (i.e. production of nematode progeny from pink bollworm cadavers) as: (cadavers yielding progeny × mean progeny per cadaver) ÷ number of larvae treated. A cadaver is a dead larva or pupa, and progeny emerged only from cadavers, although not all cadavers yielded progeny. Data were transformed with the square root function, and analysed with a three-way ANOVA (PROC GLM in SAS) with the factors of diet, genotype and nematode concentration (Table 3).

To understand more clearly what influenced nematode reproduction, we analysed the number of cadavers yielding progeny per replicate (Table 4 for *S. riobrave* and Table 5 for *H. bacteriophora*). First, we analysed this response variable with an ANCOVA using the covariate of larvae treated with nematodes per replicate. Secondly, we tested whether the number of cadavers yielding progeny was due to suitability of cadavers for reproduction or insect mortality by adding a second covariate of cadavers per replicate. For the experiment with *H. bacteriophora*, the response variable and the covariates were transformed with the log(x + 1) function to ensure normality of the residuals and homogeneity of regression slopes. For the analysis with *S. riobrave*, the response variable was transformed with the square root function. Additionally, we analysed the number of progeny produced per cadaver, transformed with the square root function, with a four-way ANOVA.

SIMULATION MODELLING

Larval mortality data from the low concentration of *S. riobrave* (three nematodes per larva) were used to parameterize a stochastic, spatially explicit model developed by Sisterson *et al*. (2004, 2005). We selected these data because *S. riobrave* induced a fitness cost of Bt resistance (see Results). We compared the rate of resistance evolution in the presence and absence of this nematode-induced fitness cost across a range of refuge sizes.

In the absence of nematodes, we set mortality from egg to pupa in refuges at 79·2% for SS and 79·3% for RR and RS, with a slight fitness cost present to balance the effect of mutation. In Bt fields mortality values were 100% for SS and RS and 79·3% for RR (Sisterson *et al*. 2004). We made the fewest possible assumptions to incorporate nematode mortality, and simply increased mortality for RR genotypes in the refuges by the same amount that nematodes magnified mortality. In the experiment with *S. riobrave*, we found that mortality with three *S. riobrave* per larva was RR = 66·1%, RS = 55·6% and SS = 58·2%. Because SS and RS did not differ statistically (see Results), we used the average value of 56·9%. Consequently, nematodes imposed 16·2% higher mortality for RR individuals [(66·1–56·9) ÷ 56·9 = 0·162]. In simulations with nematodes present, we set mortality in refuges at RR = 92·0%, RS = 79·3% and SS = 79·2%, to incorporate the 16·2% higher mortality [(92·0–79·2) ÷ 79·2 = 0·162].

We applied a sensitivity analysis by varying the percentage of non-Bt refuge from 10% to 40%. Initial *r* allele frequency was set at 0·01. Each simulation consisted of 100 randomly distributed fields. Although current regulations specify a maximum distance for refuges from Bt fields, grower compliance with the refuge requirement ranges for 66% and 100% (Bourguet *et al*. 2005; Carrière *et al*. 2005a); consequently, a random distribution of fields should provide a more complete depiction of potential spatial distributions of fields assuming variation in the level of grower compliance.

Simulations were run until the frequency of Bt resistance alleles reached 0·50 or 100 years had elapsed. Twenty simulations were run for each combination of refuge size × nematode treatment. Because the model was stochastic, parameters had a mean and variance and the distribution of fields was assigned randomly. Hence, simulations with the same refuge size and nematode treatment differed in their rate of resistance evolution. Additional details on the simulation model are provided in Sisterson *et al*. (2004).

Results

PINK BOLLWORM MORTALITY

A fitness cost of pink bollworm resistance to Bt was imposed by *S. riobrave*, but not by *H. bacteriophora*. With *S. riobrave*, pink bollworm mortality was affected by a significant interaction between genotype and nematode concentration (Fig. 1, Table 2). Mortality was significantly higher for the RR than RS genotype at the low concentration of *S. riobrave*

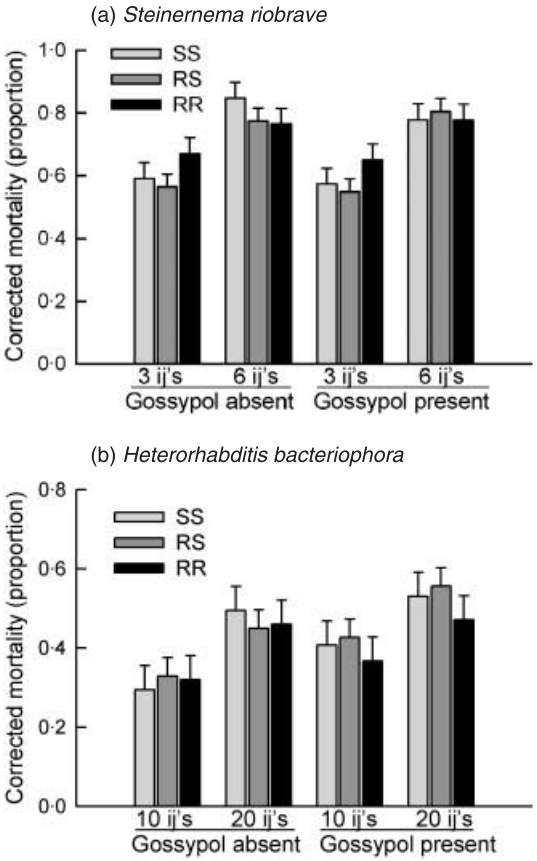

Fig. 1. Mortality of pink bollworm from (a) *Steinernema riobrave* and (b) *Heterorhabditis bacteriophora*. Bar heights are sample means and error bars the standard error of the mean for mortality adjusted with Abbott's correction. The *x*-axis describes the number of infective juvenile nematodes (ij's) to which larvae were exposed and whether larvae were raised on diet with gossypol. The legend describes the pink bollworm genotypes: homozygous resistant (RR), homozygous susceptible (SS) and heterozygous (RS).

($F = 5{\cdot}84$; d.f. 1,84; $P = 0{\cdot}019$) but not at the high concentration ($F = 0{\cdot}18$; d.f. 1,84; $P = 0{\cdot}67$), indicating that *S. riobrave* imposed a recessive fitness cost of Bt resistance at the low concentration. No significant differences were found between SS and RS genotypes at the low ($F = 0{\cdot}36$; d.f. 1,84; $P = 0{\cdot}55$) or high nematode concentrations ($F = 0{\cdot}29$; d.f. 1,84; $P = 0{\cdot}59$), indicating the absence of any dominant fitness costs. In *post hoc* comparisons, we did not detect a significant difference in mortality between the SS and RR genotypes at the low concentration of *S. riobrave* ($F = 2{\cdot}70$; d.f. 1,84; $P = 0{\cdot}10$); however, when the SS and RS genotypes were pooled their mortality was significantly less than the RR genotype ($F = 5{\cdot}21$; d.f. 1,84; $P = 0{\cdot}025$), consistent with the proposition that a recessive fitness cost was present. By contrast, genotype did not affect susceptibility to *H. bacteriophora*, indicating that *H. bacteriophora* did not affect the fitness cost of Bt resistance (Fig. 1, Table 2). For both species of nematodes, pink bollworm mortality increased significantly with nematode concentration (Fig. 1, Table 2). For *H. bacteriophora*, but not *S. riobrave*, gossypol in the diet increased pink bollworm mortality significantly (Fig. 1, Table 2).

In the controls without nematodes, run concurrently with all blocks, fitness costs were not detected. Mortality of control larvae did not vary significantly among genotype × diet combinations for the experiment with *S. riobrave* ($G = 7{\cdot}4$, d.f. = 5, $P = 0{\cdot}19$; control diet: SS = 1%, RS = 1%, RR = 2%; gossypol diet: SS = 1%, RS = 4%, RR = 7%), or with *H. bacteriophora* ($G = 10{\cdot}2$, d.f. = 5, $P = 0{\cdot}07$; control diet: SS = 4%, RS = 5% RR = 5%; gossypol diet: SS = 14%, R^S = 4%, RR = 2%). However, mortality of the SS genotype was numerically higher than the other genotypes on gossypol diet in the *H. bacteriophora* experiment; a trend also found by Carrière *et al*. (2004).

NEMATODE REPRODUCTION

Reproduction of both nematode species was affected by gossypol and nematode concentration (Table 3). For both species, the number of progeny produced per insect larva treated was significantly greater at the high nematode concentration (Fig. 2). However, the effects of gossypol differed between species: gossypol decreased reproduction significantly for *S. riobrave* but increased reproduction significantly for *H. bacteriophora* (Fig. 2).

Table 2. Analysis of variance for mortality of pink bollworm in the presence of (a) *Steinernema riobrave* and (b) *Heterorhabditis bacteriophora*. Factors in the analysis include homozygous resistant, homozygous susceptible, and heterozygous genotypes (Genotype); larvae raised on control diet or diet with gossypol (Diet); and the presence of either three vs. six *S. riobrave* or 10 vs. 20 *H. bacteriophora* per larva (Concentration). For *S. riobrave*, random factors were block ($\chi^2 = 1{\cdot}0$, d.f. = 1, $P = 0{\cdot}159$), block × genotype ($\chi^2 = 0{\cdot}5$, d.f. = 1, $P = 0{\cdot}24$), block × diet ($\chi^2 = 0{\cdot}5$, d.f. = 1, $P = 0{\cdot}24$) and block × genotype × diet ($\chi^2 = 0{\cdot}7$, d.f. = 1, $P = 0{\cdot}20$). For *H. bacteriophora*, the random factor was block ($\chi^2 = 12{\cdot}9$, d.f. = 1, $P < 0{\cdot}001$)

	(a) *S. riobrave*			(b) *H. bacteriophora*		
Source	d.f.	*F* value	*P*	d.f.	*F* value	*P*
Genotype	2,16	0·77	0·48	2,122	0·55	0·58
Concentration	1,84	79·42	< 0·0001	1,122	20·70	< 0·0001
Diet	1,8	0·18	0·68	1,122	5·22	0·02
Concentration × genotype	2,84	3·35	0·04	2,122	0·16	0·85
Concentration × diet	1,84	0·05	0·83	1,122	0·33	0·57
Diet × genotype	2,16	0·38	0·69	2,122	0·55	0·58
Diet × concentration × genotype	2,84	0·50	0·61	2,122	0·19	0·82

Table 3. Analysis of variance for reproduction by (a) *Steinernema riobrave* and (b) *Heterorhabditis bacteriophora*. Data were analysed as the number of nematodes produced per pink bollworm exposed to nematodes. Factors are defined in Table 2. See Methods for a more detailed explanation of the analysis

	(a) *S. riobrave*			(b) *H. bacteriophora*		
Source	d.f.	*F* value	*P*	d.f.	*F* value	*P*
Genotype	2	0·54	0·58	2	0·46	0·63
Concentration	1	9·86	0·002	1	6·65	0·01
Diet	1	5·68	0·02	1	5·78	0·02
Concentration × genotype	2	0·08	0·92	2	0·54	0·59
Concentration × diet	1	0·41	0·52	1	0·40	0·53
Diet × genotype	2	0·01	0·99	2	0·05	0·95
Diet × concentration × genotype	2	1·58	0·21	2	0·63	0·54
Error	115			130		

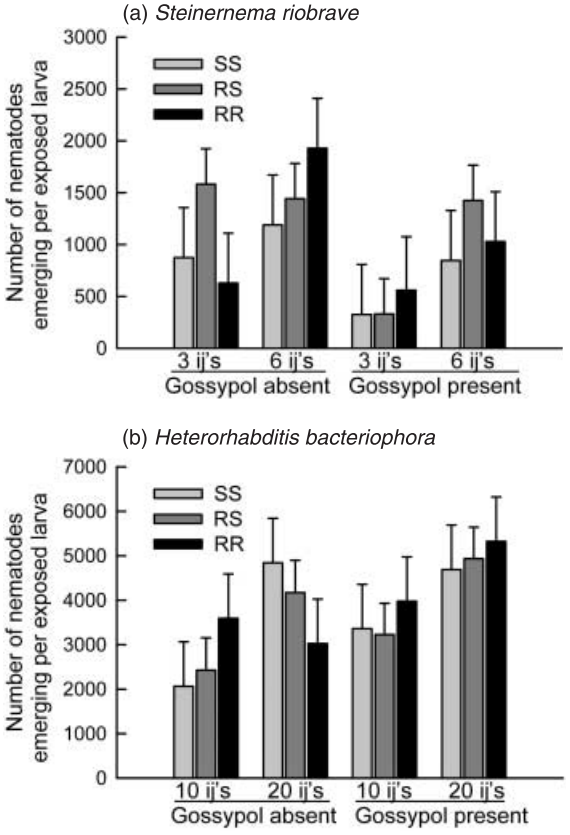

Fig. 2. Number of infective juvenile nematodes emerging per larva exposed to nematodes for (a) *Steinernema riobrave* and (b) *Heterorhabditis bacteriophora*. See Methods for a description of how values were calculated. Bar heights are sample means and error bars the standard error of the mean. The *x*-axis and legend are the same as in Fig. 1.

For *S. riobrave*, the effects on nematode reproduction resulted from differences in the number of cadavers yielding progeny but not the number of progeny produced per cadaver. The number of cadavers yielding progeny was influenced both by diet and nematode concentration (Table 4a). Per experimental replicate (10·9 larvae on average), cadavers yielding progeny increased significantly with nematode concentration (1·6 ± 0·58 vs. 3·3 ± 0·58, mean ± SE for low vs. high concentration) and when larvae fed on control diet (2·9 ± 0·58 vs. 2·1 ± 0·58, for control vs. gossypol diet). These effects were still significant when data were corrected for the number of cadavers (Table 4b), indicating that nematode reproduction was not simply a function of insect mortality, but that the suitability of cadavers for reproduction was affected by insect diet and nematode concentration. The number of pink bollworm yielding progeny per experimental replicate, corrected for mortality (7·5 cadavers on average per replicate), was greater for control diet [2·9 ± 0·56 (mean ± SE)] than gossypol diet (2·1 ± 0·56), indicating that gossypol reduced host suitability. Also, corrected means were greater at high (3·0 ± 0·57) than low (1·9 ± 0·57) nematode concentrations, an effect that likely arose because the higher concentration increased the probability that nematodes of the opposite sex would invade the host; a prerequisite for successful reproduction in this sexually reproducing species. By contrast, the number of nematodes emerging from each cadaver did not differ as a function of nematode concentration ($F = 0{\cdot}42$; d.f. 1,139; $P = 0{\cdot}52$) or diet ($F = 1{\cdot}29$; d.f. 1,7; $P = 0{\cdot}29$).

For *H. bacteriophora*, patterns of nematode reproduction also were affected by the number of cadavers yielding progeny, but not the number of progeny emerging per cadaver. Per experimental replicate (10·3 larvae on average), the number of cadavers yielding progeny increased significantly with nematode concentration (2·6 ± 0·29 vs. 3·9 ± 0·29, mean ± SE for low vs. high concentration) and when insects fed on diet with gossypol (3·0 ± 0·29 vs. 3·5 ± 0·29, mean ± SE for control diet vs. gossypol diet) (Table 5a). However, these effects were not significant when cadavers were included as a covariate (Table 5b), indicating that the number of cadavers yielding progeny could be explained entirely by patterns of insect mortality. This means that gossypol did not alter suitability of cadavers for nematode reproduction and that the high concentration of nematodes did not increase the likelihood of successful reproduction, which is expected because *H. bacteriophora* is hermaphroditic. Additionally, the number of nematodes emerging per cadaver was not affected by nematode concentration ($F = 0{\cdot}98$; d.f. 1,182; $P = 0{\cdot}32$) or diet ($F = 0{\cdot}23$; d.f. 1,8; $P = 0{\cdot}65$).

Table 4. Analysis of covariance for the number of pink bollworm cadavers yielding progeny of *S. riobrave*. Covariates in the analyses were the number of larvae treated with nematodes and the number of cadavers. Factors are defined in Table 2. For (a) cadavers yielding progeny, random factors were block ($\chi^2 = 5{\cdot}9$, d.f. = 1, $P < 0{\cdot}01$) and block × genotype ($\chi^2 = 7{\cdot}5$, d.f. = 1, $P < 0{\cdot}01$). For (b) host suitability, random factors were block ($\chi^2 = 5{\cdot}0$, d.f. = 1, $P = 0{\cdot}01$), block × genotype ($\chi^2 = 7{\cdot}4$, d.f. = 1, $P < 0{\cdot}01$), block × diet ($\chi^2 = 0{\cdot}0$, d.f. = 1, $P = 0{\cdot}5$), block × concentration ($\chi^2 = 0{\cdot}0$, d.f. = 1, $P = 0{\cdot}5$), and block × diet × concentration ($\chi^2 = 0{\cdot}8$, d.f. = 1, $P = 0{\cdot}19$)

	(a) Cadavers yielding progeny			(b) Host suitability		
Source	d.f.	*F* value	*P*	d.f.	*F* value	*P*
Genotype	2,14	0·60	0·56	2,14	0·58	0·57
Concentration	1,94	28·26	< 0·0001	1,7	8·66	0·02
Diet	1,94	9·86	0·002	1,7	8·19	0·02
Concentration × genotype	2,94	0·88	0·42	2,72	0·61	0·54
Concentration × diet	1,94	0·03	0·85	1,7	0·01	0·91
Diet × genotype	2,94	0·26	0·77	2,72	0·13	0·88
Diet × concentration × genotype	2,94	1·81	0·17	2,72	1·78	0·18
Larvae treated (covariate)	1,94	8·05	0·01	1,72	4·91	0·03
Cadavers (covariate)	–	–		1,72	2·98	0·09

Table 5. Analysis of covariance for the number pink bollworm cadavers yielding progeny of *H. bacteriophora.* Covariates in the analyses were the number of larvae treated with nematodes and the number of cadavers. Factors are defined in Table 2. For (a) cadavers yielding progeny, random factors included were block ($\chi^2 = 2{\cdot}3$, d.f. = 1, $P = 0{\cdot}06$) and block × genotype ($\chi^2 = 0{\cdot}7$, d.f. = 1, $P = 0{\cdot}20$). For (b) host suitability, random factors were block ($\chi^2 = 0{\cdot}7$, d.f. = 1, $P = 0{\cdot}20$), block × genotype ($\chi^2 = 0{\cdot}5$, d.f. = 1, $P = 0{\cdot}24$) and block × concentration ($\chi^2 = 5{\cdot}7$, d.f. = 1, $P < 0{\cdot}01$)

	(a) Cadavers yielding progeny			(b) Host suitability		
Source	d.f.	*F* value	*P*	d.f.	*F* value	*P*
Genotype	2,16	2·14	0·15	2,16	2·08	0·16
Concentration	1,105	18·38	< 0·0001	1, 8	0·89	0·37
Diet	1,105	6·03	0·02	1,96	3·48	0·07
Concentration × genotype	2,105	0·46	0·63	2,96	0·63	0·53
Concentration × diet	1,105	0·40	0·53	1,96	1·07	0·30
Diet × genotype	2,105	0·53	0·59	2,96	0·23	0·79
Diet × concentration × genotype	2,105	1·04	0·36	2,96	1·37	0·26
Larvae treated (covariate)	1,105	14·58	0·0002	1,96	0·01	0·94
Cadavers (covariate)	–	–		1,96	105·98	< 0·0001

SIMULATION MODELLING

For all refuge sizes, the average time for populations to evolve resistance was greater when a low concentration of *S. riobrave* was present than absent (Fig. 3). Delays in resistance were most pronounced at 20–40% refuge, with resistance evolving more slowly for all simulations with nematodes present than the average time to resistance with nematodes absent. In some cases, small refuge size led to rapid resistance evolution because large clusters of Bt fields were present. As demonstrated by Sisterson *et al*. (2005), isolating Bt fields from refuges can greatly accelerate resistance evolution, and consequently illustrates the need for growers to plant refuges within close proximity to Bt fields.

Discussion

Our results indicate that improved resistance management for Bt crops may be achieved with IPM strategies that incorporate entomopathogenic nematodes in non-Bt refuges; however, this effect will be likely to depend on the nematode species and its density. A recessive fitness cost of Bt resistance, measured as greater larval mortality, was caused by exposure to *S. riobrave* but not *H. bacteriophora* (Fig. 1, Table 2). No fitness costs of Bt resistance were detected in the absence of nematodes (i.e. for experimental controls). Because *S. riobrave* increased the fitness cost of Bt resistance, its presence in refuges could slow the evolution of resistance to Bt crops, a supposition that is supported through simulation modelling (Fig. 3).

Our findings are consistent with the idea that resistance to Bt often carries fitness costs (Carrière *et al*. 2001; Bird & Akhurst 2004) and that these fitness costs can vary with ecological interactions (Carrière *et al*. 2004; Janmaat & Myers 2005; Gassmann *et al*. 2006; Bird & Akhurst 2007). In general, adaptation to one environment can impose fitness costs in other environments (e.g. Ghalambor, Reznick & Walker 2004), and the magnitude of these fitness costs can vary with ecological conditions (e.g. Gassmann & Futayma 2005). From the perspective of resistance management for Bt crops, these results point to an opportunity to design refuges that maximize fitness costs, thereby slowing the evolution of Bt resistance (Carrière & Tabashnik 2001; Carrière *et al*.

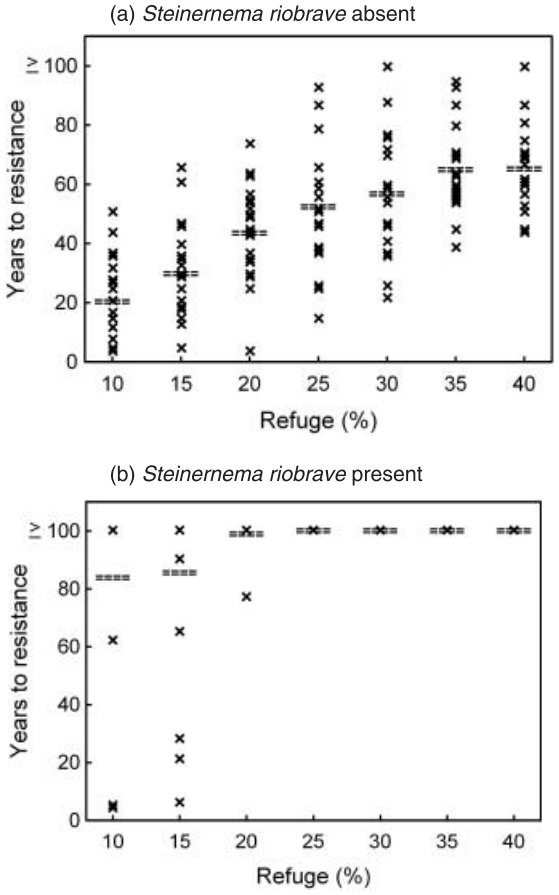

Fig. 3. Evolution of *Bacillus thuringiensis* resistance with (a) *Steinernema riobrave* absent from refuges and (b) *S. riobrave* present in refuges. A population was defined as evolving resistance once the *r* allele frequency reached 0·50. For each combination of refuge size × nematode treatment (present vs. absent), 20 simulations were run. Broken bars (= = = =): sample means; x: results of individual simulations.

2004, 2005b; Pittendrigh *et al*. 2004; Gassmann *et al*. 2006; Raymond *et al*. 2007).

The fitness cost of Bt resistance in the presence of *S. riobrave* was found only at the low nematode concentration. This result contrasts with Gassmann *et al*. (2006), in which a range of nematode concentrations magnified fitness costs. The two studies differed in the temperature at which assays were run (25 °C here vs. 20 °C previously) and suggest an interactive effect of nematode-induced fitness costs with temperature. At higher temperatures, *S. riobrave* may impose a fitness cost only at low concentrations. From the perspective of pest management in refuges, imposing only moderate levels of mortality through the application of nematodes at low levels may be acceptable because excessive mortality would reduce the population size of Bt-susceptible insects in refuges to the point that resistance evolution might be accelerated (Shelton *et al*. 2000; Sisterson *et al*. 2004).

In agricultural systems, tritrophic interactions may serve either to bolster or to diminish biological control. Tritrophic interactions among host plants, herbivores and predators and parasitoids have received considerable attention (Hare 2002; Ode 2006), but far less is known about entomopathogenic organisms (but see Cory & Hoover 2006). One of the few other studies testing tritrophic effects across nematode species reported similar patterns of larval mortality and nematode reproduction across several larval host plants, although some contrasting effects did arise (Barbercheck 1993; Barbercheck, Wang & Hirsh 1995). In this study, gossypol-induced effects on mortality caused by nematodes (Fig. 1, Table 2) and nematode reproduction (Fig. 2, Table 3) differed strongly between nematode species. Gossypol enhanced mortality from *H. bacteriophora* but did not alter mortality from *S. riobrave* (Fig. 1, Table 2). The higher mortality of gossypol-fed larvae resulted in greater reproduction for *H. bacteriophora*, but by contrast gossypol-fed larvae diminished reproduction for *S. riobrave* (Fig. 2, Tables 3 and 4). Our results suggest the potential for either synergistic or antagonist interactions of host-plant resistance with biological control by entomopathogenic nematodes. Thus, achieving complementary interactions between these trophic levels may require consideration of both the host plant and nematode species.

S. riobrave and *H. bacteriophora* differ in several aspects of their biology, which may explain why they produced contrasting results. Nematodes kill their host by releasing symbiotic bacteria, whose growth and production of immunosuppressing (Park & Stanley 2006) and otherwise toxic (Boemare 2002) compounds lead to the insect's death. Members of the genus *Steinernema* harbour bacteria from the genus *Xenorhabdus*, while *Heterorhabditis* spp. harbour bacteria from the genus *Photorhabdus*. These bacteria differ in the compounds they produce (Forst & Nealson 1996; Chattopadhyay, Bhatnagar & Bhatnagar 2004). The cadherin alleles associated with Bt resistance may increase susceptibility to *Xenorhabdus* but not *Photorhabdus*. Similarly, gossypol may enhance susceptibility of insects to *Xenorhabdus* but not *Photorhabdus*. These nematode species also differ in their host-finding behaviour. While *S. riobrave* is an intermediate forager, using both sit-and-wait tactics and cruising behaviour to find a host, *H. bacteriophora* is an active forager (i.e. a cruiser) (Campbell *et al*. 2003). Resistance to Bt may alter insect behaviour in such a way that Bt-resistant insects are more likely to encounter intermediate foragers than active foragers.

Further research on interactions among entomopathogenic nematodes, crop varieties and herbivores holds the promise of designing refuges that require fewer inputs of conventional insecticides and prolong the efficacy of Bt varieties. It is likely that the development of such IPM strategies will need to be based on specific nematode–pest interactions, as nematodes impose fitness costs only in some cases. Although tritrophic effects on fitness costs were not detected in this study, they were present for insect mortality and nematode reproduction, and it may be the case that tritrophic effects on fitness costs will arise with other combinations of species.

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