Large-scale, spatially-explicit test of the refuge strategy for delaying insecticide resistance

Yves Carrière[a,1], Christa Ellers-Kirk[a], Kyle Hartfield[b], Guillaume Larocque[c], Ben Degain[a], Pierre Dutilleul[c], Timothy J. Dennehy[d], Stuart E. Marsh[b], David W. Crowder[a,e], Xianchun Li[a], Peter C. Ellsworth[a,f], Steven E. Naranjo[g], John C. Palumbo[a], Al Fournier[a,f], Larry Antilla[h], and Bruce E. Tabashnik[a]

[a]Department of Entomology, University of Arizona, Tucson, AZ 85721; [b]Arizona Remote Sensing Center, University of Arizona, Tucson, AZ 85719; [c]Department of Plant Science, McGill University, Macdonald Campus, Sainte-Anne-de-Bellevue, QC, Canada H9X 3V9; [d]Global Insecticide Resistance Management, Bayer Crop-Science Limited Partnership, Research Triangle Park, NC 27709; [e]Department of Entomology, FSHN 166, Washington State University, Pullman, WA 99164; [f]Arizona Pest Management Center, University of Arizona, Maricopa Agricultural Center, Maricopa, AZ 85138; [g]US Department of Agriculture-Agriculture Research Service, Arid-Land Agricultural Research Center, Maricopa, AZ 85138; and [h]Arizona Cotton Research and Protection Council, Phoenix, AZ 85040

The refuge strategy is used worldwide to delay the evolution of pest resistance to insecticides that are either sprayed or produced by transgenic *Bacillus thuringiensis* (Bt) crops. This strategy is based on the idea that refuges of host plants where pests are not exposed to an insecticide promote survival of susceptible pests. Despite widespread adoption of this approach, large-scale tests of the refuge strategy have been problematic. Here we tested the refuge strategy with 8 y of data on refuges and resistance to the insecticide pyriproxyfen in 84 populations of the sweetpotato whitefly (*Bemisia tabaci*) from cotton fields in central Arizona. We found that spatial variation in resistance to pyriproxyfen within each year was not affected by refuges of melons or alfalfa near cotton fields. However, resistance was negatively associated with the area of cotton refuges and positively associated with the area of cotton treated with pyriproxyfen. A statistical model based on the first 4 y of data, incorporating the spatial distribution of cotton treated and not treated with pyriproxyfen, adequately predicted the spatial variation in resistance observed in the last 4 y of the study, confirming that cotton refuges delayed resistance and treated cotton fields accelerated resistance. By providing a systematic assessment of the effectiveness of refuges and the scale of their effects, the spatially explicit approach applied here could be useful for testing and improving the refuge strategy in other crop–pest systems.

pesticide resistance | predictive evolutionary models | pest management | resistance management

Population growth will continue to favor agricultural intensification for decades. Because agricultural intensification is associated with increased pest pressure, pesticides generally help to increase yield (1–3). Although significant progress has been made to reduce reliance on pesticides (4, 5), an increasing number of insects and mites exhibit field-evolved resistance to synthetic pesticides, *Bacillus thuringiensis* (Bt) sprays, and transgenic Bt crops (6, 7). Negative consequences of resistance include increased pesticide use, disruption of food webs and ecosystem services, increased risk to human health, and loss of profits for farmers and industry (1, 3).

One of the main strategies for delaying resistance promotes survival of susceptible pests by providing refuges, which are areas of host plants where pests are not exposed to an insecticide. Theory predicts that refuges will slow the evolution of resistance by reducing the fitness advantage of resistant individuals (7–9). Refuges can also reduce the heritability of resistance when susceptible individuals mate with resistant individuals surviving exposure to an insecticide (7). Empirical support for the refuge strategy was provided by short-term laboratory and greenhouse experiments (10, 11). Although these experiments test the hypothesis that mating between susceptible and resistant individuals delays the evolution of resistance, they do not consider several factors that affect resistance in the field (7–9), and thus

only provide partial support for effectiveness of the refuge strategy in the field. Retrospective analyses of variation in resistance evolution in the field also suggest that refuges have been effective, but these previous tests have been based primarily on comparisons among species, or qualitative comparisons within species based on a limited number of widely separated geographic areas (12, 13). In such tests, factors that vary among species or geographic areas can confound the effects of refuges. Accordingly, large-scale field tests of the refuge strategy for a single species within a geographic area where factors affecting resistance are similar are needed to test the refuge strategy more rigorously. Moreover, tests of predictive refuge strategy models are required to determine if the refuge strategy can delay resistance (14). Furthermore, to improve our ability to develop efficient refuge strategies, empirical approaches are necessary to characterize effects of refuges on resistance evolution (7, 15).

Here we tested the refuge strategy using 8 y of data on refuges and resistance to the insecticide pyriproxyfen in 84 populations of the sweetpotato whitefly (*Bemisia tabaci*) sampled in cotton fields of central Arizona. We studied the B biotype of *B. tabaci*, also known as the Asia Minor-Middle East 1 species, which is a key pest of cotton and other crops in Arizona and worldwide (16). The insect growth regulators pyriproxyfen (a juvenile hormone analog) and buprofezin (a chitin synthesis inhibitor) are selective insecticides that have been used for whitefly control in Arizona cotton (*Gossypium* spp.) since 1996 (17, 18). A single application of either insecticide on cotton when *B. tabaci* populations start to increase has substantially reduced sprays of broad-spectrum insecticides, helped to conserve natural enemies, and restored farmers' profits (18, 19). To deter rapid evolution of resistance, farmers in Arizona generally have not used pyriproxyfen to control *B. tabaci* on crops other than cotton (19, 20).

Although *B. tabaci* is polyphagous, few whitefly crops other than cotton are available in central Arizona from June to September, when pyriproxyfen is sprayed on cotton. In principle, crops that could act as refuges include spring melons (*Citrullus lanatus* and *Cucumis melo*), alfalfa (*Medicago sativa*) and cotton not treated with pyriproxyfen (referred to hereafter as untreated cotton). *B. tabaci*

movement from alfalfa and untreated cotton to treated cotton can occur throughout the period when pyriproxyfen is applied and beyond. However, because spring melons are harvested in June and July, most movement from melons to treated cotton takes place during the first half of the period when pyriproxyfen is applied.

Laboratory and field experiments show dispersal dimorphism in *B. tabaci* populations. When suitable crops are present, most individuals move within fields or short distances from fields, whereas ~6% move 2–3 km from source fields (21–23). In simulation modeling that incorporated this dispersal dimorphism, refuges of cotton and other whitefly host plants delayed *B. tabaci* resistance, whereas pyriproxyfen-treated cotton accelerated the evolution of resistance (24).

The *B. tabaci* populations monitored here for pyriproxyfen resistance were sampled before mid-October, when cotton and alfalfa suitable for *B. tabaci* occur throughout the landscape. Thus, we expected that most immigrants in the sampled cotton fields originated from fields within 3 km. To test the hypothesis that the spatial distribution of refuges and treated cotton affects the evolution of resistance to pyriproxyfen, we used Geographic Information System (GIS) technology and remote sensing data to map four types of whitefly host plants within 3 km of each of the 84 field sites: treated cotton and three types of refuges (alfalfa, melons, and untreated cotton). We used survival of *B. tabaci* eggs at a discriminating concentration of pyriproxyfen (0.1 μg of pyriproxyfen per mL) as an index of resistance to pyriproxyfen for each of the 84 field populations (see *Methods*). We analyzed the first 4 y of data to determine the spatial scale at which the crops affected resistance and formulate a statistical model that best described the association between *B. tabaci* resistance to pyriproxyfen and abundance of the host plant types near each of 46 field sites sampled from 2002 to 2005. We applied the resulting statistical model to predict resistance to pyriproxyfen in 38 populations sampled from 2006 to 2009. The comparison of predicted versus observed resistance shows that the model accurately predicted resistance evolution.

Results

In the four counties of central Arizona where *B. tabaci* was sampled from 84 sites (Fig. 1), pyriproxyfen use varied extensively among counties and through time (Table S1). No cotton was treated with pyriproxyfen in Pima County, whereas the highest abundance of treated cotton was in La Paz. The area of pyriproxyfen-treated cotton declined from 2002 to 2009 in Maricopa and Pinal, but not in La Paz (Table S1). Near the sampling sites for *B. tabaci*, cotton and alfalfa were abundant, but melon was rare and mainly found in Maricopa (Table S1 and Fig. 1).

Survival at the discriminating concentration of pyriproxyfen was 0% from 1996 to 1998 (25). Average survival was 8.0% in 2002 and increased to 45.1% by 2009 (Fig. S1), showing that resistance to pyriproxyfen progressed during this study ($F_{7,76}$ = 3.36, P = 0.0036). In Maricopa and Pinal, where most sites were located, resistance increased progressively from 2002 to 2009 (Figs. S2 and S3) despite the temporal reduction in pyriproxyfen use across these years (Table S1). Within-county variation in resistance was extensive (Fig. 1 and Figs. S2 and S3), and resistance did not differ significantly between the two counties except in 2009 (Fig. S2). Reflecting the differential use of pyriproxyfen among counties, resistance tended to be low in Pima during the 3 y it was evaluated and high in La Paz in 2008 (Figs. S2 and S3).

We used the first 4 y of data to determine which host plant types were associated with resistance and to assess the spatial scale of their effects. The acreage of each of the four host plant types was measured in 12 concentric rings around each *B. tabaci* sampling site, with the radius of the rings ranging from 250 to 3,000 m (see *Methods*). To remove among-year variation in resistance to pyriproxyfen (Fig. S2) and in areas of each crop at the different spatial scales, we analyzed data with ANOVA and used standardized residuals in subsequent analyses (see *Methods*). In these analyses, the most resistant populations in a particular year have the largest positive residuals for resistance and the least resistant populations in that year have negative residuals for resistance. Likewise, the residuals for area of a crop type were

Fig. 1. Cotton fields sampled for *B. tabaci* in central Arizona between 2002 and 2009. (*Inset*) Adjusted percentage of egg survival at the discriminating concentration of pyriproxyfen (0.1 μg of pyriproxyfen per mL) in the 84 *B. tabaci* populations sampled from 2002 to 2009. The image shows the distribution of crops that are whitefly host plants (alfalfa, cotton, melon) and nonhost crops near six sampled sites in Pinal County in 2005. A ring with a radius of 3 km is traced around GPS points for sampled sites.

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most positive for sites surrounded by the highest area of that crop type in a given year and were negative for sites surrounded by the lowest area of that crop type in that year. In each subsequent analysis, we used rank-based statistics to test for the association between the residuals for resistance and the residuals for crop area, accounting for spatial autocorrelation when needed (see *Methods*). Thus, these analyses considered spatial variation in resistance within years rather than changes in resistance across years.

We first used stepwise regression to determine crops significantly associated with resistance. Areas of alfalfa and melon were not significantly associated with resistance to pyriproxyfen at any of the 12 spatial scales from 250 to 3,000 m (one-tailed tests, $P > 0.05$ for each crop). However, at several spatial scales, area of treated cotton was positively associated with resistance to pyriproxyfen and area of untreated cotton was negatively associated with resistance to pyriproxyfen (one-tailed tests, $P < 0.05$ for each).

Based on the results of the stepwise regression, we included the residuals for the areas of treated and untreated cotton in multiple regression models and excluded the data for melons and alfalfa. The regression coefficient associated with area of untreated cotton was significant at 11 of the 12 scales, from 500 to 3,000 m (one-tailed tests, Table S2), indicating that refuges of untreated cotton at these distances influenced resistance. The regression coefficient associated with area of treated cotton was significant at four scales (1,000, 1,250, 1,500, and 2,750 m; Table S2). The average value of statistically significant coefficients associated with area of treated cotton ($n = 4$) was 0.35 (range 0.34, 0.37), whereas the average value of those associated with area of untreated cotton ($n = 11$) was −0.62 (range −0.67, −0.51).

The multiple regression results show that the coefficient of determination (R^2) varied from 8.2 to 30.1% across the 12 spatial scales (Fig. 2). The R^2 was significant (one-tailed test, $P < 0.05$) at 1,000–3,000 m, implying that resistance was significantly affected by the combined effects of treated and untreated cotton at this range of distances from sampled cotton fields.

In this type of analysis, as the area of rings begins to increase, R^2 is expected to increase if larger rings include an increasing area of plants that affect *B. tabaci* resistance in the sampled cotton fields. As the area of rings continues to increase, R^2 is expected to plateau when the land added to rings includes roughly equal areas of plants affecting resistance and plants not affecting resistance in the sampled cotton fields. Finally, R^2 is expected to decrease when the scale of analysis reaches the distance at which the area of additional plants not affecting

resistance exceeds the area of additional plants affecting resistance in the sampled cotton fields. The results show R^2 increased as the radius of rings increased from 250 to 1,750 m, peaked at 30.1% at 1,750 m, and was similar or somewhat lower at distances >1,750 m (Fig. 2). The peak in R^2 at 1,750 m shows that treated and untreated cotton located within this distance best explained spatial variation in resistance.

The multiple regression model at 1,750 m (based on ranks for all three parameters) is: resistance to pyriproxyfen = 31.6 + 0.32 (area of treated cotton) − 0.65 (area of untreated cotton) ($df = 2$, 22.4, one-tailed test, $P = 0.015$). In this model, the regression coefficient was marginally significant for the area of treated cotton ($df = 28.1$, one-tailed test, $P = 0.055$) and significant for the area of untreated cotton ($df = 22.3$, one-tailed test, $P = 0.005$).

To test the multiple regression model derived from data at 1,750 m, we substituted the areas of treated and untreated cotton within 1,750 m of the 38 sites sampled during the last 4 y of the study into the model to predict resistance to pyriproxyfen. As in the analysis of the first 4 y of data described above, the analysis of the last 4 y of data used residuals from ANOVA to remove among-year variation in abundance of crop types and resistance. The residuals for resistance reflect the variation in observed resistance to pyriproxyfen. Rank-based simple regression showed that the association between predicted and observed resistance to pyriproxyfen was positive and significant (Fig. 3, $R^2 = 16.1\%$, $F_{1,36} = 6.93$, one-tailed test, $P = 0.0062$, spatial autocorrelation was not significant in this analysis). Thus, the model formulated with the first 4 y of data adequately predicted spatial variation in the evolution of resistance in the last 4 y of the study.

Variation among counties in pyriproxyfen use and in the distribution of whitefly host plants could have contributed to the observed associations between resistance and host plant type. To exclude any such effects of variation among counties, we repeated all analyses using two-way ANOVA with county and year as classification factors to obtain standardized residuals. Such residuals are not affected by variation among counties or among years in resistance to pyriproxyfen and in area of each crop type at the 12 scales. Results from these two-way ANOVAs (SI Text and Table S3) confirm the main conclusions of the results described above from one-way ANOVAs.

Discussion

Our results show that the spatial distributions of melon and alfalfa were not associated with pyriproxyfen resistance in the sampled *B. tabaci* populations. Furthermore, the proportion of variation in resistance to pyriproxyfen accounted for by the model incorporating areas of treated and untreated cotton was
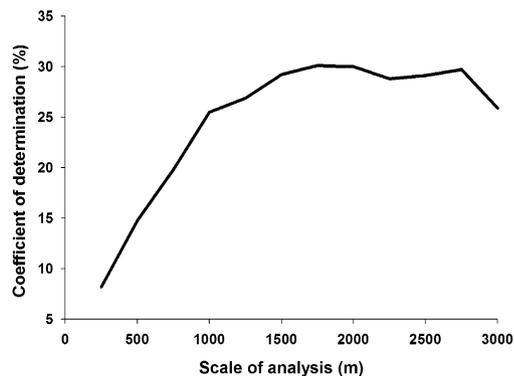
Fig. 2. Coefficient of determination (R^2) in multiple regression analyses performed at 12 scales. The response variable was resistance to pyriproxyfen (based on ranks for survival at the discriminating concentration) and explanatory variables were the area of pyriproxyfen-treated and untreated cotton (ranks) in concentric rings with a radius increasing from 250 to 3,000 m. See *Methods* for statistical details.
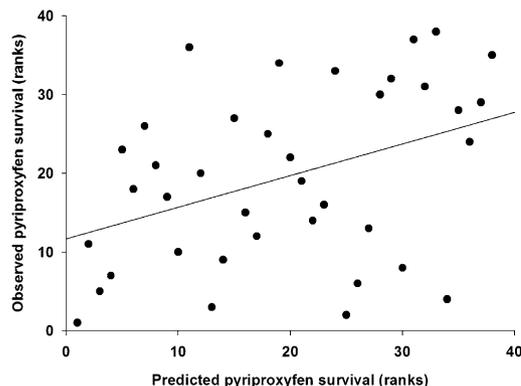
Fig. 3. Rank-based simple regression between observed and predicted resistance to pyriproxyfen in *B. tabaci* populations sampled in cotton fields during the last 4 y of the study. See *Methods* for statistical details.

similar at scales between 1,000 and 3,000 m, and the areas of treated and untreated cotton within 2,750 and 3,000 m from sampled sites were significantly associated with resistance, respectively. This suggests that cotton refuges within 3,000 m from sampled sites were effective in delaying resistance. This illustrates that our approach is suitable to identify habitats acting as refuges and to determine the spatial scale at which such refuges reduce the frequency of resistance, which are the critical elements required for development of a refuge strategy. Information on the areas of treated and untreated cotton within a certain distance from sampled sites (i.e., 1,750 or 2,500 m in the first and second analyses, respectively) was sufficient to predict spatial variation in resistance. This provides a large-scale field test of predictive refuge strategy models. The significant predictive success of these models confirms that resistance of *B. tabaci* to pyriproxyfen in central Arizona was delayed by cotton refuges and accelerated by treated cotton fields.

The refuge strategy was mandated by the US Environmental Protection Agency in 1996 to manage the evolution of resistance in some sexually-reproducing, diploid insect pests targeted by Bt corn and cotton. Simulation results suggest that the refuge strategy is useful for such pests and for parthenogenetic or haplodiploid pests such as *B. tabaci* (26). Simulation models show that three factors contribute in delaying resistance irrespective of pest reproductive mode: large refuges, low initial frequency of resistance alleles, and recessive inheritance of resistance. Nevertheless, additional factors are required to observe extensive delays when implementing a refuge strategy for haplodiploid and parthenogenetic pests (26). These factors are fitness costs, which occur when fitness of resistant individuals is lower than fitness of susceptible individuals in absence of a pesticide, and incomplete resistance, which occurs when fitness of resistant individuals is lower in the presence of a pesticide than in its absence (7, 27).

In haplodiploid pests such as *B. tabaci*, the evolution of resistance is primarily driven by selection for resistant haploid males, and reduced fitness of resistant males promotes the longest delays in resistance (26, 28, 29). In previous field experiments with pyriproxyfen-susceptible and resistant strains of *B. tabaci*, we found that males and females were equally susceptible to pyriproxyfen, resistance was partially recessive to completely dominant depending on the time elapsed between spraying and exposure, and effects of incomplete resistance were minimal (29). Furthermore, a selection experiment conducted in the laboratory did not reveal fitness costs of resistance, suggesting that costs may be absent under field conditions (30). Simulation results based on these parameters indicated that refuges of untreated cotton and other crops would delay resistance if they provided enough susceptible individuals to mate with individuals surviving exposure to pyriproxyfen (24). Nevertheless, because costs and incomplete resistance were absent, resistance evolved in <20 y in many simulations, which is consistent with the increase in survival to pyriproxyfen observed in Arizona.

Pyriproxyfen remains effective for the control of *B. tabaci* in Arizona. However, in central Arizona where non-cotton refuges do not appear to significantly delay resistance, reduced use of pyriproxyfen following the introduction of novel selective insecticides over the past decade may have been a key reason for sustained efficacy of pyriproxyfen (24). *B. tabaci* individuals surviving exposure to pyriproxyfen are subject to high predation rates because pyriproxyfen contributes in preserving natural enemies (18, 19). This may also have contributed in reducing the fitness advantage of resistant individuals on treated cotton and in delaying resistance.

Spring melons grown in California were previously found to be a source of *B. tabaci* for cotton fields up to a distance of 2.75 km, although melons were a significant source of whiteflies in only 1 of the 2 y investigated (23). This suggests that refuges of melons

were not effective in central Arizona because they were relatively rare. However, *B. tabaci* populations are regularly monitored on melons in central Arizona and suppressed with insecticides when needed. Furthermore, as noted previously, *B. tabaci* emigration from melons to cotton primarily occurs during the first half of the period when pyriproxyfen is applied on cotton (i.e., June to July). Both of these factors could have diminished the efficacy of refuges of melons. Alfalfa typically does not produce many *B. tabaci* adults from June to September in Arizona and California, possibly because harvest at close intervals during that period prevents many eggs and nymphs from completing development (31). Nevertheless, because alfalfa is rarely treated with insecticides, it was proposed that large acreages of this crop could contribute to delaying *B. tabaci* resistance to insecticides in some regions (32). Although alfalfa was as abundant as untreated cotton near sampled sites, refuges of untreated cotton delayed resistance but refuges of alfalfa did not.

Agronomic practices, abiotic and biotic ecological factors, metapopulation dynamics, and pest behavior, life history, and genetics interact to determine the trajectory of resistance evolution (7, 8, 27). When modeling the evolution of resistance, these numerous variables can be described by a limited number of parameters: the fitness of individuals with and without resistance alleles in refuges and fields where a pesticide is used, and migration and gene flow between patches (7). However, even these few parameters are not easily measured in the field. The strength of selection for resistance in a region is expected to be positively associated with the area of fields treated with a pesticide and negatively associated with the area of refuges, suggesting that areas of these habitats represent a relevant basis to model the evolution of resistance. Accordingly, the statistical approach applied here evaluated the spatial scale of effects of refuges and treated fields on pyriproxyfen resistance. This approach uses variables that are relatively easy to measure, and thus has the potential to improve refuge strategies for many pests. However, before concluding that this approach can generally provide a tractable evaluation of the global effects of relevant biological processes, additional tests are needed to see if resistance can be predicted in other species and agroecosystems.

Application of our approach for the development of refuge strategies requires spatially explicit data on resistance, location of putative refuges, and of fields where pesticides are used. Resistance monitoring data are often routinely collected for key pests (25, 33). Furthermore, the US Department of Agriculture's Natural Resources Conservation Service provides remote sensing data on crops and uncultivated habitats in most US states (34), which provides a basis to determine the spatial distribution of putative refuges. Nevertheless, spatially explicit data on pesticide-treated fields or Bt crops are often lacking (35). As such data become more accessible, we will be in a better position to improve refuge strategies for critical pests and to mitigate the economic, environmental, and social impacts of arthropod resistance to pesticides (36).

Methods

Field Sites and Pyriproxyfen Resistance. From 2002 to 2009, we sampled 84 *B. tabaci* populations in cotton fields of central Arizona (Fig. 1) from August to mid-October (mean = 10.5 populations per year, range = 8–13 populations per year, Table S1). Position of sampled fields was determined with a Global Positioning System (GPS). Cotton fields from four counties were sampled (La Paz, Maricopa, Pima, and Pinal), although in any given year *B. tabaci* populations originated from two or three counties (Table S1). Only one of the fields sampled during the course of the study was sampled twice (in 2003 and 2005).

Adult whiteflies were collected with a Makita Cordless Vacuum and brought to the laboratory within 8 h. Adults were released in cages with young cotton plants. Bioassays of the F1 progeny of these adults were conducted within 36 h of field collection by exposing newly laid eggs to various concentrations of pyriproxyfen (25). We used the adjusted percentage of eggs surviving a diagnostic concentration of 0.1 μg of pyriproxyfen

per mL as an index of resistance. For each of the 84 populations sampled, we calculated adjusted survival (%) as survival (%) at 0.1 μg of pyriproxyfen per mL divided by survival (%) at 0 μg of pyriproxyfen per mL, which is equivalent to adjusting for control mortality with Abbott's method. We use the term "resistance to pyriproxyfen" to refer to the adjusted percentage of eggs surviving the diagnostic concentration of pyriproxyfen. Resistance to pyriproxyfen was 0% from 1996 to 1998 and increased thereafter (Fig. S1 and ref. 25).

Crop Mapping. Location and shape of agricultural fields in the study area were determined with GPS or microsurveyors at a resolution of 10–100 m (37). Location of cotton fields in each year was mapped with a Geographic Information System (GIS) by the grower-funded Arizona Cotton Research and Protection Council (ACRPC), using information collected from producers and from the ground. When we validated the resulting GIS maps by checking identity of a random sample of cotton fields on the ground, we found that maps of cotton fields were 100% accurate in central Arizona (37).

We retrospectively identified alfalfa and melon fields surrounding the sampled sites with remote sensing. Crop classification maps were created with a two-step Classification and Regression Tree (CART) algorithm using the SEE 5.0 software and ERDAS Imagine (38, 39). Maps of field boundaries and three to six Landsat Thematic Mapper satellite images were used to classify the crops in each year. The two-step classification procedure began with a binary classification to separate alfalfa and melon fields. These classes were then isolated and a second classification was performed to identify alfalfa, corn, cotton, grain, fallow, orchards, and sorghum, after which the melon class from the binary classification was reinserted into the classification product.

We mapped crops from the ground in large areas of central Arizona in 2002, 2003, and 2007–2009. We used half of the data for a year to train the classifier and the other half to assess classification accuracy. When field knowledge was not available for 2004–2006, we used classification training statistics from other years. To do this, the two-step classification procedure was applied to image data from 2004 and 2005, using classification training statistics from 2002, and to image data from 2006, using classification training statistics from 2007. For the 5 y in which we had matching ground-verified training data, we achieved classification accuracies >91% for alfalfa and >70% for melon. When the classification was trained with off-year data (e.g., training with 2007 data to classify 2008 data), we achieved accuracies >90% for alfalfa and >55% for melon.

To improve classification accuracy of alfalfa and melon, corrections were made to the digital classification maps produced through the CART classifier based on expert knowledge of crop phenology patterns. We focused on all fields <3 km from sampled sites, and distinguished crops based on growth patterns depicted by sequential image data sets. The Normalized Difference Vegetation Index (NDVI) (40) highlighted phenological differences among crops that corresponded to harvesting dates (Fig. S4). We thus used the change in NDVI, and assessed every field within a distance of 3 km to correct for potential misclassification of alfalfa, cotton, and melon. This expert-knowledge observational approach likely produced accuracies close to 100% for identification of alfalfa, cotton and melon fields.

Landscape Analysis. The classified crop maps for each year were analyzed using ArcGIS (41). In a few instances, the GPS point associated with a field sampled for *B. tabaci* fell between two adjacent cotton fields on the GIS map. As it was not possible to determine which cotton field had actually been sampled in these cases, we used the GPS points as our best estimate of the position of sampled sites. We drew 12 concentric rings around each GPS point. The radius of each ring was 250, 500, 750, 1,000, 1,250, 1,500, 1,750, 2,000, 2,250, 2,500, 2,750, or 3,000 m. We measured the area (m²) of alfalfa, cotton, and melon in each ring with ArcGIS.

For each year, we used the Arizona Pest Management Center's Pesticide Use Reporting (PUR) database (42) to calculate the total acreage of cotton treated with pyriproxyfen in each county. For each year, we used the ACRPC GIS maps of cotton fields to evaluate total acreage of cotton in each county. For each year and county, acreage of cotton treated with pyriproxyfen was divided by total cotton acreage to estimate the proportion of cotton acreage treated with pyriproxyfen (pCTP). For each sampled site, the area of cotton in a ring was multiplied by pCTP to estimate the area of cotton treated with pyriproxyfen, and by 1 − pCTP to estimate the area of untreated cotton.

Statistical Analysis. *Evaluation of crop effects and predictive regression model.* To characterize effects of crops on resistance and develop a predictive regression

model, we used data from the first 4 y (2002–2005, 46 populations, Table S1). We used stepwise regression with forward selection and backward elimination to evaluate the association between resistance to pyriproxyfen and the areas of four crop types: three refuges (alfalfa, melons, and cotton not treated with pyriproxyfen) and pyriproxyfen-treated cotton. We performed regressions at each of the 12 spatial scales based on rings surrounding each sampled site with a radius of 250–3,000 m. To remove among-year variation in resistance to pyriproxyfen and in area of each crop at the 12 scales, we analyzed the data with one-way ANOVAs and used the standardized residuals (i.e., centered data divided by the SD within each year) of resistance to pyriproxyfen as the response variable and of the four crop areas as the explanatory variables in all regressions. This allowed us to pool data from the first 4 y to analyze the association between among-site variation in resistance to pyriproxyfen and among-site variation in areas of the four crop types near each sampled site. Explanatory variables that were significant ($P < 0.05$) at one or more scales were retained in the final multiple regression model, which was fitted at each of the 12 scales. In the stepwise and multiple regression analyses, we used rank-based statistics (15) that do not require the assumption of normality. We used one-tailed tests of significance for each crop type, because we expected a negative association between resistance and the area of the three refuge types and a positive association between resistance and the area of pyriproxyfen-treated cotton.
Predicted versus observed pyriproxyfen resistance. We used the multiple regression model built on the first 4 y of data (2002–2005) to predict spatial variation in pyriproxyfen resistance during the last 4 y of the study (2006–2009). We used the model based on rings with a radius of 1,750 m, because this spatial scale yielded the highest coefficient of determination. Prediction was performed by using data on the areas of cotton treated and not treated with pyriproxyfen in the last 4 y. One-way ANOVAs with year as the classification factor were performed to remove among-year variations in the areas of treated and untreated cotton within 1,750 m of each of the 38 sites sampled from 2006 to 2009. Standardized residuals from these ANOVAs were transformed to ranks, and these ranks were substituted in the multiple regression model (fitted to data from the first 4 y) to calculate predicted ranks of resistance to pyriproxyfen for the last 4 y. Similarly, standardized ANOVA residuals of resistance to pyriproxyfen in the last 4 y were transformed to ranks and used as observed values. A rank-based simple linear regression and a one-tailed test of significance were used to assess the association between predicted and observed resistance to pyriproxyfen.
Spatial autocorrelation. In each stepwise and multiple regression analysis, and in the analysis of predicted versus observed survival to pyriproxyfen, semi-variograms were computed to quantify and analyze spatial autocorrelation (43) in resistance to pyriproxyfen and other variables across sampled sites. By assessing spatial patterns in residuals (response variable) and partial residuals (explanatory variables), we evaluated potential spatial autocorrelation at any scale, including within counties. Spatial autocorrelation was accounted for in tests of significance through the use of effective sample sizes and effective degrees of freedom, in modified t and F tests performed in simple linear correlation, stepwise regression, and multiple regression analyses (43–45). Programs for these statistical analyses were written in Matlab (46).

Among-county variation in pyriproxyfen use and in the distribution of whitefly host plants could result in nearby sites having more similar resistance than sites farther apart, both within and across years. To make sure that the former possibility did not affect the outcomes of analyses, we repeated all analyses and used two-way ANOVA with county and year as classification factors to obtain standardized residuals. Such residuals are not affected by among-county and among-year variation in resistance to pyriproxyfen and in area of each crop at the 12 scales. Results from the new analyses (SI Text) were qualitatively similar to results presented herein. In the latter case, the formulation of a predictive model could be affected by spatiotemporal autocorrelation. However, Mantel tests (43) revealed no significant spatiotemporal correlation in resistance between pairs of years from 2002 to 2005 (two-tailed test, $P > 0.20$ for all pairs).

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