

3 Managing resistance evolution: the case of Bt cotton

Worldwide, pests (including insects, weeds, and pathogens) destroy 37% of all potential food and fiber crops. Cotton, in particular, is one of the crops that can be decimated by insect pests. One famous example is the devastating effect of the boll weevil (*Anthonomus grandis*) in the American south during the early part of the 20th century. Prior to the arrival of that pest, cotton had been a driving force in the economy of the southern states. The boll weevil arrived in the US in 1892, and by 1920 it was present throughout the cotton growing regions. In many former cotton-growing areas yields were reduced over 50% and it was no longer economically viable to grow cotton.

Starting in the 1920s farmers began dusting cotton with powered calcium arsenate, which provided partial control of the boll weevil. Then in the 1950s synthetic pesticides (such as DDT) were introduced which were much more successful at controlling the cotton pests. However within a few years some populations of boll weevils were already resistant to DDT. Since that time many new classes of insecticides have been developed in the continual effort to maintain control of cotton pests. In 1995, an estimated 30 million pounds of pesticide was applied to cotton in the US in an effort to control insect damage. In that year two crops, corn and cotton, accounted for over 65% of the total insecticide use in the US.

Cotton, with pink bollworm
(http://ag.arizona.edu/pubs/general/esrpt2003/article12_2003.html)



3.1 For each new pesticide or herbicide, resistance mutants always appear and lead to spread of resistant pest populations.

In addition to the expense of purchasing and applying pesticides, and the potentially large costs in terms of human and environmental health, each pesticide has a limited useful lifespan before pests become resistant. Pesticides impose strong selection on the insect populations. Any resistant mutant that arises and is able to survive on the crop will enjoy an enormous fitness advantage. Over time, the alleles for resistance will spread through the population until eventually that insecticide will no longer be able to control the pest.

Starting with the development of DDT in the 1940s many classes of synthetic insecticides have been developed. In each case resistant populations of

Table 3.1. Major classes of insecticides and the time to the first reported failure due to resistant populations of insects. Data from Georgiou (1986).

Insecticide Class	Year Introduced	First Failure
Arsenicals	1880	1940
DDT	1945	1952
Dieldrin	1954	1957
Endrin	1957	1958
Carbaryl	1959	1963
Azinphosmethyl	1959	1964
Monocrotophos	1973	1973
Phosmet	1973	1973
Phorate	1973	1974
Disulfoton	1973	1974
Carbofuran	1974	1976
Oxamyl	1978	1978
Fenvalerate	1979	1981
Permethrin	1979	1981
Fenvalerate + piperonyl butoxide	1982	1983

insects have been observed (table 3.1). There are now approximately 500 species of insects that have developed resistance to one or more pesticides. Often it is possible to switch to another class of insecticide for which that population is not resistant, but that leads to a never-ending “arms race” to develop new classes of pesticide faster than the insects can evolve resistance.

In this chapter we will examine how we can use the principles of evolutionary biology to slow the rate of evolution of resistance

3.2 Bt cotton and the pink bollworm

In the latest attempt to battle cotton pests, farmers have increasingly adopted transgenic varieties of cotton that contain the gene for a natural insect toxin, Bt. Will this be another short-lived attempt at pest control?

The Bt toxin is a protein that comes from the soil bacterium *Bacillus thuringiensis*. The bacterium can be found throughout the world and there are thousands of different strains of *Bacillus thuringiensis* that have been identified. When insects ingest the Bt protein, the protein binds to specific receptors in the lining of the insect gut. It then causes the gut lining to rupture, killing the insect within days. The Bt protein must be ingested by the insect to be effective, so only plant-feeding insects are affected. Because the insect must have the correct receptor to match the particular type of endotoxin, each Bt strain is highly specific. For example, the toxin from the Cry1Ab gene kills only insects in the order Lepidoptera (moths and butterflies). Other strains of Bt are specific only to certain beetles or to flies.

Bt toxins have been used in powdered form for about 50 years. Because they are considered “natural”, Bt sprays are often used by organic farmers to control insect pests. In 1996 the Monsanto company introduced a variety of cotton (“Bollgard”) that was genetically engineered to express the Bt toxin gene Cry1Ac. They spliced the gene to a strong promoter that expresses the toxin in all parts of the plant. In the last decade Bollgard cotton and newer varieties have been widely planted by cotton growers, and Bt cotton now accounts for over 50% of the total cotton acreage in the US.

Given the widespread planting of Bt cotton, will pests evolve resistance? The diamondback moth (*Plutella xylostella*, a pest of cabbage and broccoli) has already evolved resistance to Bt sprays in some parts of North America. Resistance alleles have been identified in populations of several species of cotton pests, such as the tobacco budworm (*Heliothus virescens*) and pink bollworm (*Pectinophora gossypiella*). So far, however, the frequency of resistance in those pests of cotton has not spread.

One important pest of cotton is the Pink Bollworm. Originally from Asia, the pink bollworm is now one of the most devastating cotton pests, affecting cotton crops worldwide. It reached the southern US cotton belt in the 1920s and is now the major cotton pest in the southwestern US.

Most of the economic damage from the pink bollworm comes from the larvae that feed on the cotton bolls. The adult is a small, inconspicuous, brown moth. Over her 2-week lifespan, each female moth will lay about 200 eggs on the calyx of the cotton flowers or boll. When the

eggs hatch, the developing larvae burrow into the cotton bolls and feed on the seeds. Larval development lasts about 2 weeks. When the larvae are mature they exit the cotton boll and pupate in the surface of the soil. After about 7-9 days, the winged adult moths emerge to mate and start the life cycle anew. There can be 4-5 generations per year.

Two aspects of this life cycle are important for understanding the evolutionary dynamics of the system. First, the larvae are relatively sedentary, with many larvae spending their whole life on a single plant. Second, the winged adults disperse prior to mating, so colonists of new fields may come from several nearby areas. Dispersal distances are generally less than 400 m, however, so any mixing is only between adjacent fields.

3.3 Selection models can describe the spread of advantageous alleles.

In order to make rational decisions about whether, and how, to best grow these new varieties of cotton, we would like to be able to predict the rate of evolution in insect pests. Ideally we would also like to find strategy that would prevent, or at least delay, the evolution of resistance. Seed companies are obviously interested in protecting their investment in new technology; farmers are interested in ensuring that their methods of controlling insect pests remain effective.

Resistance to Bt toxin is governed by a single locus. We will label the resistance allele R and the susceptible allele S. To keep the notation the same as before (where p referred to the frequency of the dominant allele), we will label the frequency of the recessive R allele as q and the frequency of the allele S as p . The allele frequencies sum to 1.0 so $p = 1 - q$.

From the previous chapter, what is the equation to predict the change in allele frequency by natural selection?

$$p' = \underline{\hspace{2cm}}$$

Using the same logic as before, how would you modify that equation to predict the allele frequency of the other allele?

$$q' = \underline{\hspace{2cm}}$$

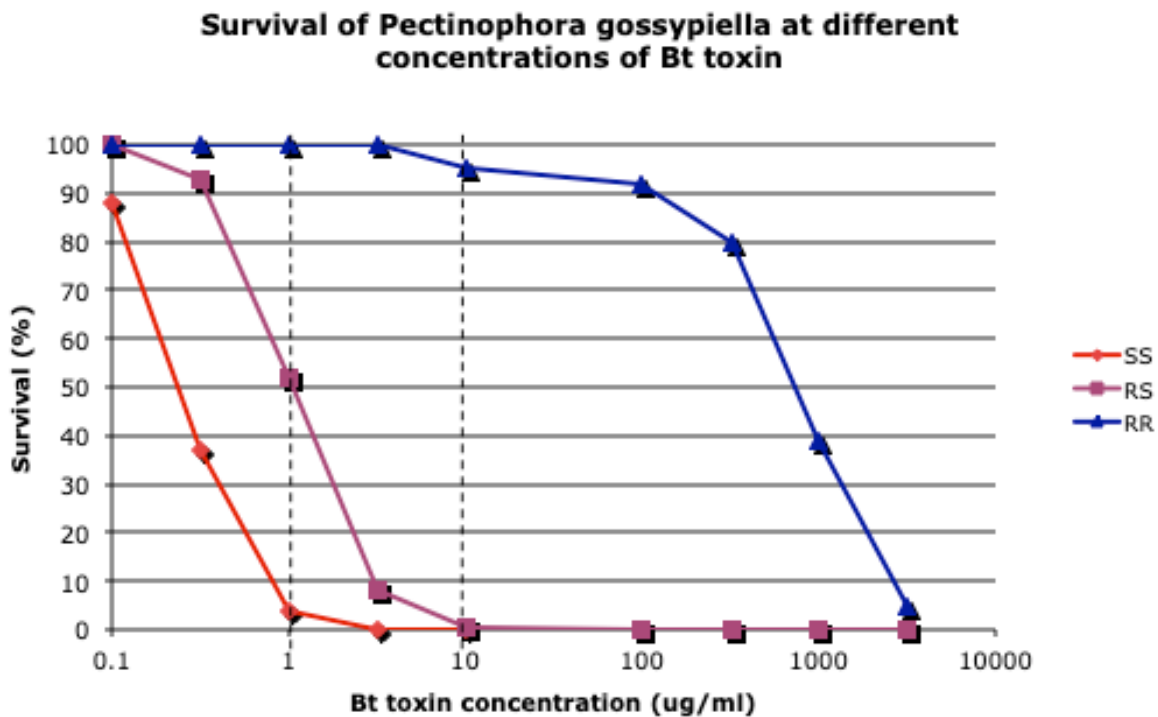
To predict changes in allele frequency, all we need to know are the fitnesses of susceptible and resistant genotypes, and the initial frequency of resistance alleles in the population. Given those few parameters we can iterate the selection equation to project the rate of increase (or loss) of a resistance allele and thus predict the ability of the pest to evolve resistance to Bt.

3.4 The fitness of resistance and susceptible bollworms depends on the environment.

As we saw in Chapter 6 the fitness of an organism can be measured experimentally as survival and fecundity. For understanding insecticides in particular, survival is the key parameter. The survival of pink bollworms depends on the interaction of their resistance phenotype (determined by the diploid genotypes) with a particular environment (presence or

absence of Bt toxin). For example, we might expect that the resistance allele confers no advantage in the absence of the Bt toxin, but it increases the fitness of insects when the toxin is present. The fitness of heterozygotes is harder to predict a priori. How can we determine the fitness of the three genotypes? Bruce Tabashnik and colleagues at the University of Arizona raised bollworms on artificial diets that contained various concentrations of Bt toxin. They then determined the survival of bollworms that were known to be homozygous susceptible (SS), heterozygous (RS), and homozygous resistant (RR) as a measure of their fitness on each diet. Their results are shown below:

Fig 3.1 Dose/mortality curves of three genotypes of pink bollworm reared on artificial diets with various concentrations of Bt toxin (Tabashnik et al 2002)



From figure 3.1 we can see that the survival of the three genotypes changes with different concentrations of the Bt toxin in the diet. Interpolating from that figure, the survival rates at 1, 3, and 10 ug/ml are as follows:

Table 3.2 Survival rates:

Bt concentration	SS	RS	RR
1 ug/ml	0.04	0.52	1.00
3 ug/ml	0.01	0.08	1.00
10 ug/ml	0.01	0.01	0.95

As before, we can use the survival of the bollworms as a measure of their fitness. What are the *relative fitnesses* of the three genotypes at 10 ug/ml?

How would you describe the dominance or recessiveness of the resistance allele at the three toxin concentrations? At 1 ug/ml, the fitness of the heterozygote is approximately intermediate between the two homozygotes. Therefore resistance allele effectively additive: having two copies of the R allele increases survival approximately twice as much as having only one copy of the R allele. However when tested at 10 ug/ml Bt toxin, neither the SS nor the RS genotypes can survive. Under those conditions, resistance is a recessive trait because only the recessive RR homozygotes can survive.

Looking again at Figure 3.1, is the R allele additive, dominant, or recessive at the very lowest concentration (0.1 ug/ml)?

The key point here is that “recessiveness” depends on both allele and environment. It is not an absolute characteristic of the gene. It is simply a description of the expression of that allele in heterozygotes compare to homozygotes in a particular environment.

3.5 How fast will resistance spread?

As a warm-up exercise, let's assume that the bollworm population starts with a moderate frequency of the resistance allele ($q = \text{freq}(\text{R allele}) = 0.1$) and that the effective dosage of Bt toxin is 1 ppm. Using eq 10.1 and fitnesses from Table 3.2, it is straightforward to predict the change in allele frequency:

$$q' = \frac{(0.1^2 \cdot 1.0) + (0.9 \cdot 0.1 \cdot 0.52)}{(0.9^2 \cdot 0.04 + 2 \cdot 0.9 \cdot 0.1 \cdot 0.52 + 0.1^2 \cdot 1)} = 0.417$$

In one generation the frequency of the R allele is predicted to increase over 4-fold, from 0.1 to 0.417, because of the large fitness difference between the resistant and susceptible genotypes.

- Now do the same using the set of fitness at the highest pesticide concentration (10 ug/ml). In that case the survival rates of the three genotypes are {0.01, 0.01, and 0.95}.

$q' =$ _____

For these conditions, stronger selection favoring the R allele led to a greater increase in allele frequency.

Now let's repeat that exercise using a range of initial allele frequencies, at two different Bt toxin concentrations:

Tables 3.3. Increase in the R allele frequency for three different starting frequencies of the R allele and two selection scenarios.

Low dose: 1 ug/ml survival={0.04, 0.52, 1.0}

	Frequency of the R allele		
Initial frequency (q)	0.001	0.01	0.10
Frequency after 1 generation (q')	0.012	0.106	0.417
Frequency after 2 generations (q'')			

High dose: 10 ug/ml survival={0.01, 0.01, 0.95}

Initial frequency	0.001	0.01	0.1
Frequency after 1 generation	0.001	0.019	0.536
Frequency after 2 generations			

Complete the last rows of the tables to predict the R allele frequency in the second generation.

There is an apparent paradox in the high-dose environment. Selection favoring the R allele is stronger, so if it is present in moderate frequency it will increase to fixation very rapidly. But, the R allele is also completely recessive. If the initial frequency is low, the frequency of homozygous RR genotypes is extremely low (q^2). Almost all of the copies of R are in heterozygous individuals. Because there is no difference in fitness between the SS and RS genotypes, selection favoring R is very weak. The only individuals that have increased fitness are the RR homozygotes and they are extremely rare. It may take a long time for the R allele to increase to a high enough frequency to produce an appreciable number of RR homozygotes.

This illustrates the general principle that *advantageous recessive alleles will increase extremely slowly when they are rare*.

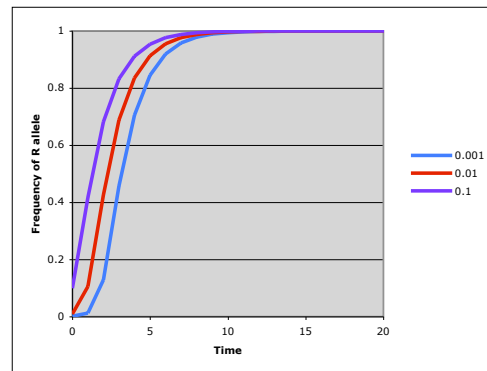
At low concentrations of Bt the selection pressure is weaker, but the resistance phenotype is approximately additive (the heterozygote has intermediate fitness). When the R allele is moderately common ($q=0.1$) it takes 6 generations to reach 95% frequency instead of only 2 generations at the high dose (Figure 3.2). When R is very rare ($q=0.001$) it still takes about the same time (7 generations).

3.6 Two approaches for controlling the evolution of resistance: High Dosage and Refuges

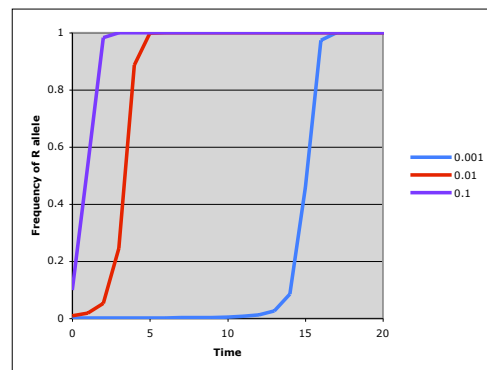
One lesson from our exercise above is that the frequency of a *rare* advantageous allele will increase extremely slowly if it is *completely recessive*. Field surveys have shown that resistance alleles are indeed rare in natural populations of the pink bollworm. We also saw that recessiveness depends on the dosage of Bt. Therefore, if the dosage of Bt toxin can be kept high enough that both RS and SS individuals are killed, we may be able to slow the increase in resistance. One of the breakthroughs in breeding Bt cotton was the ability to produce strains that expressed the toxin in high enough concentration to kill both SS and RS individuals. For example, the widely planted “Bollgard” cotton variety produces Bt toxin concentrations of 10-20 ug/g dry wt, which from figure 3.1 is sufficient to kill both RS and SS bollworms.

The second approach is to provide unselected refuges that provide a reservoir of susceptible individuals. Farmers do that by planting fields of non-Bt cotton (where susceptible bollworms can survive) alongside their Bt cotton crop. Refuges can have two effects on the rate of resistance evolution. First they reduce the difference in fitness between SS and RR individuals, so the rate of increase in R alleles will be slower. For example, imagine that there is a 10x difference in fitness of SS and RR individuals in the Bt crop ($W_{ss}=W_{rr}=1$), and they have equal fitness in the refuge ($W_{ss}=W_{rr}=1$). If there is a 50:50 mix of Bt crop and refuge, the net fitness of the two genotypes averaged across both environments will be 0.55 and 1.0. The inclusion of refuges reduces the fitness advantage of RR from 10x to less than 2x. Because the overall selective advantage of R is reduced, it will spread somewhat more slowly.

Fig. 7.2 a. Low dose conditions (1 ug/ml): R allele is additive. By six generations the resistance allele is nearly fixed, no matter what the starting allele frequency is.



b. High dose (10 ug/ml): R allele is completely recessive. If the allele starts out rare (0.001) there is a long delay before it increases in frequency. If it starts out common (0.1), it increases faster because selection is stronger.



However a much more powerful effect of the refuge occurs when the resistance allele is recessive. In that case the refuges provide a reservoir of susceptible individuals that help ensure that most R alleles are present in susceptible RS heterozygotes, rather than resistant RR homozygotes. As we saw above, when the R allele is rare, q^2 is extremely small so there will be very few RR insects that are able to survive in the Bt treated crops. Most of the insects will be killed by the Bt toxin so very few adults will emerge from the Bt crop. The pest density in refuge is expected to be much higher than in the Bt crop. Almost all of the insects that survive to reproduce will be SS individuals from the refuge. As long as the two populations are well mixed, any surviving RR individuals are likely to mate with the much more common SS individuals, producing offspring that are RS heterozygotes. Those offspring will be killed by the high dose of Bt. In principle the evolution of resistance can be delayed indefinitely if there is a sufficient refuge to keep the S allele in the population and ensure that most R alleles are found in the heterozygotes.

3.7 Apply this theory to real crops:

The promise of the high dose + refuge approach has led the US regulatory agencies to mandate that farmers who plant Bt cotton maintain a certain percentage of non-Bt cotton as a refuge to ensure population of susceptible insects. The required refuge percentage is currently set at 5% of the total area, if the refuges are embedded within Bt cotton fields. Will that high dose/refuge approach be effective in preventing the evolution of resistance in populations of the pink bollworm? That depends on if the resistance allele starts out sufficiently rare and on the amount of refuge that is needed.

What is the initial frequency of resistant insects? Researchers in Arizona monitored fields for the frequency of R alleles for several years following the introduction of Bt cotton. They collected pink bollworms from various fields and raised them in the lab on diets with 10 ppm Bt toxin. Only RR genotypes can survive on that concentration of Bt. From the frequency of survivors (q^2) they could then calculate the frequency of the R allele (q). They found some variation in frequency from year to year, but during seven years of monitoring the frequency of resistance in natural populations of bollworms averaged $q = 0.004$ (Tabashnik 2005).

For $q=0.004$, what is the expected frequency of RR homozygotes? _____

What fraction of buffer crop is needed to prevent the evolution of resistance for at least 20 generations? To keep our model simple, we will assume complete mixing of insects between the Bt crop and the refuge. In that case the net fitness of each genotype will simply be the average of its fitness in the two environments, weighted by the frequency of the crop and refuge. If r is the fraction of non-Bt refuge then $(1-r)$ is the fraction of Bt crop and the overall net fitness of each genotype will be:

$$w = r \cdot w_{\text{refuge}} + (1-r) \cdot w_{\text{Bt}}$$

Using the high dose survival values from Table 3.2 the fitnesses of the three genotypes on Bt cotton are $w_{Bt} = \{0.01, 0.01, \text{ and } 0.95\}$ for SS, RS, and RR genotypes. We'll assume that all three genotypes survive on non-Bt cotton, so the fitness of all three genotypes is 1.0 in the refuge.

For example, imagine that 20% of the cotton fields were non-Bt refuges and 80% contain Bt cotton. In that case the overall fitness of susceptible bollworms would be

$$w_{SS} = 0.20 \cdot 1.0 + (0.80) \cdot 0.01 = 0.208$$

Table 3.4

Proportion Refuge	Weighted Average Survival		
	SS	RS	RR
0	0.01	0.01	0.95
0.05	0.059	0.059	0.952
0.10			
0.20	0.208	0.208	0.960

- Fill in the third row of the table: Calculate the weighted average fitness when 10% of the fields are non-Bt refuges and 90% are Bt crops.

Now we are (finally!) ready to calculate the rate of evolution of resistance. Using the weighted average fitness and an initial allele frequency of $q=0.004$, and assuming that 10% of the fields are non-Bt refuges, what will be the frequency of R after one generation of selection?

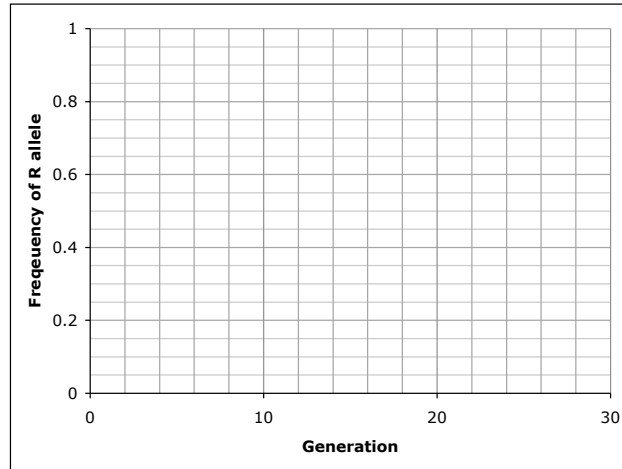
$$q' = \underline{\hspace{2cm}}$$

We can continue to project the changes in the frequency of the R allele over several generations of selection, as shown in the table below.

Table 3.5

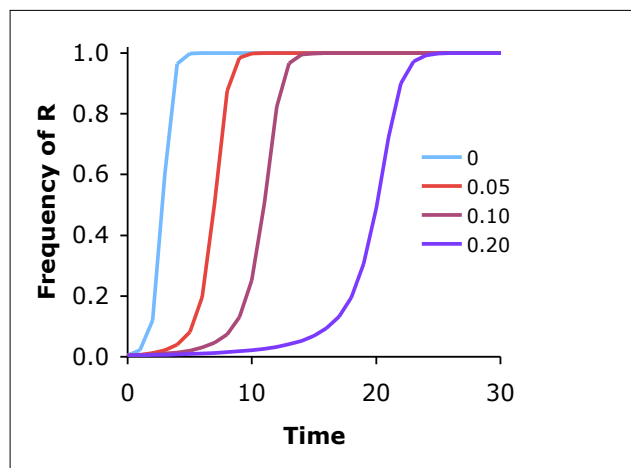
Refuge %	Generation									
	0	2	4	6	8	10	15	20	25	30
0	0.0040	0.0083	0.0343	0.6666	0.9999	1.0000	1.0000	1.0000	1.0000	1.0000
0.05	0.0040	0.0045	0.0052	0.0060	0.0072	0.0089	0.0202	0.2034	1.0000	1.0000
0.10	0.0040	0.0043	0.0045	0.0049	0.0053	0.0057	0.0072	0.0098	0.0149	0.0293
0.20	0.0040	0.0041	0.0042	0.0044	0.0045	0.0047	0.0051	0.0056	0.0062	0.0069

Sketch a graph of the predicted rate of resistance evolution for various percentages of refuges and crops as shown in Table 3.5. How effective are the refuges in preventing the evolution of resistance to Bt cotton?



Will refuges work if there is only a partial dose of Bt? Weak expression of the Bt gene in cotton plants may result in low concentrations of the Bt toxin in the leaf tissue. In that case, the resistance gene is only partially recessive. Imagine the fitness of bollworms on plants with low quantities of Bt toxin are 0.01, 0.05 and 1.0 for SS, RS, and RR genotypes. The RS heterozygotes now has a slightly higher fitness than the SS homozygote in the presence of Bt. All three genotypes have a fitness of 1.0 in the non-Bt refuge. As before we can calculate the weighted average fitness for various proportions of crop and refuge and graph the spread of the resistance allele (Figure 3.4).

Figure 3.4 Evolution of resistance with "low dose" Bt, for different proportions of crop and refuge. The four lines show refuge proportions of 0% to 20%.

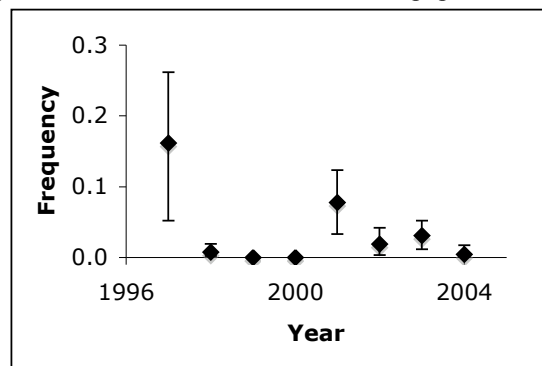


Are the refuges still effective in preventing the evolution of resistance?

3.8 Does the High Dose / Refuge strategy actually work?

The problem of resistance evolution was studied intensively before Bt cotton was released for commercial use. To implement the high dose / refuge strategy, a mandatory minimum refuge percentage of 5% non-Bt cotton was required for all farmers who used the transgenic Bt seed. That, coupled with the fact that many growers continued to plant non-Bt cotton, meant that the actual refuge percentage in Arizona ranged from 10% to 70% in different areas of the state. After 10 yrs of exposure to Bt crops, the frequency of resistance is still rare (Tabashnick 2005).

Figure 3.5 Frequency of the Bt resistance allele in Arizona populations of the Pink Bollworm.



3.9 Your turn:

Our model made various assumptions about the life cycle of the bollworms. For example, we assumed that the larvae remain on a single type of plant their whole life, and that there is complete mixing of insects between the non-Bt refuges and the Bt crops prior to mating. What will happen if those assumptions do not hold? For example it is probably important to consider the spatial arrangement of refuges and crops. Consider how the predictions about resistance evolution will change under these two scenarios:

1. Very fine scale intermixing of Bt and non-Bt plants, so larvae feed on multiple kinds of plants.
 - Imagine an insect where the larvae are mobile and feed on many different individual plants. What happens if there is very fine-scale intermixing of Bt and non-Bt crops (perhaps alternating rows or by planting a mixture of Bt and non-Bt seed)? In that case, individual larvae will feed on a random combination of Bt and non-Bt plants, effectively getting only a partial dose of Bt toxin. How will that affect the evolution of resistance? ¹
2. Very coarse scale separation of Bt crops from the refuges, so adults cannot intermix.
 - At the opposite extreme, how would resistance evolve if the Bt crops and refuges were widely separated, so insects do not move between refuge and field? In this case the adult insects would mate and lay eggs within the same field where they developed. How will resistance evolve in the buffer and in the crop?

For further reading:

Gould, F. 1998. Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. *Ann. Rev. Entomology* 43: 701-726

Rausher, M. 2001. Co-evolution and plant resistance to natural enemies. *Nature* 411:857-864

Tabashnik, B. E., T. J. Dennehy, and Y. Carrière. 2005. Delayed resistance to transgenic cotton in pink bollworm. *PNAS* 102;15389-15393.

¹ Pink bollworm larvae do not often move between plants so this scenario is unlikely to be important for pink bollworms. Other pests such as corn borers and potato beetles have much more mobile larvae, so fine-scale intermixing would not be a good strategy to control those pests.

Answers

p 3: $p' = \frac{p^2 w_{UU} + pq w_{US}}{\bar{w}}$, $q' = \frac{q^2 w_{RR} + pq w_{RS}}{\bar{w}}$

p 5: dominant; $q' = 0.53$, a 5x increase

p 6: low dose: 0.128, 0.427, 0.682; high dose: 0.001, 0.052, 0.983

p 8: 1.6×10^{-5}

p 9: 0.109, 0.109, 0.955; $q' = 0.0041$

p 10:

