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The evolution of resistance to two-toxin pyramid transgenic crops

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Abstract. Pyramid transgenic crops that express two *Bacillus thuringiensis* (Bt) toxins hold great potential for reducing insect damage and slowing the evolution of resistance to the toxins. Here, we analyzed a suite of models for pyramid Bt crops to illustrate factors that should be considered when implementing the high dose–refuge strategy for resistance management; this strategy involves the high expression of toxins in Bt plants and use of non-Bt plants as refuges. Although resistance evolution to pyramid Bt varieties should in general be slower, resistance to pyramid Bt varieties is nonetheless driven by the same evolutionary processes as single Bt-toxin varieties. The main advantage of pyramid varieties is the low survival of insects heterozygous for resistance alleles. We show that there are two modes of resistance evolution. When populations of purely susceptible insects persist, leading to density dependence, the speed of resistance evolution changes slowly with the proportion of refuges. However, once the proportion of non-Bt plants crosses the threshold below which a susceptible population cannot persist, the speed of resistance evolution increases rapidly. This suggests that adaptive management be used to guarantee persistence of susceptible populations. We compared the use of seed mixtures in which Bt and non-Bt plants are sown in the same fields to the use of spatial refuges. As found for single Bt varieties, seed mixtures can speed resistance evolution if larvae move among plants. Devising optimal management plans for deploying spatial refuges is difficult because they depend on crop rotation patterns, whether males or females have limited dispersal, and other characteristics. Nonetheless, the effects of spatial refuges on resistance evolution can be understood by considering the three mechanisms determining the rate of resistance evolution: the force of selection (the proportion of insects killed by Bt), assortative mating (deviations of the proportion of heterozygotes from Hardy-Weinberg equilibrium at the total population level), and male mating success (when males carrying resistance alleles find fewer mates). Of these three, assortative mating is often the least important, even though this mechanism is the most frequently cited explanation for the efficacy of the high dose–refuge strategy.

Key words: Bacillus thuringiensis; *bollworm; Bt corn; Bt cotton; budworm; European corn borer; heterozygotes vs. homozygotes; rootworm; transgenic crops; two-gene resistance mechanisms.*

Introduction

Transgenic insecticidal crops that carry one or more of several toxin genes from *Bacillus thuringiensis* are now grown extensively in the United States. These Bt varieties are highly effective against many of their target pests and have been a boon to agriculture, capable of reducing crop damage and use of chemical pesticides. Nonetheless, they carry the risk that the target pests will evolve resistance to the Bt toxins, thereby diminishing the benefits that Bt varieties are currently providing. Indeed, the focus of U.S. government oversight of Bt crops is on management strategies to slow or reduce the risk of resistance evolution (U.S. EPA 2001, Matten et al. 2004). The cornerstone of resistance management is the "high dose–refuge" strategy in which highly toxic, high-dose Bt varieties are planted as monocultures, and non-Bt varieties are planted in either separate fields or separate sections of the same fields as refuges in which there is no selection for resistance (Gould and Tabashnik 1998). Theoretical studies have shown the efficacy of the high dose–refuge strategy for the case in which resistance occurs as a recessive, single-locus resistance allele (Tabashnik and Croft 1982, Onstad and Gould 1998, Peck et al. 1999, Caprio 2001); this is the form of resistance expected for Bt varieties that cause high (>99%) mortality (Gould et al. 1995, Gould 1998). The absence of widespread resistance evolution to Bt crops where this strategy has been used provides circumstantial evidence that it does work (Tabashnik et al. 2008).

A new generation of Bt crop products is currently being brought to market in which two Bt genes are transformed into the same variety to produce a "pyramid" of toxins. For pyramid products in which both Bt genes are highly toxic, it is assumed that

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resistance will be governed by two single-locus, diallelic genes, with full resistance requiring insects to have recessive resistance alleles at each locus (Mani 1985, Comins 1986, Gould 1986, 2006, Caprio 1998, Roush 1998, Tabashnik et al. 2004). These pyramid Bt products should give both greater crop protection against target pests (e.g., Jackson et al. 2004) and reduced risk of resistance evolution (Mani 1985, Gould 1986, 1991, 2006, Roush 1998, Zhao et al. 2003, Gahan et al. 2005), provided the resistance genes segregate independently and exhibit two independent modes of toxicity against target pests (but for empirical examples of cross-resistance, see Gould et al. 1992, McGaughey 1994, Gould 1998, Greenplate et al. 2003, Jackson et al. 2007).

Pyramid varieties have brought calls for modifications to current resistance management policies set by the U.S. Environmental Protection Agency. In particular, three modifications might be considered to reduce the burden placed on farmers by the current high dose–refuge strategy. First, the required proportion of refuge could be reduced, because less refuge is required to slow resistance evolution to acceptable rates. Second, the central feature of the high dose–refuge strategy, that refuges be spatially separated from areas of Bt varieties, could be removed, with non-Bt and Bt varieties being planted as seed mixtures. Third, the requirement that non-Bt varieties be used, either as spatially separated refuges or in seed mixtures, could be lifted.

Here, we conducted a theoretical exploration of factors that might affect the durability of pyramid Bt products against insect resistance evolution when they are deployed in either seed mixtures or spatial refuges. Several pyramid Bt products have already been developed that target different insect pests on different crops, and more varieties are likely to be developed. Therefore, rather than tailoring our models for a specific Bt product or target pest, we instead performed a theoretical analysis designed to highlight general factors affecting resistance evolution that should be considered when devising resistance management strategies. Our models are generic and are designed to reveal the mechanisms underlying resistance evolution. While the specific quantitative results we present are not intended to give recommendations for any specific Bt product or target pest, the theoretical comparisons we make should give a general guide outlining which management strategies are most likely to be effective under different general assumptions about the system in question.

Because pyramid products could allow reductions in the area of refuge in the high dose–refuge strategy, we first investigated the consequences of reducing refuge area on resistance evolution. As found for single-Bt gene (non-pyramid) products (Ives and Andow 2002), we show for pyramid products that there are two distinct modes of resistance evolution depending on whether or not a population of purely susceptible insects could persist across the landscape; as the amount of refuge is reduced below the threshold required to maintain a purely susceptible pest population, the rate of resistance evolution increases rapidly. By comparing a pyramid product to a single-gene product, we show that there is nothing unique about the pyramid product in terms of slowing the rate of resistance evolution; the same general mechanisms explain resistance evolution to both single-gene and pyramid products. Furthermore, when the available refuge is reduced below the point that a purely susceptible population can persist, factors that might be expected to greatly reduce the rate of resistance evolution no longer have strong effects; for example, a cost of resistance (i.e., when resistant insects feeding on non-Bt plants show lower survival or fecundity) exhibits weaker protection against resistance evolution.

We then investigated the use of seed mixtures in resistance management. The attraction of seed mixtures to farmers is that they eliminate the need to have separate management plans for Bt and non-Bt fields or sections within fields. Furthermore, the non-Bt plants within mixtures may benefit from being surrounded by Bt plants if insect larvae move among plants; those larvae on Bt plants will be killed, thereby reducing attack on non-Bt plants. However, movement of larvae within seed mixtures has two known consequences for resistance evolution. First, it causes greater mortality from the Bt toxins, and hence, greater selection for resistance. Second, larval movement will likely decrease the recessiveness of a resistance gene, and this will speed resistance evolution by increasing the survival of resistant-susceptible (RS) heterozygotes (Mallet and Porter 1992, Peck et al. 1999, Davis and Onstad 2000). We show that these disadvantages of seed mixtures apply to pyramid Bt varieties just as they do for single-gene Bt varieties.

Finally, we investigated the consequences of the spatial location of refuge fields for the high dose–refuge strategy. Current management regulations in the USA require spatial refuges to be within relatively (depending on the crop and target pest) close proximity to Bt fields to ensure that susceptible adults emerging from the refuge can mate with potentially resistant insects emerging from Bt fields (Anonymous 1998, FIFRA 1998). The common justification for this regulation is that resistance management requires SS individuals from refuges to mate with RR individuals from Bt fields, thereby producing heterozygote RS larvae that are killed in Bt crops and removing R alleles from the population. Closer examination of theoretical models for single-gene Bt varieties, however, shows that this explanation for the efficacy of the high dose–refuge strategy is incomplete (Ives and Andow 2002). There are three factors that combine to determine the rate of resistance evolution. First, assortative mating can be caused by spatial structure (global nonrandom mating) that leads to a greater proportion of RR homozygotes (relative to Hardy-Weinberg equilibrium) than would occur if there were complete mixing between refuge and Bt fields, and this acts to increase the rate of resistance evolution.

Second, if females tend to remain in natal fields, then those females emerging from refuges will more likely stay within refuges, reducing mortality caused by Bt toxins and thereby reducing the force of selection for resistance (although also increasing insect population size in the refuges and leading to greater crop damage). Third, if males tend to remain in their natal fields, then heterozygous RS or resistant RR males in Bt fields will have reduced mating success; the few males emerging in Bt fields will have to compete with the males immigrating from the much larger populations in refuges. This effect of limited male movement can greatly reduce the rate of resistance evolution. These three factors make it difficult to draw simple conclusions about the importance of spatial structure and movement for resistance evolution, especially because they act through different mechanisms on females (mortality) vs. males (mating success) and may work in opposition to each other.

We show that the same types of complexities also confound simple conclusions about the consequences of spatial structure and adult movement for the case of pyramid Bt products. For this we used a spatially explicit model of resistance evolution in which male and female movement rates can be changed independently. We show that male and female movement have different effects on resistance evolution, and that these effects combine in nonadditive ways to give the net effect of movement. A key feature of rapid resistance evolution is the existence of "hotspots" of high resistance allele frequencies that appear when there is limited movement (Peck et al. 1999, Storer et al. 2003*b*); resistance then develops in local areas and spreads spatially, as opposed to developing uniformly throughout space as is the case when there is long-range movement and a well-mixed population.

We show all of these patterns using strategic examples rather than either providing formal mathematical proofs or extensive numerical simulations. This approach is in keeping with our goal of highlighting the general factors that need to be considered when considering resistance management strategies for pyramid Bt products targeting specific pests. Our goal is not to be comprehensive, but instead be illustrative of the complexities of managing resistance evolution.

Model

Our base model kept track of both allele frequencies and insect densities, and was similar to the single-locus models analyzed by Ives and Andow (2002). We first describe the base model and then the modifications for seed mixtures and larval movement among plants, and for explicit spatial structure and limited adult movement.

Base model

The base model assumes that adults have high movement rates among fields and therefore are effectively uniformly distributed among refuge and Bt fields in proportion to their areal extent; each generation a proportion Q of the adult population occurs in refuge and $1 - Q$ in Bt fields. Mating within fields is random, with females producing F offspring. Resistance to each of two Bt toxins is governed by diallelic, independently segregating loci, with R_1 and S_1 denoting resistant and susceptible alleles to Bt toxin 1, and R_2 and S_2 denoting the resistant and susceptible alleles to Bt toxin 2. Thus, there are nine genotypes of offspring whose frequencies within fields are at Hardy-Weinberg equilibrium. The survivals of offspring with genotypes R_1R_1, R_1S_1, and S_1S_1 from Bt toxin 1 are given by s_{1RR}, s_{1RS}, and s_{1SS}, and similarly s_{2RR}, s_{2RS}, and s_{2SS} give the survivals associated with Bt toxin 2. Survivals on plants containing both of the Bt toxins are assumed to be multiplicative, as is expected if toxins have independent modes of action (Raymond et al. 1989). For example, the survival of an $S_1S_1S_2S_2$ individual on Bt plants is $s_{1SS} \times s_{2SS}$, and the survival of an $R_1S_1R_2S_2$ individual is $s_{1RS} \times s_{2RS}$. For simplicity, throughout the analyses, we assumed $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, and $s_{1SS} = s_{2SS} = 0.01$; for these values, the dominance of both resistance alleles is $h = 0.05$. Except when we considered a cost of resistance, we assumed that survival of all genotypes on non-Bt plants is 1. When we incorporated a cost of resistance (Gould 2006), we considered two cases. In the first, we assumed both resistant homozygotes and heterozygotes experience a cost, with survivals of $w_{1RR} = w_{2RR} = 0.99$, $w_{1RS} = w_{2RS} = 0.99$, and $w_{1SS} = w_{2SS} = 1$ on non-Bt plants. In the second, we assumed only resistant homozygotes experience a cost, but they experience a large cost, with survivals of $w_{1RR} = w_{2RR} = 0.5$, $w_{1RS} = w_{2RS} = 1$, and $w_{1SS} = w_{2SS} = 1$.

Following any mortality caused by Bt, we assumed there is density-dependent survivorship given by $(1 + x)^{-1}$ where x is the density (all genotypes) of surviving larvae within a field. The specific form of this survival function makes little difference for any of our qualitative or quantitative conclusions. Because the model explicitly keeps track of the number of individuals of different genotypes, rather than just genotype frequencies, density-dependent survival changes the rate of resistance evolution. To investigate this effect, we also considered a frequency-only model that does not keep track of population densities, only gene frequencies. The frequency-only model is the same as the model including density dependence, except the term $(1+x)^{-1}$ is removed and genotype densities are converted to frequencies each generation. The frequency-only model is essentially identical to two-toxin models analyzed previously (Mani 1985, Gould 1986, 2006, Roush 1998). Note that in our formulation, density dependence occurs at the scale of individual fields. If density-dependent survival were caused solely by natural enemies, and if the natural enemies were globally dispersing, then density dependence could act at the global rather than local scale, giving results corresponding to the frequency-only model.

To run simulations, we assumed initial resistance allele frequencies were 0.001 for both resistance genes (Roush 1998). Failure of Bt crops (i.e., when the insect population is resistant) was assumed to have occurred when both resistance alleles exceed a frequency of 0.5. In some scenarios, in particular, when the proportion of refuges (or non-Bt plants in seed mixtures) was very small, insect densities can be very low when this criterion for Bt failure is reached. Nonetheless, once resistance allele frequencies reach 0.5, the resistant population recovers from low density very rapidly, so using this criterion to assess resistance failure gives similar results to those obtained by using a threshold density of insects.

Seed mixtures

For the case of seed mixtures, we assumed that all fields are the same and contain a fraction q of non-Bt plants and $(1 - q)$ of Bt plants, with females depositing eggs such that larvae initiate on non-Bt and Bt plants in proportion to their prevalence. We followed Mallet and Porter (1992) in assuming that larvae have two stages (a = young and b = old) and move between plants with probability μ between stages. When they move, they move to non-Bt or Bt plants with probabilities q and $(1 - q)$. For comparison among cases with different larval movement probabilities μ, we assumed that survival on Bt plants of susceptible genotypes in different larval stages, s^a and s^b, is divided equally between stages. Thus, the total survival of a susceptible larva to Bt toxin 1 that remains on a Bt plant is $s_{1SS} = s^a_{1SS} \times s^b_{1SS}$, and the total survival of a heterozygous larva to Bt toxin 2 that remains on a Bt plant is $s_{2RS} = s^a_{2RS} \times s^b_{2RS}$. Similarly, the total survival of a heterozygous larva to Bt toxin 2 that moves from a Bt to a non-Bt plant is $s_{2RS} = s^a_{2RS} \times w^b_{2RS}$. These survivals to the two toxins are then combined as before to give the total survival from both toxins; thus, the total survival of a $S_1S_1R_2S_2$ larva that moves from a Bt to a non-Bt plant is $s^a_{1SS} \times w^b_{1SS} \times s^a_{2RS} \times w^b_{2RS}$. Finally, density-dependent mortality occurs at the scale of individual plants after mortality has been caused by Bt; thus, the survival of insects on a plant is given by $(1 + x_p)^{-1}$ where x_p is the density of second-stage larvae per non-Bt or Bt plant.

Spatial structure

The spatially structured model is built on a 50-by-50 grid of same-sized fields, with a proportion Q being refuge fields and a proportion $(1 - Q)$ being Bt fields. By assuming that the entire landscape is made up of refuge or Bt fields, we ignored the possibility of fields of other crops or non-crop habitat. Biologically, this is equivalent to assuming that, even though different types of habitats may be available on a real landscape, these habitats are permeable to dispersing adults who move through them as if they were not there. Refuges were distributed randomly on the grid, and crop rotation was included by randomly rearranging refuges on the grid. We considered the extreme cases in which fields are either rotated every insect generation or never rotated; simulations with rotation every two or three insect generations produced intermediate results (not presented).

We assumed that when males disperse from their natal fields, they do so before mating, whereas females disperse from natal fields following mating. We assumed that the probability of dispersing a linear (Euclidean) distance x from natal fields is proportional to d^x, so dispersal drops off exponentially with distance; the fraction of adults remaining in their natal field is proportional to $d^0 = 1$. In the simulations we used values of $d = 0.2$, 0.4, and 0.9, and had a maximum dispersal distance of 12 fields; these correspond to mean dispersal distances of 1.0, 2.1, and 6.0. At $d = 0.9$, the results from the spatially explicit model are almost identical to the base model that assumes complete spatial mixing of adults. Finally, we gave the grid of fields "wrap-around" boundaries (i.e., we placed the grid on a torus), so that insects dispersing off one side of the grid appear on the opposite side; this assumption makes the dynamics on the 50-by-50 grid better approximate the dynamics expected for a much larger grid while remaining computationally manageable. We compared simulations on the 50-by-50 grid to those on a 100-by-100 grid, and there was no effect of grid size on the conclusions (results not presented).

Within fields, we made the same assumptions as in the base model. There was random mating and no movement of larvae among plants. Following mortality from Bt toxins, survival depends on the density within each field according to the equation $(1 + x_{ij})^{-1}$, where x_{ij} is the density of larvae in the ijth field.

Results

We first considered the case of random dispersal of male and female adults from natal fields and asked how reduction in the proportion of refuge Q changes the rate of resistance evolution. Resistance evolution shows two distinct modes depending on the existence of density-dependent survival within fields. As the proportion of spatial refuge decreases from $Q = 0.1$, the time to failure of Bt crops initially decreases slowly (Fig. 1a). In contrast, the population size (measured at the start of simulations when resistance alleles have frequency $p = 0.001$) decreases rapidly (Fig. 1b). At the point at which the susceptible population would go extinct, the time to failure starts to decrease rapidly, indicating a change in the mode of resistance evolution. We show this pattern for three different values of female fecundity ($F = 50$, 100, and 200) that give different values of the proportion of refuges Q at which the susceptible population would go extinct. Even though the time to failure is much greater when $F = 50$ than when $F = 200$, as long as susceptible populations persist, when both populations would go extinct, the time to resistance evolution is the same for $F = 50$ and $F = 200$.

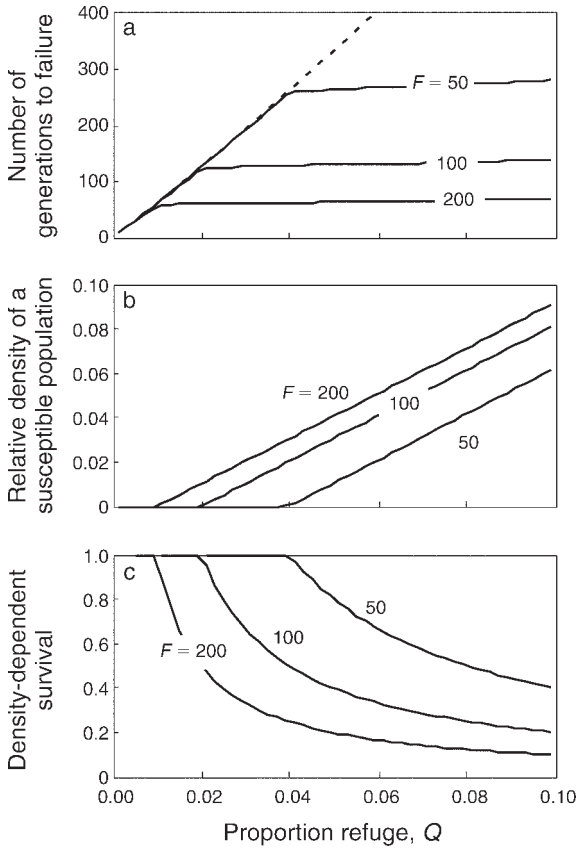

FIG. 1. Effect of persistence of a purely susceptible population on resistance evolution when adults disperse uniformly among Bt and refuge fields. (a) Generations to control failure (frequency of both resistance alleles > 0.5) as a function of the proportion of refuge Q for pest insects having fecundity $F = 50$, 100, and 200. The dashed line gives the case of a frequency-only model. (b) Corresponding densities of purely susceptible populations. (c) Density-dependent (post-selection) survival of larvae in refuges. The results are identical for the case of seed mixtures under the assumptions that larvae do not move among plants.

The change in mode of resistance evolution when the proportion refuge Q becomes too small to support a susceptible population can be explained by considering density-dependent larval survival. The rapid decreases in time to failure with decreasing Q when susceptible populations cannot persist matches the decrease in time to failure observed in the frequency-only model in which only allele frequencies (not also population densities) are modeled (Fig. 1a). When susceptible populations are going extinct, densities are very low, and therefore, there is little density-dependent mortality (Fig. 1c). In contrast, when Q is large and populations persist, density-dependent mortality decreases the survival of susceptible larvae in refuge fields. This decrease in survival of unselected insects increases the selection for resistance, because resistant insects in Bt fields have relatively higher fitness. This flattens the curves in Fig. 1a as Q increases. The decrease in density-dependent survival of susceptible individuals explains why the time to Bt failure is lower than in the case of the frequency-only model, and why the time to failure increases only slowly with increasing proportion of refuge Q. It also explains the shorter times to failure for populations with higher fecundity F (Fig. 1a); higher fecundity causes greater density-dependent mortality of susceptibles, which increases selection for resistance.

When there is a cost of resistance, resistance is slowed, but it still exhibits two modes of evolution (Fig. 2a). The switch point for the change in the model of resistance evolution occurs at the same value of Q regardless of the cost of resistance, because the cost of resistance does not affect the persistence of a susceptible population (Fig. 2b). As Q drops below this point, the time to resistance decreases rapidly, with the time to failure for a population with a cost of resistance quickly approaching that for a population with no cost of resistance. Thus, when the susceptible populations cannot persist, the value of a cost of resistance for slowing resistance evolution diminishes, and when refuges are very rare, a cost of resistance has little benefit.

How does the rate of resistance evolution to a pyramid Bt variety compare to resistance evolution to a single-gene variety? To address this, we considered four hypothetical cases describing resistance to a single-

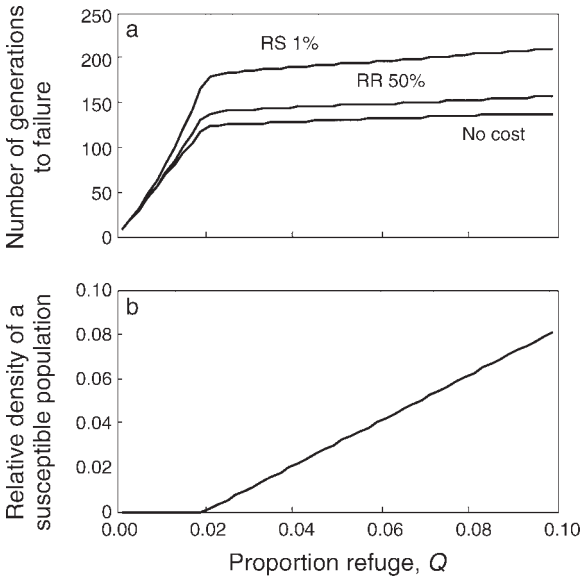

FIG. 2. For varying proportions of refuge fields Q, the effect of a cost of resistance on (a) generations to control failure (frequency of both resistance alleles > 0.5) and (b) the densities of purely susceptible populations. Costs of resistance were modeled either by assuming a 1% mortality of RR (resistant homozygote) and RS (resistant-susceptible heterozygote) individuals for either resistance trait (labeled RS 1%), or a 50% mortality of RR individuals for either trait (labeled RR 50%). We assumed survivals of RR resistant, RS heterozygote, and SS susceptible individuals for loci 1 and 2 are $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, and $s_{1SS} = s_{2SS} = 0.01$ (see *Base model* subsection for clarification).

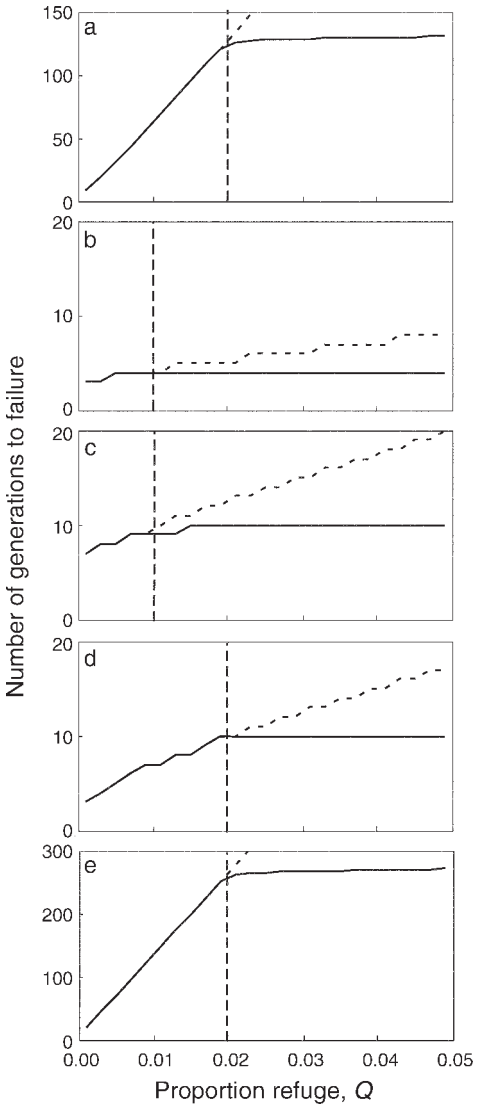

FIG. 3. Comparison among times to control failure for (a) a pyramid Bt crop variety expressing two Bt proteins; (b) a single Bt-protein variety in which the insect resistance gene is identical to one of the resistance genes in the pyramid variety and has the same initial frequency ($p = 0.001$); (c) the same resistance gene as in panel (b) but having an initial frequency of $p = 10^{-6}$; (d) the same as in panel (c) except the survival of the SS susceptible insects is $s_{SS} = 10^{-4}$ to conform to the survival of purely susceptible insects in the case of a pyramid crop variety, with the level of dominance of heterozygote expression $h = 0.05$; and (e) the same as in panel (d) except $h = 0.0005$ so that the RS heterozygote has the same survival as the $R_1S_1S_2S_2$ and $S_1S_1R_2S_2$ heterozygotes in the case of a pyramid Bt variety. In each panel the vertical dashed line gives the value of Q below which a purely susceptible population goes extinct, and the diagonal dashed line gives the results of the frequency-only model. For the baseline pyramid case [panel (a)], we assumed $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, $s_{1SS} = s_{2SS} = 0.01$, and $F = 50$.

gene variety; all assume that there is a diallelic, single-locus resistance gene, but they differ in susceptibility of the SS homozygotes, the initial resistance allele frequency, and the degree of dominance of expression in the RS heterozygotes. The first single-gene case is identical to either one of the two resistance genes we have considered for the case of a pyramid Bt variety, with $s_{RR} = 1$, $s_{RS} = 0.0595$ and $s_{SS} = 0.01$, and has an initial frequency of $p = 0.001$. Thus, the evolution of a resistance gene is identical to the evolution of either of the resistance genes to the pyramid Bt variety if the other resistance gene were already fixed for R resistance. Comparing the one-gene and two-gene cases (compare Fig. 3b to 3a) shows that resistance requiring two genes is much slower. This repeats the well-known result (Mani 1985, Gould 1986, 1991, 2006, Roush 1998, Caprio 2001, Zhao et al. 2003, Gahan et al. 2005) that the benefits of a pyramid Bt product will be overridden if there has been prior resistance development for one of the two genes in the pyramid, as demonstrated empirically by Zhao et al. (2005).

The second single-gene case has the same values of s_{RR}, s_{RS}, and s_{SS} as either of the resistance genes to the pyramid Bt variety, but has an initial frequency of $p = 10^{-6}$; thus, the initial frequency of RR resistant individuals in the population is equal to the initial frequency of $R_1R_1R_2R_2$ double homozygotes ($p_1p_2 = 10^{-6}$) in the case of the pyramid variety. Reducing the initial frequency of the resistance allele causes only a small increase in the time to Bt failure (compare Fig. 3c to 3b), and is still much shorter than for the two-gene case (compare Fig. 3c to 3a). From this we conclude that the performance of the pyramid variety is not due simply to the initial rarity of resistant $R_1R_1R_2R_2$ insects.

The third single-gene case also starts at an initial frequency of $p = 10^{-6}$, but the survival of the SS susceptibles is $s_{SS} = 10^{-4}$, the same as the survival of $S_1S_1S_2S_2$ double homozygotes for the two-gene case of the pyramid Bt variety; for this case, we assumed that the dominance of expression of resistance in the RS heterozygote was $h = 0.05$, the same as either of the resistance alleles in the case of the pyramid variety. Decreasing the survival of the SS susceptibles had almost no effect on the time to Bt failure (compare Fig. 3d to 3c). Therefore, the performance of the pyramid variety was not due to the very low survival it caused for $S_1S_1S_2S_2$ insects.

The fourth single-gene case was identical to the third except we assumed that the dominance of expression of resistance in the RS heterozygote was $h = 0.0005$; this made the survival of RS heterozygotes equal to that of $R_1S_1S_2S_2$ and $S_1S_1R_2S_2$ single heterozygotes in the case of the pyramid variety. For this single-gene case, resistance evolution was slower than the two-gene evolution of resistance to the pyramid Bt variety (compare Fig. 3e to 3a). This suggests that the performance of the pyramid Bt variety is due in large part to the lower survival of heterozygous insects, as found for frequency-only models (e.g., Mani 1985).

This conclusion can be verified by calculating the asymptotic rate of resistance evolution when resistance

alleles are rare. For the simple but informative situation in which both resistance genes confer the same survivals (i.e., $s_{1RR} = s_{2RR} = s_{RR}$, $s_{1RS} = s_{2RS} = s_{RS}$, and $s_{1SS} = s_{2SS} = s_{SS}$), under the assumptions that $s_{SS} < 1$ and $h > 0$ the asymptotic rate of resistance evolution is

$$\begin{cases} \dfrac{(1-Q)hs_{SS}}{Q} & \text{if } Q \le \dfrac{2}{F} \\ F(1-Q)hs_{SS} & \text{if } Q > \dfrac{2}{F}. \end{cases} \quad (1)$$

These expressions are the same as those for the case of a single resistance allele when setting $s_{SS} = 1$ (Ives and Andow 2002). Thus, the two-gene case has an asymptotic rate of evolution equal to its single-gene counterpart in which dominance is reduced from h to hs_{SS}. This implies that pyramiding two Bt toxins in the same crop variety slows evolution mainly by reducing the survival of individuals heterozygous for one or the other resistance allele. This approximation only holds when resistance allele frequencies are low for the case of high dose ($s_{SS} < 1$) and incomplete recessiveness ($h > 0$); when these assumptions do not hold, other differences between single-gene and pyramid Bt varieties arise. Nonetheless, the equivalence of these asymptotic rates of resistance evolution underscores the similarity between one-gene and pyramid Bt varieties. Evolution of resistance to a pyramid Bt variety does not greatly differ from evolution of resistance to a single-gene variety that causes high mortality of susceptible insects and for which a resistance allele has very low expression in heterozygotes.

Seed mixtures

We modeled seed mixtures by assuming adult insects disperse uniformly among fields that contain a fraction q of non-Bt plants. Larvae initiate feeding on non-Bt and Bt plants in proportion to their presence in the fields, and then with probability μ move to a different plant that with probability q is a non-Bt plant. For the special case in which larvae always remain on their initial plant ($\mu = 0$), the model is identical to the two-locus base model. This provided a starting point for comparison; the seed mixture model with no larval movement gave exactly the same times to Bt failure as the base model with spatial refuges and complete mixing of adults among fields (Figs. 1–3).

When all fields are seed mixtures, introducing larval movement has two effects (Fig. 4). First, if susceptible larvae move from their initial plants, they have a greater probability of being killed by Bt, which lowers the overall density of larvae (Fig. 4b). This occurs because the probability that larvae encounter at least one Bt plant is $q[1 + \mu(1 - q)]$, which is an increasing function of μ. Because increasing μ increases exposure to, and hence, mortality from Bt plants, it will increase selection for resistance that, by itself, would decrease the time to Bt failure. Second, larval movement increased the dominance of resistance expressed over larval development (Fig. 4c), which also decreased the time to Bt failure (Mallet and Porter 1992, Peck et al. 1999, Davis and Onstad 2000). These two effects therefore combine to increase the rate of resistance evolution due to larval movement. Furthermore, the effect of larval movement on dominance increases with increasing proportion of non-Bt plants q (Fig. 4c). This causes the time to failure to decrease with increasing q, provided q is large enough that a purely susceptible population can persist. Thus, larval movement can give the unintuitive result that increasing the proportion of non-Bt plants actually speeds resistance evolution.

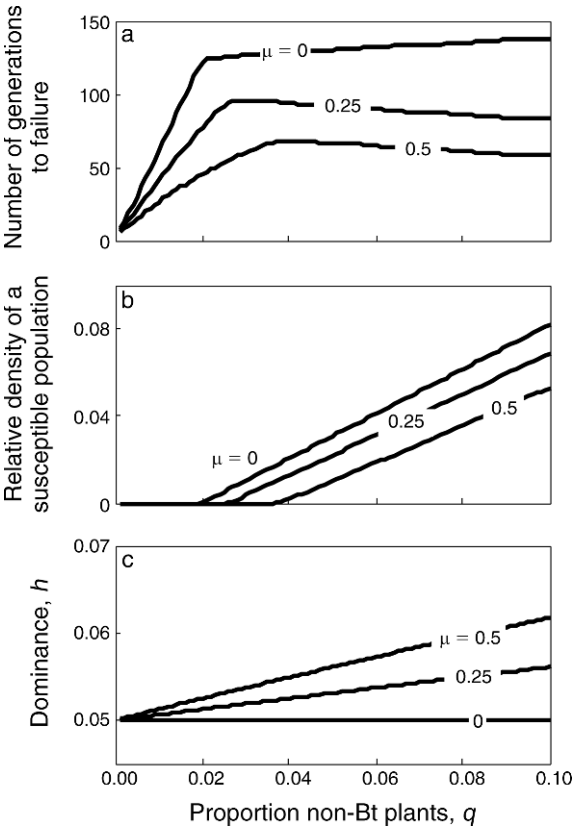

FIG. 4. Effect of larval movement within mixed-plant fields on (a) generations to control failure (frequency of both resistance alleles > 0.5), (b) the densities of purely susceptible populations, and (c) the effective dominance in expression of each resistance trait, h. The parameter μ gives the proportion of larvae leaving the plant upon which they were initially oviposited. Adults are assumed to disperse randomly among fields. We assumed $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, $s_{1SS} = s_{2SS} = 0.01$, and $F = 50$.

Spatial structure

To investigate the explicit spatial arrangement of refuge and Bt fields, we constructed a model with a 50-by-50 grid of fields. We first considered the case in which fields are rotated every insect generation. This removes the possibility of populations building up in

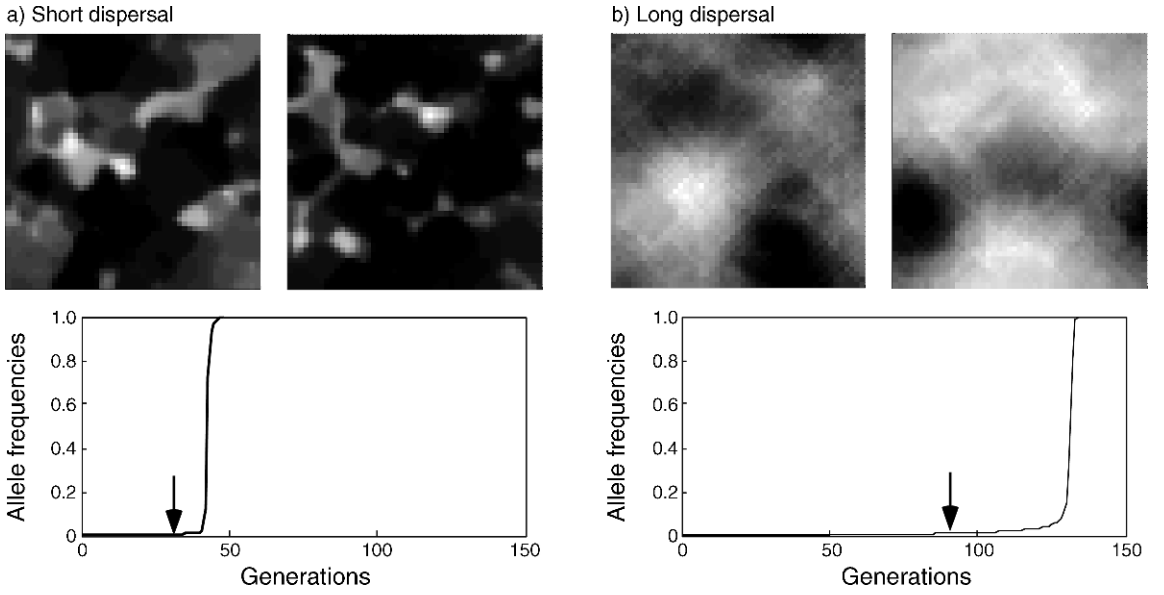

FIG. 5. Spatial distribution of resistance allele R_1 and time course of resistance evolution when male and female adults show (a) short-range ($d = 0.2$) and (b) long-range ($d = 0.9$) dispersal from their natal fields. Refuges are assumed to make up 10% of fields and are rotated each insect generation. Spatial distributions are shown for two consecutive generations once allele frequencies reach 0.01 [generations 36 and 89 for panels (a) and (b), respectively, marked by arrows]. We assumed $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, $s_{1SS} = s_{2SS} = 0.01$, and $F = 50$.

refuges when females remain in their natal fields, because refuges are pulled out from under sedentary populations. For the case of complete field rotation, limited dispersal distances of males and females nonetheless create local but transient "hotspots" of high-resistance allele frequency (Fig. 5a) similar to the results of models for single Bt crop varieties (Peck et al. 1999, Storer et al. 2003*b*). The hotspots are shared by resistance alleles of both resistance genes, so in Fig. 5 we have only displayed the frequency of one of them. Due to limited dispersal, these hotspots tend to remain in the same location for two to three generations, allowing the local build-up of resistance alleles. In contrast, for longer-range dispersal (Fig. 5b) there are no hotspots, and resistance allele frequencies change spatially from one generation to the next, with the spatial distribution of frequencies determined by the proportion of Bt fields in the region (defined roughly as the maximum distance moved by dispersing adults in a generation).

The effects of male and female dispersal distance on the rate of resistance evolution differ (Fig. 6). When females having unlimited dispersal and Q is above the threshold of 0.02 that allows a purely susceptible population to persist, limited male dispersal (smaller d) generally increases the time to Bt failure (Fig. 6b). Thus, limited male dispersal distance slows resistance evolution. This is caused by two effects of limited male dispersal distance: it decreases the proportion of heterozygotes in the population (thereby speeding resistance), and it reduces the mating success of males carrying the resistance alleles (thereby slowing resistance). Of these effects, the one on male mating success is the more important, leading to slower resistance evolution as male dispersal decreases.

When males have unlimited dispersal, limited female dispersal distance changes the distribution of larvae among fields but does not produce assortative mating (Fig. 6c). Therefore, the decrease in the time to resistance caused by limited female dispersal (smaller d) is caused by an increase in the strength of selection for resistance. This is also seen in the value of Q at which a purely susceptible population would go extinct and the mode of evolution switches; this occurs at about $Q = 0.04$ for $d = 0.2$ and $Q = 0.02$ for $d = 0.9$, which indicates that more refuge is required to sustain a population with short female dispersal distances because mortality of susceptibles is higher. Both of these patterns are caused by the rotation of fields when there is a small proportion of refuge in the landscape. If there is limited female dispersal, when their natal field rotates from refuge to Bt, they cannot disperse in high numbers into refuge fields, leading to increased mortality of stranded susceptible females.

When both males and females have limited dispersal distance (Fig. 6a), moderate dispersal distances ($d = 0.4$) slow, while short dispersal distances ($d = 0.2$) speed, the rate of resistance evolution relative to the case of long dispersal ($d = 0.9$); a similar result that intermediate dispersal distance leads to slowest resistance evolution was found for the one-locus model of Caprio (2001). The complicated consequences of limited dispersal distance of both males and females is not surprising, given that limited male dispersal generally slows resistance while

limited female dispersal speeds resistance. The combined effect of limited dispersal of both sexes depends on the interaction between these two effects.

These results have been for the case of complete field rotation every insect generation. To address the opposite extreme, we considered the case of no rotation, so refuges remained in the same place after they were initially randomly assigned locations on the 50-by-50 grid (Fig. 7). We also considered two different types of dispersal to separate the effects of insects remaining in their natal field from the effects of the distance they disperse once they leave their natal field. The first type of dispersal (Fig. 7a–c) is the same as previously considered, with the probability of an insect dispersing to fields proportional to d^x where x is the distance of a field from the natal field. In this case, if insects do not disperse far from their natal fields, there is also a greater chance that they remain in the natal field. In the second type of dispersal (Fig. 7d–f), all insects leave their natal field and then disperse different distances; they cannot return to their natal field. While the second type of dispersal is not realistic, comparing it to the first type of dispersal reveals the effect of remaining in the natal field by separating out the effect of dispersal distance once the natal field is left.

When insects can remain in their natal fields, limited dispersal distance of both sexes greatly slows resistance (Fig. 7a); this was similarly found by Sisterson (2005*a*) for a one-locus model. This appears to be due mainly to the effect of limited dispersal of females (Fig. 7c); limited male dispersal distance has a much smaller and more complicated effect (Fig. 7b). The situation is very different when insects are forced to leave their natal fields. Resistance evolution occurs more rapidly, and limited dispersal distance speeds rather than slows resistance evolution (Fig. 7d). Furthermore, the rate of resistance evolution when both sexes show the same dispersal distances appears to be driven more by the effects of limited male dispersal distance (Fig. 7e) than female dispersal distance (Fig. 7f). Thus, the importance of remaining in natal fields is illustrated by the striking contrasts between the two types of dispersal in which insects are allowed to stay in their natal fields (Fig. 7a–c) or forced to leave (Fig. 7d–f).

Most of these differences are a consequence of populations building up in refuges when adults are allowed to remain in their natal fields. Allowing females to stay in their natal fields reduces the number of larvae that are exposed to Bt fields and therefore experience selection for resistance. The effect of distance dispersed once individuals leave their natal field is the reverse, with shorter dispersal distances increasing the rate of resistance evolution. When females or males disperse only short distances, hotspots of high-resistance allele frequencies are more likely to occur, resulting in faster resistance (Fig. 7d–f). Thus, for the case of spatially fixed refuges, dispersal needs to be divided into two different components: Limited dispersal from natal fields slows resistance, while limited dispersal distance once the natal field is left speeds resistance.

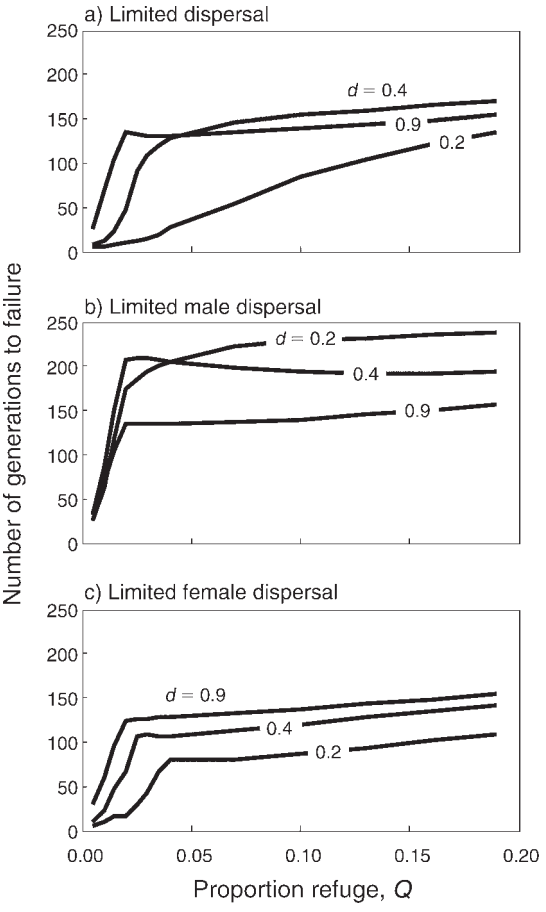

FIG. 6. Effect of limited dispersal of (a) males and females, (b) males only, and (c) females only on generations to control failure. Each line represents the average of five stochastic replicate runs of the model on a 50-by-50 grid of fields. We assumed that fields are rotated randomly between Bt and non-Bt varieties each insect generation, and $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, $s_{1SS} = s_{2SS} = 0.01$, and $F = 50$.

Overall, the spatial model gives a complex picture of the factors affecting resistance evolution. There are large differences between the cases of refuge rotation vs. no rotation, between male vs. female dispersal, and between dispersal from natal field vs. dispersal distance once the natal field is left. Although we only considered the extremes of rotation every insect generation and no rotation, the problem becomes more complicated with intermediate levels of rotation (e.g., every three insect generations) that give results intermediate between the two extremes. As a simplifying assumption, in our model we assumed that males disperse before mating, while females disperse after mating. This means that the effects of male dispersal are to change the distribution of heterozygotes in the population (assortative mating) and change the frequencies of resistance alleles through variation in male mating success, while the effect of

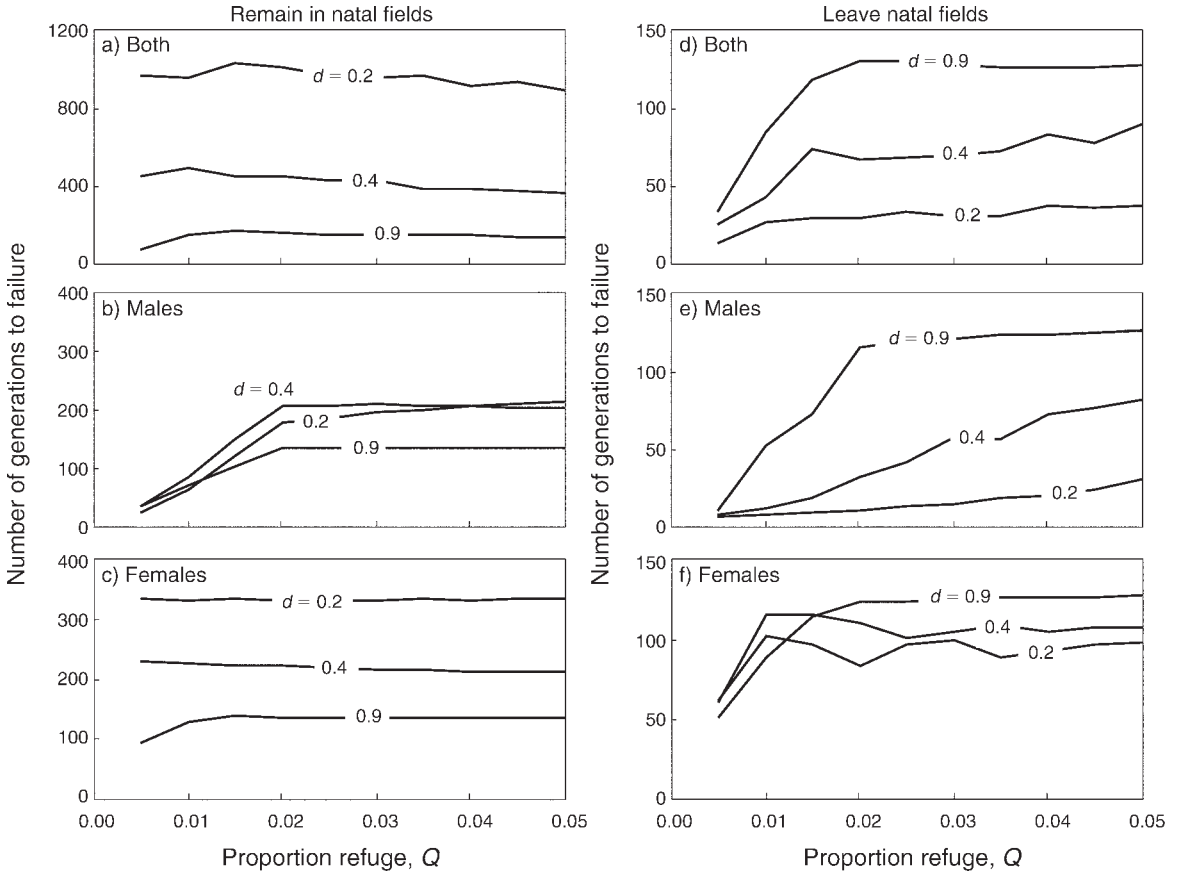

FIG. 7. For the case of no refuge rotation, the effect of limited dispersal of (a, d) males and females, (b, e) males only, and (c, f) females only on generations to control failure. For panels (a–c), limited dispersal has two effects: it reduces the fraction of individuals leaving their natal fields, and it reduces the distance traveled by those that leave. For panels (d–f), all individuals leave their natal fields. Each line represents the average of five stochastic replicate runs of the model. We assumed $s_{1RR} = s_{2RR} = 1$, $s_{1RS} = s_{2RS} = 0.0595$, $s_{1SS} = s_{2SS} = 0.01$, and $F = 50$.

female dispersal is to change the distribution of larvae among fields and hence change the proportion of the population under selection (in Bt fields).

Even though the effects of dispersal on resistance evolution are complex, they can nonetheless be intuited by considering the three processes affecting resistance evolution: selection, assortative mating, and male mating success (Ives and Andow 2002). Furthermore, in our simulations, global assortative mating is the least important of the three. This finding is striking, because random mating (the necessity for susceptible males to leave refuges, mate with resistance females in Bt fields, and thereby produce heterozygotes that are killed by high-dose expression of Bt) is generally viewed as the main process underlying the efficacy of the high dose–refuge strategy of resistance evolution management.

DISCUSSION

To explore the factors that may affect the speed of resistance evolution to pyramid Bt crop varieties, we used a suite of related, general models. Our goal has not been to give detailed predictions or recommendations for resistance management of any one crop or pest, but instead to give illustrative examples of factors that should be considered routinely when designing specific resistance management programs. One of the themes repeated in our examples is that resistance evolution to pyramid Bt varieties is not very different from resistance evolution to varieties that express only a single Bt protein. The main (though not sole) effect of pyramiding Bt proteins is to increase the mortality of heterozygotes in the insect population; heterozygotes in either of the resistance genes, but not both, are strongly selected against, and this removes resistance alleles from the population. A highly toxic, single Bt protein variety could have the same or better performance than a pyramid variety in terms of resistance evolution if it caused expression of resistance in heterozygotes to be highly recessive. Thus, pyramid products should not be considered as silver bullets that abrogate the need for resistance management, and a pyramid product should not by itself enable new approaches to resistance management.

For all of the models we considered, two modes of evolution of resistance were found depending on the ability of a purely susceptible population to persist. When a susceptible population cannot persist, resistance evolution is well described by a frequency-only model in which the time to resistance decreases rapidly as the amount of refuge (Q) or non-Bt plants in seed mixtures (q) decreases. When susceptible populations can persist, the individuals on non-Bt plants experience density-dependent survival; by decreasing the survival on non-Bt plants, this increases the relative survival of resistant individuals on Bt plants, thereby increasing the selective differential and speeding resistance evolution. This contrast between frequency-only and density-dependent models is based on the assumption that density dependence operates at the scale of individual plants or fields, and not at the regional scale. If higher regional densities lead to uniform decreases in survival in all fields (Bt and refuge), as might occur if density-dependent survival were determined solely by a regionally dispersing natural enemy that caused spatially uniform mortality, then a frequency-only model would be appropriate. Nonetheless, we suspect that density dependence operating only at the regional scale is unlikely, so the density-dependent model is more likely appropriate for resistance management.

In our density-dependent models, the switch between the two modes of resistance evolution is typically abrupt, with resistance evolution occurring much more rapidly once populations reach a threshold below which susceptible populations cannot persist. This leads to a clear caution for resistance management. If the population of a target pest drops to very low densities, then evolution of resistance might become much more rapid. Furthermore, at these very low levels, factors that are generally thought to slow resistance evolution, such as a cost of resistance, may be greatly weakened.

One of our main conclusions is that management strategies must be designed to allow the persistence of a population of susceptible insects. While a Bt crop might be seen as extremely successful if it greatly reduces pest densities (Carriere et al. 2003), it is exactly when the pest is disappearing that the risk of resistance is greatest. This theoretical result leads to a very simple adaptive management strategy for resistance management. Pest populations should be monitored in both Bt and refuge fields, or on both Bt and non-Bt plants in seed mixtures, and the proportion of non-Bt plants in the landscape increased immediately if pest populations become small, regardless of whether resistance is detected in the population. This adaptive management strategy is less risky than any a priori guess about the proportion of refuge that should be required for resistance management. Also, it provides a valuable complement to direct monitoring of the frequency of resistance alleles in a population (Tabashnik et al. 2005, Stodola et al. 2006, Downes et al. 2007, Liu et al. 2008, Xu et al. 2009). While resistance allele frequency monitoring can provide essential information to track the emergence of resistance once resistance allele frequencies exceed 10^{-3} (Andow and Alstad 1998), at this point rapid changes in management strategies would be needed to substantially prolong the use of a Bt crop (Andow and Ives 2002). In contrast, managing the high dose–refuge strategy to sustain a threshold abundance of pests in the system provides a way to maintain the essential feature of the high dose–refuge strategy: that a susceptible population can persist. Our theoretical analyses show that many factors will affect the rate of resistance evolution, and it will be hard to devise a priori requirements that are appropriate across the geographical range of any given pest species.

Deciding whether to deploy non-Bt plants in either seed mixtures or in spatial refuges depends on a set of advantages and disadvantages for either approach. Setting aside issues of the ease of management from the farmers' perspective, for single-Bt crops, seed mixtures can have the well-known disadvantage of speeding resistance evolution if larvae move among plants (Mallet and Porter 1992, Peck et al. 1999, Davis and Onstad 2000). Larval movement has the effect of increasing the dominance of expression of resistance in heterozygotes, thereby increasing the rate of resistance evolution. We showed the same results for pyramid Bt crops.

The effects of spatial structure and limited dispersal (the proportion of insects leaving natal fields and the distance they disperse if they leave) on resistance evolution were complex in our models. The rate of evolution depends on the frequency of refuge rotation, male vs. female limitations on dispersal, the proportion of individuals leaving natal fields, and the distance they disperse once they leave. Although the patterns of resistance evolution are complex, they can nonetheless be understood by considering the three processes that underlie resistance evolution: the strength of selection for resistance (the proportion of the population killed by Bt), assortative mating (departures of the proportion of heterozygotes from Hardy-Weinberg equilibrium in the entire population), and male mating success (when males emerging from Bt plants obtain fewer mates than those emerging from non-Bt plants) (Ives and Andow 2002). One of the interesting conclusions from the simulations is that the slowest resistance evolution occurs when insects (especially females) have low dispersal and refuges are planted in the same fields year after year. In this case, the population can be maintained in the refuge. While this increases crop damage in the refuge, it also ensures that resistance is greatly slowed for insects with low dispersal (Sisterson 2005*b*). A corollary of this result is that spatially large refuges may be better than small refuges (e.g., section of fields planted in a non-Bt variety) if this helps to maintain a susceptible population.

Should regulations require spatial refuges to be planted within close proximity of Bt fields to ensure

movement of insects from refuges into Bt fields? Given the complex effects of limited dispersal, it is impossible to answer this question with a simple yes or no. In our simulations, for insects that leave their natal field type (either by dispersing or by refuge rotation) and that have long-distance male dispersal, increasing the dispersal distance of females slows resistance evolution. This suggests that greater mixing among Bt and refuge fields is advantageous, and requiring closer proximity between Bt and refuge fields would be beneficial. Conversely, if females have long-distance dispersal, increasing the dispersal distance of males increased the rate of resistance evolution, suggesting that closer proximity between Bt and refuge fields would be a disadvantage. The differing results under different scenarios suggest that it is unlikely that regulations requiring close proximity of refuge and Bt fields will be necessary for all pest species, even those that show limited dispersal. The value of refuge proximity, or lack thereof, will likely depend on specific ecological conditions of the pests and crops under consideration.

All of our inferences are based on theoretical comparisons of different resistance management strategies; under a common set of assumptions about survival of different genotypes, initial resistance allele frequencies and so forth, we asked what strategy gives the lowest rate of resistance evolution. Furthermore, we intentionally selected parameter values that gave relatively long times to resistance (often more than 100 insect generations). While we recognize that it would be helpful for policy makers and seed companies to have specific predictions in terms of exactly when resistance is likely to occur under different management strategies, these predictions are impossible without information that is generally unknown, such as the initial frequency of resistance and the expression of resistance in heterozygotes (Andow 2001, Storer et al. 2003*a*). Nonetheless, comparing different strategies and identifying the factors that affect the rate of resistance evolution provide essential guidance for the development of any specific management plan. Models can be used to help design resistance management plans without expecting the models to predict exactly when resistance will occur.

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