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Abstract

In this review, we evaluate the intentional mixing or blending of insecticidal seed with refuge seed for managing resistance by insects to insecticidal corn (*Zea mays*). We first describe the pest biology and farming practices that will contribute to weighing trade-offs between using block refuges and blended refuges. Case studies are presented to demonstrate how the trade-offs will differ in different systems. We compare biological aspects of several abstract models to guide the reader through the history of modeling, which has played a key role in the promotion or denigration of blending in various scientific debates about insect resistance management for insecticidal crops. We conclude that the use of blended refuge should be considered on a case-by-case basis after evaluation of insect biology, environment, and farmer behavior. For *Diabrotica virgifera virgifera, Ostrinia nubilalis*, and *Helicoverpa zea* in the United States, blended refuge provides similar, if not longer, delays in the evolution of resistance compared to separate block refuges.

Key words: IPM, seed mixture, seed blend, Bt corn, refuge

In the 40 years since the publication of seminal papers by Comins (1977) and Georghiou and Taylor (1977), management of insect resistance to insecticides and insecticidal crops has become more complicated and diverse. Insect resistance management (IRM) is the scientific approach to managing pests over the long term so that resistance does not interfere with the ability of stakeholders to achieve their goals (Onstad 2014). One of the cornerstones of IRM for insecticidal crops is the deployment of refuge (areas planted with crops not expressing insecticidal traits) to produce susceptible insects (Onstad and Knolhoff 2014). Refuges can be natural areas, separate blocks of the crop, within-field strips, or randomly distributed refuge plants within a field produced by planting a blend or mixture of refuge and insecticidal seed. Although this review focuses on the influence of blended or block refuge on the evolution of resistance, the effects of refuge on other aspects of long-term IPM should not be ignored before implementation of a strategy (Onstad 2014, Grettenberger and Tooker 2015).

About 25 years ago, entomologists began to rigorously evaluate the intentional mixing or blending of insecticidal seed with refuge seed for managing resistance to insecticidal crops (Wilhoit 1991, Mallet and Porter 1992). The pros and cons of seed blends will be discussed throughout this review, but two of the benefits of seed blends are the maximization of adult mixing and the certainty that refuge will be deployed because all the refuge seed is in the same bag as the seed expressing the insecticidal trait. Separate areas of refuge

are most effective when 1) the treatment kills more than 99.99% of the wild-type pests (high dose), 2) the resistance allele is rare (circa 0.1% or less), 3) the resistance is mostly, if not completely, recessive in its expression, and 4) non-assortative mating occurs with adults mixing across the refuge and insecticidal crop (Onstad and Knolhoff 2014). The choice to use a blend versus separate refuge should be based on careful evaluation of the trade-offs for each case involving a pest species, farming practice, and landscape (Onstad et al. 2011, Onstad and Carrière 2014, Carrière et al. 2016).

This review has six major sections. In Population Biology of the Pest section, we describe the pest biology that will contribute to weighing these trade-offs. In Comparison of Models section, we compare biological aspects of several abstract models to guide the reader through the history of modeling, which has played a key role in the promotion or denigration of blending in various scientific debates about IRM for insecticidal crops. In Farmer Behavior section, the importance of farmer behavior is discussed. In Hypotheses section, we describe the two hypotheses that currently explain why seed blends can be less useful than block refuges in delaying evolution of resistance. Case Studies Regarding Insecticidal Corn section contains a few case studies that we hope will demonstrate how the trade-offs will be different in different systems. Case studies for *Ostrinia nubilalis, Diabrotica virgifera virgifera*, and *Helicoverpa zea* are presented. Conclusions section provides some conclusions. Note that we will primarily discuss transgenic insecticidal corn (*Zea*

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mays), but the concepts pertain just as much to other forms of host plant resistance (Onstad and Knolhoff 2014).

Because IRM involves management, not simply the study of pests and their environments, we must focus on more than the evolution of resistance. Although stakeholders tend to try to simplify problems so that they are tractable, unfortunately, except in extreme situations, a fully informed decision to deploy a particular type of refuge will involve answering many questions about pest biology and farming practices (Tables 1 and 2). For example, intermediate rates of pest survival and movement and moderate levels of farmer compliance with block refuge requirements do not support simple or easy decision making. There may be trade-offs and interactions that only mathematical models can help decision makers evaluate. Our review attempts to demonstrate the complexity of both the pest systems and the decision-making process.

Population Biology of the Pest

One significant difference between studies of seasonal, traditional IPM of herbivorous pests and of long-term IPM that includes IRM is the need to understand larval and adult behavior and their effects on evolution and IRM (Onstad 2014). With a seasonal perspective, average mortality and phenology seem to be the most common processes studied. To manage a pest likely to evolve resistance, we also need to measure when, where, and how larvae and adults feed, move,

Table 1. Questions about larval behavior and survival that should be considered in most studies of seed blends for IRM

What is survival of genotype after leaving Bt plant and arriving on refuge?
What is survival of genotype after leaving refuge and arriving on Bt plant?
What is survival of genotype due to movement?
What is probability of leaving insecticidal plant?
What is probability of leaving refuge plant?
What are movement rates at various pest densities?
How many moves from plant to plant with feeding in between?
What is the timing of movement(s) relative to age of larva?
Does first movement occur without feeding first?
Are there differences in movement for insect genotypes?
What is probability of feeding on fertilized plant tissue after pollination?
What is survival of genotype on fertilized plant tissue?
How does cross-pollination with insecticidal trait affect survival in refuge?
Are there differences in any parameter due to growth stage of crop?

Table 2. Questions about adult behavior that should be considered in most studies of block and blended refuge for IRM

Do females or males mate before dispersing out of natal field?
What is proportion of each gender that mates before dispersing?
Can each gender mate more than once?
Over how many days can each gender mate more than once?
How far can a male or female disperse before mating?
How does pest density influence behavior?
Does the female oviposit mostly in natal field?
Are there differences in adult dispersal for insect genotypes?
Are there differences in emergence for insect genotypes?
Does adult emergence and behavior differ in the refuge and insecticidal fields?
Are there differences in any parameter due to growth stage(s) of crop(s)?

and mate (Onstad et al. 2011). In evaluating many pest systems, it is striking how little is known (at least quantitatively) about the behavior of many major pests (Gould 1991).

Tables 1 and 2 display some of the questions that should be considered when studying pest biology relevant to IRM, particularly decisions about refuge configuration. We use these lists to indicate the complexity of the pest systems that must be recognized as stakeholders address refuge deployment. Note, however, that every real study will have its limitations and will rarely answer most of these questions by the time a decision must be made about refuge configuration and deployment. Some biological aspects have not yet been elucidated even for some of the most extensively studied corn pests. At the beginning of an IRM project, stakeholders should determine what answers exist in the literature or in unpublished sources. Then observations should be made on some of the listed processes. The crop and insect species will determine how to prioritize the set of questions. For instance, if larvae do not feed on corn ears, then the three questions pertaining to fertilized tissue and cross-pollination will not need to be answered (Table 1). In fact, entomologists can choose between measuring feeding on fertilized tissue or simply measuring survival on the whole plant after cross-pollination (more discussion below). Again, in many cases, stakeholders will need to make decisions without answers to some questions because of constraints on methods, time, and funds. For example, all questions about genotypes other than those common in wild populations will likely require the development of a colony of resistant insects. Or measurements may require the existence of final insecticidal product, which may be available only for a year or two before IRM decisions are made.

Survival

Survival is the most important biological parameter to understand the impacts of seed blends for IRM including survival before and after movement, on vegetative and reproductive tissues, and during movement (Table 1). However, most people think first about larval movement when seed blends are being considered. It is the differential survival between susceptible homozygotes and heterozygotes (Gould 1994) and the reduced survival of susceptible insects in blended refuge relative to block refuge (Carrière et al. 2004) that will determine the success of blended refuge for IRM for insecticidal crops. Therefore, survival will need to be measured differently for every targeted insect species depending on the insect's crop environment and preferences. For instance, the crop environment will determine how predation and desiccation or the impact of rainfall will affect survival during larval movement from plant to plant or to and from hiding/feeding places on a plant. Also as a crop matures, this environment and the availability of succulent and nutritious plant tissues change. Most importantly, the environment includes the concentration and effectiveness (dose) of the insecticide. As discussed below, dose may differ across various plant tissues and over time. For example, survival may increase over the growing season if the dose of insecticide declines in the reproductive stage.

Insect ecologists have measured survival of larvae in seed blends. Erasmus et al. (2016) artificially moved larvae at different ages from plant to plant to assess survival. Other examples are presented in several sections below. Few studies have measured differential survival of susceptible and resistant larvae in seed blends because few field ecologists have access to colonies of resistant insects. Also, resistant insects cannot be released in the environment requiring that studies must be performed under strict insect containment.

For pests feeding on corn kernels in the field, survival may be influenced by cross-pollination in seed blends. Much of the effect of

cross-pollination on insects that feed on reproductive tissue is determined by how much maternal plant tissue is consumed compared to fertilized tissue. Fertilization in flowering plants such as corn often occurs by a process called double fertilization (Berger et al. 2008). It is a complex process that leads to the production of the endosperm and the embryo, the yellow and white parts of the kernel, respectively. Fertilized tissues such as the endosperm and the embryo differ from maternal tissues, such as the husk, silk, shank, and cob, which are not affected by cross-pollination. In situations with 100% cross-pollination of a refuge plant by insecticidal plants, circa 50% of the kernels per corn ear will express the trait (50% hemizygous), while 50% of kernels will not inherit the gene due to open pollination and gene segregation in the ear. This scenario is actually very close to reality in the field as rates of natural self-pollination in corn are as low as 5% (Waller 1917).

In essence, to estimate the durability of insecticidal traits for insects that feed on pods, seeds, kernels, fruit, and flowers, we need to know 1) the proportion of the population that moves to or is oviposited on these tissues during reproductive growth stages of the plant, 2) the ratio of maternal and fertilized tissues in the diet of these larvae, 3) the toxicity of fertilized tissue, and 4) the toxicity of pollen coming from insecticidal plants. Or we can simply measure survival on the entire plant under normal behaviors and avoid the difficult tasks of measuring specific processes. Obviously, if only maternal tissue is eaten or if a very low proportion of the population on refuge plants feeds on these tissues, then durability is mostly determined by feeding on non-toxic tissue in the blended refuge. If only older larvae feed on fertilized tissues then even susceptible larvae may not be harmed much by the dose of insecticidal trait (Pannuti et al. 2016).

Studies of *O. nubilalis* provide an example of how feeding influences survival on reproductive-stage corn. Greenhouse tests indicated that survival of *O. nubilalis* resistant to Cry1Ab may vary depending on plant stage (Crespo et al. 2009). No resistant larvae were found on vegetative-stage corn, but resistant larvae survived on reproductive-stage corn (Crespo et al. 2009). A seasonal analysis of tissue-specific expression indicated that Cry1Ab levels in leaves of insecticidal plants were not reduced during the reproductive stage, which suggests that Cry1Ab-resistant *O. nubilalis* surviving reproductive stage corn likely consumed other plant parts with lower levels of Cry1Ab expression such as pollen, silk, kernels, and ear shank (Crespo et al. 2009). In addition, results from pollen and silk assays corroborated on-plant assays (Crespo et al. 2009), suggesting that resistant insects were able to survive on tissues with lower Cry1Ab expression. The results obtained with *O. nubilalis* were consistent with the Cry1Ab expression profile of reproductive tissues of various Cry1Ab corn hybrids (Koziel et al. 1993, Crespo et al. 2009). Similar results were obtained with *Ostrinia furnacalis*, which exhibited some ability to survive after 3 d of feeding on silk and kernels of Cry1Ab plants but no survival on leaf tissue (Wang et al. 2004).

Larval Movement

We need to understand movement from plant to plant, and sometimes, within a plant. If many of the larvae feed on both vegetative and reproductive tissue, we also need to quantify timing and rates of movement to and from these tissues. Usually, we assume that larvae do not move from field to field. Movement by the same species will be different on different plant species, at different growth stages, and on plants of different sizes and densities. Smaller plants may have higher pest densities that promote movement away from crowded conditions, but larger plants may overlap in a crop canopy permitting more frequent plant-to-plant movement. Few studies have

measured the influence of pest density on pest movement in insecticidal crops or refuge (Berger 1992, Wangila 2013). Wangila et al. (2013) observed greater movement when larval density of *Diatraea saccharalis* was higher on a plant.

Many details of larval movement can influence IRM. Timing of movement relative to feeding and the age of the larva are important. For example, if movement occurs after all feeding has ended and the larva is moving to a pupation site then movement will have no influence on resistance evolution. The probabilities of leaving both a refuge plant and an insecticidal plant must be measured. Greater movement away from insecticidal plants compared with departure rates from refuge plants can reduce selection pressure (Onstad et al. 2011, Lopez et al. 2013). In general, it is important to know whether movement consists of discrete steps with each interval of feeding followed by movement or whether movement is mostly one long, continuous step from the natal plant to the final destination with no feeding between points. Most of these measurements can be made for plant-to-plant movement and within plant movement for pests that feed on reproductive tissues. If a colony of resistant insects is available then any differences in movement between phenotypes can be measured in a cage or greenhouse.

Studies of larval movement by two corn borers, *O. nubilalis* and *Chilo partellus*, are worth highlighting. Ross and Ostlie (1990) measured dispersal and survival by young larvae of *O. nubilalis* in vegetative corn. After day 2, mean dispersal rate was a maximum 2 cm/d until day 30 (Ross and Ostlie 1990). The maximum distance moved after 2 d increased as the larval density increased from one to four egg masses per plant. However, Ross and Ostlie (1990) found no relationship between density (one to four egg masses) and probability of leaving a plant (51–56%). Note that the mean and maximum dispersal rates were much higher for neonates within 2 d of hatching (Neiswander and Savage 1931, Beck 1956). Neiswander and Savage (1931) concluded that older larvae of *O. nubilalis* move before entering diapause, especially at high densities, but do not feed afterwards.

Davis and Onstad (2000) concluded that larval dispersal of *O. nubilalis* in cornfields is greater due to exposure to insecticidal corn. Goldstein et al. (2010) discovered that most neonates are able to detect the insecticidal protein within 24 h and that dispersal is greater from insecticidal corn (75%) than from refuge corn (42%). Razze and Mason (2012) also observed higher dispersal rates from insecticidal corn than from refuge corn within the first 4 h after eclosion in the laboratory and in the field. Razze et al. (2011) measured movement and feeding during the first 48 h after hatching. They found that 50% of the larvae had moved off of the plant within 6 hours and that only 5% of these had eaten. Davis and Coleman (1997) also reported little feeding during the first 24 h. Razze et al. (2011) observed that *O. nubilalis* neonates hatching on insecticidal corn consumed less plant tissue than those on refuge corn.

Hari and Jindal (2010) directly observed movement of *C. partellus* larvae in corn in India. First, they closely observed neonates 10–240 min after manually infesting 2-wk-old plants in a screen house. Then they gathered observations of more extensive movement by infesting 2-wk-old plants in the field and recording locations after 2 and 7 d. They even compared infestations of neonates with manual infestations of egg masses to determine any effects of manual infestation on behavior. Berger (1992) performed a complementary study of *C. partellus* and concluded that larval movement was different for corn and sorghum and changed with larval density and larval age.

Some researchers have based their knowledge of larval movement on indirect measurements and plant injury (Carroll et al. 2013,

Erasmus et al. 2016). One advantage of this approach is that it is easier and likely less expensive to simply add larvae or eggs to a central plant and then return circa 3 wk later to record all the damage caused by the given species to all the plants in the plot. This kind of study along with an intensive set of observations of actual movement would be a good combination. However, without some observation of actual larval behavior, it may not be possible to know the timing of feeding and movement and the repellency, if any, of insecticidal plants. Also, care must be taken to ensure that damage caused by similar species is not mistaken for injury caused by the species that is the focus of the experiment.

Adult Behavior

To appreciate the value of seed blends relative to separate block refuges, we need to know several aspects of adult behavior (Table 2). Here we discuss the five most common questions about dispersal and mating. First, do females or males mate before dispersing out of natal field? If they mate in the natal field in which they emerged then separate block refuges likely will provide less value to IRM because assortative mating will occur. Seed blends will permit greater mixing of phenotypes in the natal field. Females mating in and remaining in the block refuge can be valuable for IRM because the susceptible offspring survival is at higher levels in the refuge. However, the opposite is the case for males. Males that disperse to some extent permit mixing and mating of susceptible and resistant individuals. Second, what proportion of each gender mates before dispersing? Sometimes, the location of mating is variable within a population and different between genders. *Diatraea grandiosella* females mate in the natal field and males mate inside and outside the natal field (Guse et al. 2002). Most *O. nubilalis* mate outside the natal field (Showers et al. 2001, Dalecky et al. 2006, Dorhout et al. 2008) except when cornfields are irrigated. In this case, a higher proportion of adults typically remain in the humid natal field with much less dispersal out to adjacent dry vegetation (Hunt et al. 2001, Guse et al. 2002). The *D. v. virgifera* females mate in the natal field within a day or two of eclosion (Spencer et al. 2013). The protandrous males of this pest mostly mate locally but can slowly disperse beyond the natal field for mating (Spencer et al. 2013).

At least three other quantitative factors in adult behavior complicate the trade-offs between seed blends and block refuges. If a male or female can mate more than once, then mating could happen both locally and far from the natal field as noted with the males of *D. grandiosella*. The distance between multiple matings depends on the number of days during which each gender can mate. Finally, we need to know how far a male or female can disperse before mating. For some migratory insects, such as *Spodoptera frugiperda*, no published report has determined how many mate locally and how many mate after migration.

Because adult behavior usually occurs over larger areas and mating may happen over a few hours, experiments involving adults are likely more challenging than those involving larvae (at least those above ground). In addition, adult insects may change their behavior when entomologists try to observe them in the field. The intensive study of mating during the first hours or days after eclosion should indicate how soon the adults can mate after emergence, whether the females produce a pheromone to attract males before dispersal, and when mating begins. Some of these observations can be performed in a laboratory setting, but they should be confirmed with field observations. Investigations of dispersal timing and distance are always challenging, especially because of typically low recapture rates in mark-release and recapture studies. They must be done under realistic conditions in the field that can vary over time and

space. Furthermore, the researcher needs to determine whether mating could occur at the end of smaller steps that combine to produce the total distance dispersed.

Separate Block Refuge

Modeling has shown that a well-positioned block refuge is most effective when it remains in the same location year after year and the males disperse widely for random mating while females remain in natal refuge to mate and lay eggs (Guse et al. 2002, Onstad et al. 2014). These factors allow the susceptible pests to increase in numbers in the refuge with dispersing males ensuring the production of heterozygotes in the insecticidal crop after mating with any heterozygous- or homozygous-resistant females.

Natural refuge can consist of the same plant species as the insecticidal crop or it may consist of other plants (alternate hosts) that are effective habitat for the pest. This type of refuge is also called unstructured refuge because it is not planted as part of the structure of the insecticidal cropland. Alternate hosts may be more or less effective in producing susceptible pests depending on how well the larvae survive and on the insect's oviposition preference for these plants. Even when the plant species is the same, the unstructured refuge may not be managed in a way that maintains the quality of the pest's environment the same as it is in the typical insecticidal crop field (see Farmer Behavior section). For example, the phenology of the unstructured refuge may not be synchronized with the insecticidal crop, which will reduce the effectiveness of the unstructured refuge. In addition, the proximity of natural refuge to insecticidal crop fields must be taken into consideration when developing IRM plans and making predictions about durability of insecticidal crops.

Comparison of Models

Mallet and Porter (1992) and Tabashnik (1994) used abstract models of an insect population under selection by an insecticidal crop to explore the value of seed blends versus separate block refuges. They assumed random mating at the landscape level in all scenarios. They assumed that the larvae moved once with feeding before and after movement. They also assumed that probability of movement off of refuge plants is the same as that from insecticidal plants and that all genotypes move equally. As expected for initial studies of a new concept, these two studies only considered the simplest model. The main point of Tabashnik (1994) was to show that seed blends can delay resistance compared with scenarios without refuge. He also explained the paradox presented by Mallet and Porter (1992) that seed blends used in a landscape with significant levels of natural refuge could actually reduce durability relative to scenarios with only natural refuge. Tabashnik (1994) concluded that this paradox is based on their core assumptions described above and the basic foundation of the high dose/refuge strategy: rare initial levels of resistance, recessive resistance, and very high mortality caused by the insecticidal trait. As these assumptions are relaxed or converted to others, the paradox disappears and seed blends may increase durability even in the presence of significant amounts of natural refuge (Tabashnik 1994).

Although never creating a purely abstract model to explore seed blends in IRM, Onstad has taken a general approach to the modeling and evaluation of seed blends and block refuges for corn IRM. Onstad and Gould (1998) followed by Davis and Onstad (2000) used a model that included random mating at the landscape level and larvae moving once, with feeding before and after movement. Unlike earlier models, the probability of movement off of refuge plants was lower than that from insecticidal corn. On the basis of preliminary

observations in Gould's laboratory (Gould 1994), Onstad developed a concept called 'predispersal tasting survival'. This was proposed to deal with situations in which neonates move without fully feeding on insecticidal tissue (Onstad and Gould 1998). In other words, some neonates may taste the plant tissue before moving and the survival rates due to this tasting differ by a small fraction in susceptible homozygotes and heterozygotes.

Carroll et al. (2012, 2013) modeled the evolution of insects in landscapes of corn expressing a toxin from *Bacillus thuringiensis* (Bt). Their abstract models did not include natural refuge. They assumed that mating was random only in the natal field. Two types of cornfields existed in the modeled landscape: 1) Bt only and 2) cornfields with refuge and Bt corn. Larval movement and survival were calculated in the following manner. The model allows a variety of distinct movements or steps during the larval stage; for *D. grandiosella*, the authors assumed three steps. During each step or interval, feeding and movement from plant to plant occur. Carroll et al. (2012, 2013) used two movement parameters. The base movement rate is the proportion that moves from refuge corn, but it is included in calculations for all movement rates. The genotype-specific movement rate is the probability of moving off of Bt corn for each insect genotype. Carroll et al. (2012) chose to make this probability equal to (1 – daily genotype survival on Bt corn). Thus, according to Carroll et al. (2012), movement is greater from Bt corn than non-Bt corn, and homozygous susceptible individuals are more likely to move from Bt corn than heterozygotes- or homozygous-resistant individuals. Carroll et al. (2013) used the model to calculate estimates of refuge productivity and compared them to productivities observed in the field.

Carroll et al. (2012) also included a movement penalty (mortality during movement). They found that mortality due to movement significantly delayed evolution of resistance because of the high rates of movement off of Bt corn. In scenarios with single insecticidal traits, including movement mortality increased Bt corn durability in seed blends relative to fully compliant block refuge. When mortality during movement was included for populations in both blended and block refuge, Onstad (2006) also found that increasing this density-independent mortality delayed the evolution of resistance in his model.

Caprio et al. (2016) developed three detailed models that emphasized the feeding and movement of larvae on cross-pollinated corn kernels expressing variable doses of insecticides. In their models, the crop only has reproductive tissue and the pest feeds only on this tissue. Thus, the model does not represent pests that feed on vegetative tissue or on other crops. Caprio et al. (2016) found that as larvae spent more time initially feeding on corn silks (maternal tissue), durability of the traits increased in seed blends. Their results indicated that increasing larval movement rates off of insecticidal kernels tended to increase durability of seed blends. Because they assumed random mating in the landscape and 100% compliance (see Farmer Behavior section), there was no detriment to the use of block refuge in their models.

Farmer Behavior

Whether we call this subject farming practices or farmer behavior, the main point is the same: choices made by farmers influence the relative effectiveness of refuge (Hurley and Mitchell 2014). Some of these choices are directly related to the planting of a refuge, while others pertain to farming practices that can influence the trade-offs between types of refuge.

Separate block refuges should be planted at a recommended level (or greater) to effectively manage pest resistance. However,

farmers may not 'comply' with these recommendations or contractual requirements (Hurley and Mitchell 2014). Surveys have shown that compliance rates are often below 100% (Onstad et al. 2011, Hurley and Mitchell 2014, Trumper 2014, Pan et al. 2016, Reisig 2017). Sometimes the size of a refuge is too small. In other cases, the refuge is planted too far away from the insecticidal crop field. The quality of a separate refuge is influenced by the planting and subsequent management of the crop. If this different quality causes the refuge to be less attractive as a mating site or for oviposition or reduces survival of immature pests, then the block refuge will not contribute as much as intended to resistance management (Onstad et al. 2011). The most important and obvious practice that will directly reduce the effectiveness of block refuge is the greater use of insecticides in refuges relative to use in insecticidal crops (Onstad et al. 2011). Even if the insecticide is not targeted against the primary pest of the insecticidal crop, as long as it is lethal to that pest, it will reduce the effectiveness of the refuge.

Three other farming practices that influence the value of refuge have been the focus of publications: technology adoption, seed saving, and weed management. Adoption of the insecticidal crop in the market is the proportion of the cropland that is planted with an insecticidal crop (and its required refuge). Until adoption is 100%, farmers make the choice not to plant insecticidal crop for a variety of reasons (Hurley and Mitchell 2014). Required refuge may be less valuable when adoption is low because a large amount of cropland does not express the insecticidal trait anyway.

Seed saving and weed management can complicate or worsen attempts to manage resistance (Krupke et al. 2009, Onstad et al. 2012). For the purposes of this paper and IRM, seed saving means that a farmer collects seed from insecticidal and refuge crops at the end of a growing season and plants them either separately or mixed together in the next season. Thus, accidental blending may occur. More importantly, the dose of insecticide expressed in the crop may not be consistent in the next season (Onstad et al. 2012). When seeds of an insecticidal corn crop germinate in the next season and produce weedy or volunteer corn because they are not removed, the weedy corn plants may cause problems for IRM (Krupke et al. 2009). The main concern is that lower doses will be expressed in some of the new crop or weeds compared to the expression expected in hybrid insecticidal seed.

Hypotheses

Here we present two main hypotheses that have been promoted to explain why seed blends may not delay the evolution of resistance as much as a separate block refuge of the same proportional area. Of course, neither hypothesis by itself allows one to determine whether blends or blocks are better for IRM. A complementary analysis of block refuges is also needed. Fewer formal hypotheses have been promoted to explain why block refuges may not delay the evolution of resistance as much as blended refuge.

Tabashnik (1994) and Carrière et al. (2004) were early proponents of the 'lower productivity' hypothesis. They postulated that an average refuge plant in a seed blend may not produce as many susceptible insects as the average plant in a block refuge. Feeding during part of the larval stage on insecticidal plants in the seed blend would reduce the survival of susceptible homozygotes (and possibly heterozygotes). They noted, however, that if larvae leave insecticidal plants more than they do refuge plants then it is possible that, on a per plant basis, the refuge in a seed blend could be more productive (effective) than a block refuge (Carrière et al. 2004). Later, a concern for cross-pollination of refuge plants and subsequent reduction in

survival due to feeding on insecticidal seed was added to the concerns within this perspective. Survivors from the refuge plants are needed to mate with resistant homozygotes surviving on the insecticidal plants to produce heterozygotes, which are killed by the insecticide. A number of studies have observed reductions in refuge productivity (live insects per refuge plant) in seed blends compared with blocks of refuge (Carroll et al. 2013, Oyediran et al. 2016). Some have observed no differences (Wangila et al. 2013).

The second main concern is expressed in the 'differential survival' hypothesis (Mallet and Porter 1992, Gould 1994). In this view, the most important influence on evolution is the difference in survival between the susceptible homozygotes and the heterozygotes moving to and from refuge and insecticidal plants (Brevault et al. 2015).

Because there are so many processes and factors that determine the production of susceptible homozygotes and heterozygotes in a seed blend (Table 1), it is likely that some cases will be explained by one hypothesis, some will be explained by the other hypothesis, and it is possible that other hypotheses will be postulated in the future. Furthermore, because these two hypotheses are not mutually exclusive, it is possible that cases will be explained by more than one hypothesis.

Case Studies Regarding Insecticidal Corn

Here we present three case studies to highlight the types of evaluations that can contribute to decision making. Published case studies for insecticidal corn mostly have concluded that seed blends are satisfactory, whereas most for insecticidal cotton (*Gossypium hirsutum*) have drawn the opposite conclusion (Heuberger et al. 2011, Ramalho et al. 2014). In Brazil concerns about the high larval movement rates of *S. frugiperda,* and the presence of natural corn refuge have suggested that seed blends should be avoided. However, still more needs to be learned about this pest.

O. nubilalis in the United States

The random mating by *O. nubilalis* in rain-fed landscapes in the United States is described above in the Pest Biology section. Compliance by growers with block refuge requirements is high, but not 100% (Onstad et al. 2011). Onstad and Gould (1998) based their model on unpublished and never-described preliminary measurement of larval movement and survival. Davis and Onstad (2000) performed a field study to evaluate the Onstad and Gould model and concluded that the amount of plant-to-plant movement and differential survival by *O. nubilalis* were likely significant enough to make seed blends riskier than separate blocks. Several years later, Kang et al. (2012) measured survival and larval movement before feeding more carefully. They concluded that for *O. nubilalis*, the survival rates of susceptible homozygotes leaving insecticidal corn and refuge corn were the same (Kang et al. 2012). Burkness et al. (2011) determined that feeding on the cross-pollinated ears resulted in only moderate (40–57%) reduction in survival caused by pollen from Bt11 (Cry1Ab) sweet corn.

Kang et al. (2012) were the first to explicitly model the effects of cross-pollination of refuge plants on IRM. They concluded that because 1) few *O. nubilalis* larvae move to the corn ear, 2) feeding on ear occurs only in one of two annual generations, and 3) survival on cross-pollinated ears in blended refuge is only moderately reduced, cross-pollination is not an important issue for IRM against *O. nubilalis*.

Kang et al. (2012) assumed that no natural refuge exists in the modeled rain-fed corn landscapes. On the basis of all the assumptions described here, they demonstrated that the value of blended refuge is similar to that of block refuge. Given that mating is non-random in dry, irrigated landscapes (described above), blended refuge should be even more valuable for IRM under these conditions.

D. v. virgifera in the United States

It is not easy to study movement of larvae that are underground. However, Hibbard et al. (2003, 2004, 2005) overcame these difficulties and measured the movement of *D. v. virgifera* larvae in a series of experiments. They concluded that two movement periods were possible during the larval stage. Onstad (2006) analyzed their data to create parameters for his model of the beetle in seed blends, but because of the variability in the observations he did not believe that the data provided clear evidence of secondary movement after the first establishment of the larvae. Therefore, he used the general approach introduced when modeling *O. nubilalis* (Onstad and Gould 1998). Onstad (2006) concluded that seed blends were better than block refuges when all mating occurs in the natal field.

In 2007–2008, Murphy et al. (2010) observed more adults emerging from block refuge plants than from blended refuge plants when the refuge was 20% of the Cry3Bb1 cornfield, but the difference declined when the refuge was 10%. In 2010–2011, Zukoff et al. (2012) infested the central plant of a three-plant cluster with eggs. They found no significant difference between larvae recovered on the two adjacent refuge plants when the central plant was either insecticidal corn (Cry3Bb1 and Cry34Ab1/Cry35Ab1) or refuge corn at each of the five plant stages studied, indicating that the movement rate off of the two infested plants was similar.

Pan et al. (2011) created a more realistic model using new data on adult and larval behavior. They used the single-movement model for larvae (Onstad 2006) but changed the values of two parameters based on the data of Binning et al. (2010). Pan et al. calculated 100% predispersal tasting survival for larvae starting on insecticidal corn. They also calculated a probability of movement away from insecticidal corn slightly greater than that for refuge corn. Because adults do not fly very far before mating (Spencer et al. 2013, Hughson and Spencer 2015), mating is not random across large blocks of corn, but it is considered random in seed blends (Hughson and Spencer 2015). Because of this non-random mating and less than full compliance by farmers in planting block refuge, modeling results indicated that the seed-blend scenarios in many cases produced equal or greater durability than block refuges that were relocated each year (Pan et al. 2011).

Thus, for *D. v. virgifera*, simulations have demonstrated significant value in blending refuge. This conclusion was also supported by an economic analysis of *D. v. virgifera* IRM (Onstad et al. 2014).

H. zea in the United States

Survival and Larval Behavior of *H. zea*

Because the larvae of *H. zea* have low rates of movement from plant to plant in corn (Burkness et al. 2015), the main concern about corn earworm in seed blends is the influence of cross-pollination and intermediate doses of insecticidal traits in fertilized tissue in kernels on refuge corn ears (Crespo et al. 2015, Pan et al. 2016). First, we need to understand how frequently this pest feeds on the various tissues on corn ears. According to Capinera (2017), 'On corn, its most common host, young larvae tend to feed on silks initially, and interfere with pollination, but eventually they usually gain access to the kernels. They may feed only at the tip, or injury may extend half the length of the ear before larval development is completed'. Wiseman (1999) stated, 'Both young and old larvae feed on silks; those that penetrate the ear destroy many of the developing kernels'.

Horner et al. (2003) published observations of larvae on corn ears on Bt and non-Bt corn in the US states of MD and NC. They stated on page 920,

> Furthermore, exposure to the expressed toxin may be related to the condition of silk tissue at the time of egg hatch. Larvae hatching from eggs laid on wilted or brown silks may have a greater chance of surviving intoxication episodes because of reduced toxin expression in the senescent tissue. Plant stress may also contribute to the varied responses among locations. The NC site, which showed the lowest Bt-induced effects, was not irrigated, and thus rapid dry-down of silk tissue and possible acceleration of kernel maturation may have lowered toxin expression. All of these factors together can influence the efficacy of MON810 Bt corn in reducing *H. zea* infestations and ear damage.

Horner et al. (2003) also described how *H. zea* larvae can taste or perceive toxic kernels and choose to feed on non-toxic tissue on corn ears. Thus, commonly observed silk feeding can increase survival on cross-pollinated corn ears. Feeding on the ear tip also provides less or non-toxic maternal tissue for the larvae. Horner et al. (2003, Figure 3) observed wide variation from site to site and year to year in percentage of corn ears with >5 cm² of kernel damage. For refuge ears, the range is 7–48% with an average of circa 20%. Kernel area consumed varied much more from site to site and year to year than within replicates (Horner et al. 2003, Figure 2), suggesting an influence of weather or other variables on *H. zea* feeding.

To understand the impact of cross-pollination on *H. zea*, it is necessary to consider the sequence of events that occurs during the life history of this pest on corn plants (Crespo et al. 2015). During the reproductive stage of corn, *H. zea* is primarily an ear-feeding pest where it lays its eggs mostly on silks. The refuge ear is composed of fertilized kernels and maternal tissues (husk, silk, shank, and cob). Only kernels originating from cross-pollination will express insecticidal traits. *H. zea* first feeds on silks, and the feeding on kernels is more frequent only when insects reach the third instar (Archer and Bynum 1994, Caprio et al. 2016). It is not uncommon to find *H. zea* feeding on aborted ovules or the cob before larvae move to kernels. Bioassays with *H. zea* showed that older larvae were significantly less susceptible to insecticidal protein than neonates (Ali and Young 1996). As a consequence, it is likely that *H. zea* larvae become less susceptible to an insecticidal trait as they mature; and the selection differential in favor of more resistant insects also declines. Development of *H. zea* larvae feeding on blended refuge plants was not significantly delayed compared with the development of larvae in block refuge (Crespo et al. 2015).

It is also possible that a larva could avoid lethal exposure by feeding exclusively on maternal tissue in the ear tip or feeding only on kernels that do not express Bt proteins (Horner et al. 2003, Crespo et al. 2015). *H. zea* moves extensively among kernels (Caprio et al. 2016). The pattern of kernel damage caused by intoxicated *H. zea* on MON810 Bt corn was characteristically different with spatial patterns of kernels damaged showing scattered, discontinuous patches of partially consumed kernels, which were arranged more linearly than the compact feeding pattern on non-Bt ears (Horner et al. 2003). Bioassays with Cry1Ac and Cry2Ab revealed that *H. zea* selected a diet with lower concentrations of Cry1Ac, but the avoidance of Cry2Ab was not as noticeable as that observed for Cry1Ac (Gore et al. 2005). Therefore, the survival by *H. zea* depends upon larval-feeding behavior, including 1) the detection and avoidance of Bt proteins, 2) the make-up of proteins in a pyramid of two or more insecticidal traits, and 3) susceptibility of the larvae to the toxin (Crespo et al. 2015).

Survival of *H. zea* Feeding on Cross-Pollinated Refuge Corn Ears

For *H. zea*, the information from 14 published, replicated field trials that measured survival of larvae on refuge plants adjacent to Bt corn demonstrates that the effect of cross-pollination on larval survival varies over time and location (Table 3). We believe that the effects of cross-pollination and our ability to observe these effects are likely influenced by many factors including weather, corn hybrid, cultivation practices, *H.zea* behavior, and synchrony of insect flights with a certain plant development stage that vary from season to season and site to site. Furthermore, the susceptibility of *H. zea* to insecticidal traits differs substantially across locations (Siegfried et al. 2000). For a long-term study of resistance evolution, the mean effect will likely give us a reasonable understanding of consequences for evolution and IRM. In the southern United States, cross-pollination affects larvae only in the second generation on corn and only in one of the

Table 3. Summary of replicated field studies measuring survival of *H. zea* on cross-pollinated corn ears in blended refuge relative to survival on ears pollinated in blocks of pure refuge corn

Infestation	Location	Insecticidal traits[a]	Relative survival[b]	Reference
Natural	Minnesota	A	0.63	Burkness et al. 2011
Artificial	Minnesota	L	1	Burkness et al. 2015
Artificial	Minnesota	L	1	Burkness et al. 2015
Natural	Georgia	L	0.92*	Crespo et al. 2015
Natural	Mississippi	L	0.58	Crespo et al. 2015
Artificial	Iowa	L	0.83*	Crespo et al. 2015
Artificial	Iowa	L	0.86*	Crespo et al. 2015
Natural	Louisiana	B	1	Yang et al. 2014b
Natural	Louisiana	B	1	Yang et al. 2014b
Natural	Louisiana	B	1	Yang et al. 2014b
Natural	Louisiana	B	1	Yang et al. 2014b
Artificial	Louisiana	B	0.37	Yang et al. 2014a
Artificial	Mississippi	C	0.67	Babu 2013
Artificial	Mississippi	C	0.64*	Babu 2013

[a]Insecticidal traits are A = Cry1Ab, B = Cry 1A.105 × Cry2Ab2 and Cry1F, C = Cry 1A.105 and Cry2Ab2, and L = Cry1Ab × Cry1F × Vip3A.

[b]Proportion surviving on blended refuge plant relative to those surviving on plant in block refuge. For values <1, an asterisk indicates no significant difference between the number of larvae found on refuge plants in block refuge versus refuge plants in blends.

four generations per year selected by insecticidal traits (two on corn, two on cotton).

Table 3 summarizes the published field trials concerning survival in cross-pollinated blended refuges. Yang et al. (2014a) observed only 37% survival in a seed blend with Cry 1A.105/Cry2Ab2 and Cry1F relative to the block refuge. In this field trial, they placed two neonates from a lab colony 'on the top of each ear' after removing all larvae naturally infesting the ears. It is not clear what effect starting the neonates on significantly manipulated corn ears had on feeding behavior. When all 14 field trials are considered, the mean survival is 0.82 (Table 3). Note that 6 out of 14 trials had survival of 1 indicating at least as many larvae surviving on cross-pollinated refuge corn as on the pure refuge. When we separated results by insecticidal trait or type of infestation, no clear patterns were identified (Table 3).

Synthesis of Knowledge about *H. zea*

Pan et al. (2016) created a model of *H. zea* and simulated its evolution in several landscapes of the southern United States. Natural refuge varies from state to state and compliance by farmers with planting a required block refuge is under 50% (Pan et al. 2016). In the model, *H. zea* mates randomly in the crop landscape. Only a small proportion of larvae move from plant to plant (2–16%, Burkness et al. 2015). Using Figure 3 of Pan et al. (2016), we can find the durability of MON810 × DAS-01507-1 × MIR162 in Mississippi when survival due to cross-pollination in corn crops is 0.82 (the mean calculated from Table 3). Durability is estimated to be from 18 to 23 yr depending on the amount of blended corn refuge. This range of durability is essentially the same as that modeled for block refuge with maximum compliance rate of 44% reported by Reisig (2017).

Conclusions

Mallet and Porter (1992) concluded that as IRM researchers investigate real systems, it is likely that the IRM plan will be situation dependent. They stated that 'More information on insect life histories, movement between plants, avoidance of toxins, stage-specific mortalities, possible gene dominance, the effect of multiple loci, and the actual strength of selection are needed. More complex models will also be required to understand more realistic situations'. This conclusion that the use of blended refuge should be considered on a case-by-case basis is supported by others (Onstad et al. 2011, IRAC 2013, Trumper 2014, Carrière et al. 2016). As this review has indicated, farmer behavior must also be considered on a case-by-case basis before developing a refuge strategy.

Even if we cannot a priori determine which pests should be managed with one kind of refuge, can we identify any real systems for which seed blends are not likely to be helpful? If a crop canopy has significant overlap of stems and leaves from adjacent plants, it is likely that pests would feed on both refuge and insecticidal plants. Unless we believe that a pest can constantly monitor its food and sense its substrate and move to refuge as necessary, it is not likely that these crops would provide the right environment for blended refuge. If farmers are unlikely to grow a separate block refuge for economic or cultural reasons, then blended refuge may be the only choice unless significant unstructured, natural refuge exists in the landscape. As Head and Savinelli (2008) indicated, the local cultural and agronomic conditions must be considered when developing an IRM strategy.

The decision to pyramid multiple insecticidal traits is not exempt from the complexities highlighted in our review. Under ideal conditions, pyramids can delay the evolution of resistance relative to the use of single traits. But with regard to refuge deployment and configuration, we believe that pyramids are not unique. They should be evaluated for impact on genotypic survival just like any single insecticidal trait. Then this information should be combined with other knowledge of pest biology and farming practices to make a decision (Carroll et al. 2012).

In any case, IRM is never simple or easy (Carrière et al. 2016). Data on behavior of adult or immature insects cannot be collected inexpensively. Researchers need to remain persistent and dedicated to understanding nature. Given the complexity of the factors influencing the trade-off between blended refuge and separate block refuges, it is likely that modeling will continue to play a key role in the evaluation of refuge strategies.

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