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Agricultural production: assessment of the potential use of Cas9-mediated gene drive systems for agricultural pest control

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ABSTRACT

To highlight how gene drives could be useful for control of agricultural insect pests, we selected species that are pests of animals (New World screwworm), plants (spotted wing *Drosophila*, diamondback moth, *Bemisia tabaci* whitefly), or stored grains (red flour beetle). With the exception of whitefly, routine methods for delivering DNA to the germline and selecting for genetically modified insects have been developed. The traditional approach in agriculture has been to suppress insect pest populations using insecticides and other farming practices. Similarly, we suggest the main use of gene drives in agriculture will be for population suppression through targeting essential genes. We provide examples of gene drives that target specific genes including female-essential genes. Further, we discuss issues related to containment in the laboratory and eventual field testing of strains harboring a Cas9-mediated gene drive system.

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Introduction

Insect pests of crops, livestock, and agricultural products have long been a threat to food production. Global losses due to arthropod crop pests (mostly insects and mites) are estimated to total $470 billion annually (Culliney 2014). Similarly, insects are major economic pests of livestock. For example, in the United States the horn fly is estimated to cause losses of $1 billion annually for the cattle industry (Kunz et al. 1991). Historically, efforts to control these pests and reduce losses have relied on extensive physical labor, changes in agricultural practices or the application of chemicals that kill the offending insects. However, chemical controls are fraught with their own challenges. Some of the most potent pesticides were later discovered to hurt benign and beneficial insects and vertebrates. Meanwhile, target pests develop resistance over time, requiring ever-increasing doses of poison until the pesticide loses all effectiveness. Thus, new pesticides must be

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continuously developed which effectively control the target insect, while limiting the impact on non-target species (Casida and Quistad 1998).

Using knowledge of pest genetics to control pests, either by reducing pest populations or limiting their ability to do damage, is a particularly attractive idea that has historically been limited by technological feasibility. The sterile insect technique (SIT), which involves radiation-induced chromosome breakage, has been successfully used in a small number of species to produce adult males that can be released in the field to mate with wild females that then produce inviable embryos (Klassen and Curtis 2005). To substantially reduce a pest population, this technique requires the continual release of large numbers of irradiated males. Thus SIT is particularly effective if the pest population is low. A number of more complex genetic approaches for manipulating pests were described and explored theoretically and empirically, starting in the 1940s (Klassen and Curtis 2005). For human disease vectors, an alternative approach to eradication is to replace the population with insects that cannot transmit disease as they carry a gene refractory to transmitting a human pathogen. Curtis suggested the concept of linking the refractory gene to a genetic element (translocation) that could drive the desired gene into a wild population (Curtis 1968). Genetic drive elements with super-Mendelian inheritance (more than 50% of the offspring inherit the genetic element) abound in nature, ranging from sequences capable of replicating themselves in a genome (e.g. transposons, aka 'jumping genes') to systems that kill any offspring that fail to inherit the element (e.g. Medea; Beeman, Friesen, and Denell 1992). However, adapting natural gene drives has proven to be technically difficult as the mechanisms are not generally well understood or easily adapted. Nevertheless, artificial drives based on the principles of natural elements have been developed such as synthetic Medea (Chen et al. 2007). Artificial drives based on the CRISPR/Cas9 gene-editing system seem the simplest and most effective to build. Briefly, Cas9 is an RNA-guided nuclease discovered in bacteria to function as part of an adaptive immune system (Bhaya, Davison, and Barrangou 2011). Short RNA sequences guide Cas9 to a target, where Cas9 introduces a double-stranded break. Given appropriate guides, Cas9 is also capable of introducing double-stranded breaks in eukaryotic genomes, including those of insects and vertebrates, where the breaks can be repaired randomly (non-homologous end-joining, NHEJ) or based off a template (homology-directed repair) (Jinek et al. 2012; Mali, Esvelt, and Church 2013). The latter mechanism can be exploited to introduce the coding sequence for Cas9 and guides into the genomic site targeted by those guides. This results in a self-replicating insertion that could continuously mutate a target gene every generation or carry an introduced gene into the population (Esvelt et al. 2014). More details for the design of Cas9 gene drives are discussed elsewhere in this volume (Min, Smidler, and Esvelt 2017).The purpose of this paper is to discuss the potential use of Cas9-mediated gene drive systems for control of several agricultural insect pests.

Gene drive systems and agricultural insect pests

One of the simplest goals for gene-based pest control would be population suppression, i.e. decreasing the number of insects in an area, thereby reducing the damage done to crops or livestock. One way a Cas9-based gene drive could be used for population suppression is to target a gene that is essential for survival in the field but unimportant in a rearing facility

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(e.g. a gene essential for vision). Alternatively, a Cas9-based gene drive could be placed within a gene necessary for female development, survival, or fecundity. In theory, releasing a few insects carrying a very efficient drive could be sufficient to suppress a pest population after many generations. In practice, growers would likely desire a faster response, which could be achieved by releasing more insects carrying the gene drive. For example, a ratio of 1:10, modified:wild-type insects could lead to pest suppression in 10–20 generations. However, rearing a large number of insects carrying a gene drive that targets an essential gene for females would be difficult in practice, since the drive would be expected to suppress the captive population. The simplest way to overcome this limitation would be to manually cross the males of the drive strain back to wild type (non-drive) virgin female insects each generation, but this would also be very labor intensive. It may be possible to obtain sufficient virgin females through mechanical sorting of males and females at an early stage of development. Sorting could be based on differences in size between the sexes (Carvalho et al. 2014) or sex-specific expression of a fluorescent marker gene (Catteruccia, Benton, and Crisanti 2005).

We suggest that an attractive alternative is to control Cas9 activity such that it can be suppressed in the insect rearing facility, but would be active in the field. There are a variety of conditional expression systems that can be used to switch Cas9 production on or off. The most effective systems involve the use of a chemical added to the insect food which blocks gene activation. For example, the tetracycline transactivator (tTA)/tetO conditional expression system (Gossen and Bujard 1992), which uses tetracycline in the insect diet to suppress gene expression, is currently used for suppression of female-lethal genes in engineered strains designed for genetic control programs (Koukidou and Alphey 2014; Scott 2014), as will be discussed below. The protein, tTA, binds a very specific genetic sequence, tetO, and can activate genes found down stream of tetO sequences. However, in the presence of tetracycline, tTA does not bind to DNA. An example of the design of a conditional Cas9 gene drive system is shown in Figure 1.

It should be noted that conditional expression systems, like tTA/tetO, would not prevent accidental release of modified insects (Akbari et al. 2015), a particular concern for gene drives, since the drive would be active when the insects feed on a diet that lacks tetracycline (i.e. most food options found in the wild). However, if tTA was replaced with reverse tTA (rtTA) (Urlinger et al. 2000), the system would only drive on diet that contains tetracycline, as rtTA will only bind DNA in the presence of tetracycline. This could provide a means for containment while evaluating gene drive systems, in addition to physical barriers and other strategies (e.g. split drive with Cas9 and gRNA components separated) (Akbari et al. 2015). In addition to the proposed containment strategies for evaluation of gene drives, we also support the parallel development of reversal drives with recoded target genes (Esvelt et al. 2014). As discussed elsewhere in this volume (Burt et al.), there are several issues that would need to be addressed through studies in containment (e.g. influence of genetic background) prior to consideration of a field release. Further, we have focused only on using Cas9-mediated gene drives for population suppression. They could also be used to drive anti-pathogen genes into an insect population that carries diseases. While there may be cases where this would be more useful than population suppression, primary damage from pest insects is typically the greater concern for agriculture. The purpose

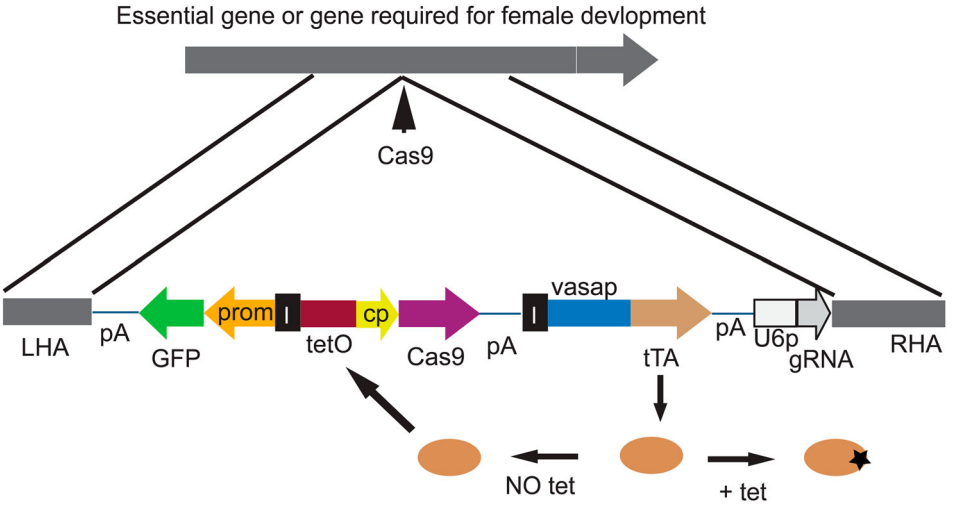

Figure 1. *A conditional Cas9-mediated gene drive*. The tetracycline transactivator (tTA) is expressed in the germline using the *vasa* gene promoter. In the absence of tetracycline in the diet, tTA will bind to tetO and activate expression of Cas9. Expression of a gRNA complementary to an essential gene is controlled using a U6 gene promoter. Following Cas9 digestion of the essential gene, homology-directed repair will lead to insertion of the multi-gene cassette bracketed by left (LHA) and right (RHA) homology arms. A GFP marker gene would facilitate initial identification of genetically modified insects. Insulator elements (I) prevent tTA bound to tetO from enhancing its expression from the vasa gene promoter, which could reduce fitness. Additional containment measures should be used for drive evaluation such as physical barriers and splitting the drive by not including the vasa-Cas9 or U6-gRNA gene in the construct shown.

of this paper is to examine the potential for using gene drives for population suppression of selected agricultural pests.

The new world screwworm: a devastating obligate parasite of livestock

The New World screwworm fly, *Cochliomyia hominivorax* is a serious pest of warm-blooded animals (Krafsur 1998; Alexander 2006). Females lay their eggs in open wounds or a natural orifice. The hatched larvae then feed on the animal's living tissue. Animals with severe screwworm infestations may die if untreated. While most cases are less severe, they can still have an economic impact (Vargas-Teran, Hofmann, and Tweddle 2005), as the animal suffers weight loss, and carcasses and hides are damaged. Screwworm populations are limited in their range by cold seasonal temperatures: the insect cannot survive freezing temperatures and cannot overwinter successfully under temperate conditions. The pre-eradication range was between 35 and −35 latitudes in the Western Hemisphere.

Edward Knipling realized that if large numbers of sterile males could repeatedly be released into wild populations, it would eventually eliminate population reproduction and lead to eradication (Knipling 1960, 1955). This genetic control method is now generally referred to as the SIT. The program initiated by Knipling and his colleague, Raymond Bushland, began with releases of sterilized insects in Florida in the late 1950s.

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Subsequently, the SIT approach was used to eradicate screwworm from all of the USA (Mastrangelo and Welch 2012). However, Texas farmers faced an ongoing threat of invasion of screwworm from Mexico. To alleviate this threat, SIT was used to eradicate the fly from that country in a joint program with the Mexican government. Subsequently, the program was extended to eradicate screwworm from all of Central America (Vargas-Teran, Hofmann, and Tweddle 2005). Currently, to prevent re-infestation from South America, sterilized flies are being released in a 'buffer zone' in Eastern Panama and along the border with Colombia (Scott et al. 2017). The screwworm mass rearing facility is in Pacora, Panama, and is run by the U.S.-Panamanian Commission for the Eradication and Prevention of Screwworms (Comisión Panamá–Estados Unidos para la Erradicación y Prevención del Gusano Barrenador del Ganado or COPEG). About 15 million flies are reared each week. The annual producer benefit from eradication of screwworm is estimated to be $1.3 billion annually (Vargas-Teran, Hofmann, and Tweddle 2005). Due to the success of the screwworm SIT program, SIT has since been used for control of several species of tropical fruit flies (e.g. Mediterranean fruit fly), olive fly, and some Lepidopteran species (e.g. codling moth) (Klassen and Curtis 2005). So why was the screwworm SIT program so successful? Are there any lessons for future control programs based on Cas9 gene drive systems?

SIT is often said to be a 'numbers game' because success depends on the released sterile males outnumbering the wild-type males, preferably by at least 10:1 (Klassen and Curtis 2005). Fortunately, the screwworm fly is naturally present at low densities (Knipling 1955). In addition, economical mass rearing methods were developed (Melvin and Bushland 1936; Chaudhury and Skoda 2007), sterilization by radiation had only a small negative effect on male fitness (Crystal 1979), and there were no significant mating barriers across the eradication zone (Lachance et al. 1982). More recently, efficient methods were developed for distributing chilled sterile flies by air using modern GPS-guided navigation (Tween and Rendon 2007; Scott et al. 2017). Other factors that contributed to the success of the program that we think are also applicable to future gene drive programs include:

- The technology was never seen as a 'silver bullet' that by itself would eradicate the pest population. Within the eradication zone a large monitoring network of veterinarians and others was established, and any infested animals were identified and promptly treated (Mastrangelo and Welch 2012).
- As the eradication zone spanned several countries, agreements between neighboring countries were essential, as insects will cross national boundaries (Wyss 2000).
- The eradication program was effectively managed by a team that did not have other responsibilities.
- From the beginning, the screwworm program sought to engage the public and address any concerns regarding the release of radiation-sterilized flies.

Given the success of using SIT to eradicate screwworm, should we consider developing gene drive systems for agricultural pest population suppression? Screwworm remains a problem throughout most of tropical South America and on some Caribbean islands, notably Cuba and Jamaica (Scott et al. 2017). It may not be necessary to use a gene drive system for eradication and/or suppression of populations on islands and in

regions in South America west of the Andes. In these locations, a more efficient SIT approach could be successful. The efficiency of SIT can be increased several fold by releasing only sterile males (Rendon et al. 2004). However, to date this has not been possible in the screwworm program. Recently, the corresponding author's laboratory, along with USDA-ARS collaborators, have succeeded in creating transgenic 'male-only' screwworm strains that carry a conditional female-lethal gene that is suppressed by addition of tetracycline to the larval and adult diets (Concha et al. 2016). The best of these strains are now being evaluated under semi mass rearing conditions. Field trials of sterilized male flies have also been planned, but will require regulatory approval.

An efficient SIT program may not be able to eradicate or suppress screwworm populations east of the Andes, which presents a considerable geographic area with ideal habitat for screwworm. With no appreciable geographic barriers, there would be little to stop wild-type flies from invading an area being targeted by aerial releases of sterile screwworm flies. In contrast, the eradication of screwworm from North and Central America benefited from a narrowing of the eradication zone as the program moved progressively southward from Texas to Panama. In South America, a Cas9 gene drive might prove more useful; flies carrying an efficient gene drive system may only have to be released once throughout the region to potentially serve as an effective means for population suppression. Consequently, prior to release, approval should be sought from all countries that have endemic screwworm flies, regardless of whether they see actual releases, since the drive effect will spread beyond the original release zones over the course of multiple generations. In contrast, for the Screwworm Eradication Program, it was only necessary to obtain agreements with countries over which sterile flies were to be released (Wyss 2000).

What gene should be targeted for a Cas9 gene drive in screwworm? Modeling suggests that it would be advantageous to target genes that are essential for females but not males, or required for germ-cell development or reproduction in one sex (Burt 2003; Deredec, Burt, and Godfray 2008). One possibility would be to target the *transformer* (*tra*) gene, which is essential for female development in screwworm, as in most Diptera (Li et al. 2013). An unknown Y-linked male-determining gene switches off *tra* in males (Figure 2). In females, *tra* switches the *doublesex* and *fruitless* genes into their 'female-mode', which leads to female development and behavior (Scott, Pimsler, and Tarone 2014). In the absence of *tra* activity, XX females develop as phenotypic males. We don't know if XX males are fertile, but in the Australian sheep blowfly, a close relative of screwworm, the Y chromosome does not appear to encode any factors essential for male fertility (Concha and Scott 2009). Thus it is possible that transformed XX screwworm males will be fertile. If so and the XX males are competitive, a gene drive that would transform all females to males could be very effective for suppression of the insect pest population. One potential problem with this approach is that *tra* may play an essential role in the female germline. If so, it would be desirable to limit Cas9 expression, and thus gene drive, to the male germline, assuming a suitable gene promoter can be found.

What would be the ecological impact of screwworm eradication from the Western hemisphere? Screwworm is an ectoparasite of warm-blooded animals, including humans. The eradication of screwworm from Texas correlated with an increase in white-tailed deer populations. However, other factors such as the absence of large predators (Wolverton, Kennedy, and Cornelius 2007) also likely contributed to the increase in

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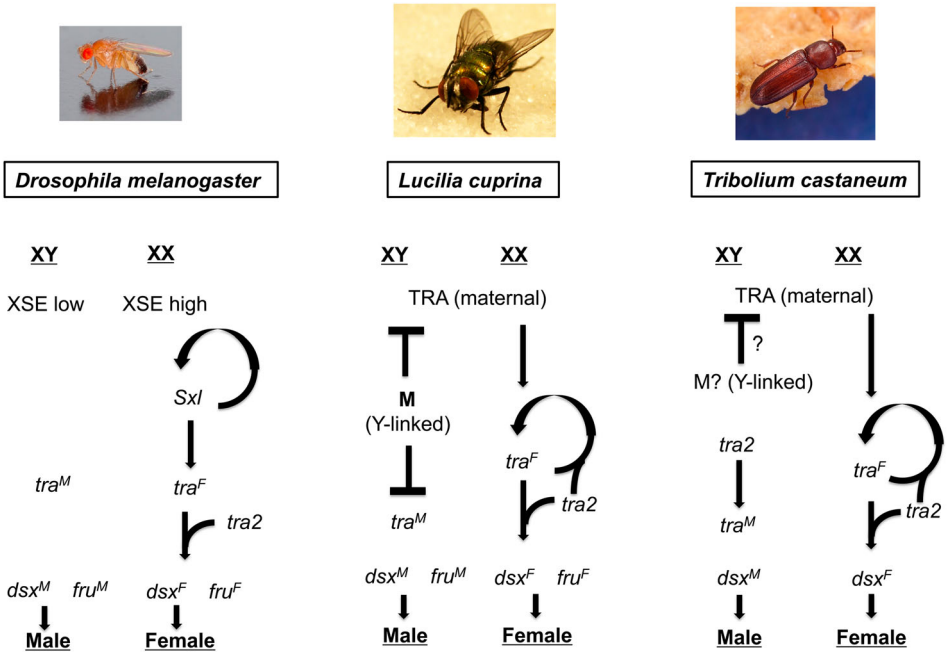

Figure 2. *A comparison of the genetic mechanisms that determine sex in Drosophila melanogaster, Lucilia cuprina and Tribolium casteneum*. In female *D. melanogaster* embryos, a high dose of X signal elements (XSE) leads to activation of the master gene *Sex lethal* (*Sxl*). *Sxl* both autoregulates its expression and switches *transformer* (*tra*) RNA splicing into the female mode. Only the female form of *tra* mRNA codes for functional protein. TRA combines with TRA-2 to switch *doublesex* (*dsx*) and *fruitless* (*fru*) into the female modes of splicing. DSX and FRU control the expression of many genes that influence sexual development and behavior. The bottom of the regulatory hierarchy in *L. cuprina* appears to be the same as *Drosophila*. However, it is *tra* RNA splicing that is autoregulatory in females. The presence of the Y-linked M factor inhibits the female-mode of *tra* RNA splicing in male embryos. As in *Drosophila*, only the female form of *tra* mRNA codes for functional protein. In female embryos, maternal TRA likely contributes to the initiation of the female mode of *tra* splicing. The sex determination regulatory mechanisms appear to be identical in *L. cuprina* and *C. hominivorax*. Remarkably, the genetic mechanisms that determine sex in the red flour beetle, *T. casteneum*, appear to be very similar to the *L. cuprina* regulatory hierarchy. Adapted from Scott, Pimsler, and Tarone (2014) and Shukla and Palli (2013).

deer populations in Texas, as well as across North America (VerCauteren 2003). There is insufficient literature regarding the ecological impact of eradication of the screwworm, which likely reflects that researchers have placed the primary focus of their limited resources on the interaction of the pest with livestock rather than with native hosts. Nevertheless, there is an opportunity to carry out ecological studies on screwworm and its hosts in the large Caribbean islands and/or South America before commencing a screwworm eradication/suppression program. Further, the screwworm experience highlights the need for more basic ecological studies for other pest insects before and after a population suppression program.

Lastly, there is a potential for resistance to develop to a Cas9-based gene drive system (Esvelt et al. 2014; Unckless, Clark, and Messer 2017). For example, resistance could arise due to repair errors or rare alleles with nucleotide polymorphisms in the PAM sequence. Consequently, it may be advantageous to follow a gene drive release with a male-only SIT,

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if the gene drive is effective at significantly reducing the population in a few generations. SIT is particularly effective if the population size of the pest species is low (Klassen and Curtis 2005). Thus the gene drive could provide a means for suppressing pest populations to the low levels at which SIT would be effective and economical. Further, SIT could eradicate the pest before resistance to the drive mechanism leads to a rebound in the population.

Spotted wing *Drosophila*: an invasive pest of soft-skinned fruits

Drosophila suzukii (commonly called spotted-wing *Drosophila*) larvae are capable of infesting a wide range of host fruit but appear to be most significant pests in stone fruits (peach, cherry, and plum) and berries (raspberries, blackberries, blueberries, and strawberries) (Lee et al., "In Focus," 2011; Asplen et al. 2015). Unlike most other *Drosophila* species, female *D. suzukii* use their highly developed serrated ovipositor to pierce the skin of soft fruits and lay their eggs inside the fruit (Lee et al., "The Susceptibility of Small Fruits," 2011). *D. suzukii* has a short generation time and multiple generations per year (Tochen et al. 2014). In contrast, the larger tephritid fruit flies (*Rhagoletis* sp.) native to North America and Europe have only one generation a year. *D. suzukii* is endemic in Asia but in 2008 the fly was found in California and Europe. Since then, *D. suzukii* has spread rapidly and is now found in temperate regions in North America and Europe (Cini et al. 2014). In the USA, any fruit that contain developing *D. suzukii* larvae can cause an entire shipment to be rejected. Growers are currently using broad-spectrum insecticides to protect fruit from damage caused by *D. suzukii*. Growers in North Carolina are using more insecticide to manage this invasive fruit fly, but the effectiveness of these treatments is weather dependent (Diepenbrock et al. 2016; Diepenbrock, Hardin, and Burrack 2017). It is also anticipated that *D. suzukii* will develop resistance to some of the more commonly used insecticides. Non-chemical means for controlling *D. suzukii* are needed.

Since several tropical fruit flies (e.g. the Mediterranean fruit fly) have been successfully controlled using SIT (Enkerlin 2005), the corresponding author's lab and others are working on developing transgenic male-only strains. As discussed above for screwworm, these strains could be used for an efficient SIT program. Further, if the strains carry dominant female-lethal genes, it would not be necessary to sterilize the males before release. This is because all of the female offspring of the released males will die and the male offspring will pass on the dominant female-lethal gene. However, since any fruit containing a single *D. suzukii* maggot (wild type or transgenic) would be rejected, it is unclear if this approach would be acceptable to growers. Assuming a transgenic strain can be made with the desired properties and is approved by regulators, would an efficient SIT program be successful and economical for controlling *D. suzukii*? We are not aware of accurate measurements of *D. suzukii* population sizes, but trapping data suggest populations can be high, particularly in the warmer months. Consequently, a genetic control program would most likely be attempted early in the growing season when populations appear to be relatively low and in conjunction with intensive application of conventional methods for controlling *D. suzukii*.

As discussed above for screwworm, a Cas9-mediated gene drive targeting an essential gene could provide an effective means for population suppression. A high-quality

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reference genome sequence for *D. suzukii* is available (Chiu et al. 2013), which will facilitate gRNA design. Germ-line transformation of *D. suzukii* is relatively straightforward using procedures developed for *D. melanogaster*, a close relative that is a widely studied model genetic organism. Further, the engineering should be easier than for screwworm, as genetic tools developed for manipulating *D. melanogaster* should function in *D. suzukii*. Indeed, we have found that the *D. melanogaster vasa* and *U6:3* gene promoters can be used to drive expression of Cas9 and gRNAs respectively in *D. suzukii* embryos (Li and Scott 2016). We have shown that Cas9 can be used to make loss of function mutations in the *D. suzukii white* and *Sex lethal* genes. The latter would appear to be a good target for a gene drive system since *Sex lethal* is essential for female *Drosophila* but not male. However, in *D. melanogaster Sxl* is required for female germ-cell identity (Schupbach 1985). If drive is very efficient, females would all be sterile. This would require that drive occur in the male germline. However, since the *Sxl* gene is on the X chromosome, if males carrying the mutant *Sxl* gene with the Cas9 gene cassette are mated with wild-type females, all of the male offspring will inherit a wild-type X chromosome. Thus the gene drive could not occur. An alternative would be to target the *D. suzukii tra* gene, which is essential for female development but not required in the female germline (Schupbach 1982) and is not on the X chromosome. The ecological consequences of *D. suzukii* suppression in North America and Europe are unknown. However, since it is a relatively recent invader from Asia, the ecological impacts are not be predicted to be significant.

In areas where *D. suzukii* is a recent invader and localized, such as on an island or around a port/airport, it may be reasonable to deploy a gene drive strategy aimed at population eradication rather than population suppression. For recent incursions, populations may be sufficiently localized and genetically homogeneous to achieve an eradication outcome. As for any biological control project, species specificity of the gene drive construct would need to be ensured. As discussed above, the gene drive would target a gene essential for female development or viability. Thus, the drive should be self-limiting. However, given the invasive nature of *D. suzukii*, additional measures should be taken to ensure containment of the drive and eradication of the species only in the non-native range. For example, both human-assisted and natural movement must be considered (Webber, Raghu, and Edwards 2015).

Diamondback moth

The diamondback moth (DBM), *Plutella xylostella*, feeds on plants in the mustard family (*Brassicaceae*). These plants include cabbage and an array of vegetable crops related to it (e.g. broccoli, collards, cauliflower, Brussels sprouts). Beyond vegetables, this moth also feeds on canola and wild mustards. The global economic cost of the DBM is estimated at between $4 and $5 billion, annually (Zalucki et al. 2012).

The DBM cannot overwinter in cold climates but has continuous generations in more tropical areas. Because each generation can be two-weeks to a month and each female can lay a total of approximately 150 eggs, the caterpillar densities on a crop can increase rapidly. Although the DBM has many biological control agents, the typical response to an infestation is application of synthetic insecticides, often applied more than once a week (Grzywacz et al. 2010). The DBM is notorious for evolving resistance to synthetic insecticides, with loss of control often emerging within a few years of commercialization

of a new class of insecticide (Wang and Wu 2012). Although DBM cannot withstand cold winters, it is still a major, if sporadic, pest in temperate geographic areas because of its ability to migrate hundreds of miles, and in some cases over a thousand miles (Chapman et al. 2002; Fu et al. 2014). Broccoli has been genetically engineered to produce toxins from *Bacillus thuringiensis* (Bt toxins) that are effective against many DBM populations, but commercialization does not appear to be likely in the near future (Grzywacz et al. 2010). It is also important to note that the DBM was the first insect found to have resistance to Bt toxins. This occurred based on overuse of *B. thuringiensis* bacterial spores as a pesticide in Hawaii (Tabashnik 1994).

The problem of crop losses due to DBM, as well as concerns about human health and environmental impacts of insecticide use have persisted for over four decades (Talekar and Shelton 1993; Furlong, Wright, and Dosdall 2013). This made the concept of using area-wide suppression based on SIT (Knipling 1955) very appealing for this pest. Sutrisno and Hoedaya (1993) reported on early efforts at inducing sterility using gamma radiation of DBM pupae. They found a dose of radiation that could cause substantial sterility in the moths and the offspring of treated moths. They tested the efficacy of this treatment on moth populations, initially using small cages in the laboratory. After success at this level of testing, they moved on to field cages, and then to open field plots. With replicated single 9:1 releases of irradiated to normal males and females they reduced field cage populations by approximately 50% after one generation. In the field experiment the release ratio was 14:1 and the reduction was about 40%. The second generation moths also showed some sterility and in 9:1 releases caused about 20% population reduction. These results were somewhat encouraging. Furlong, Wright, and Dosdall (2013) indicate that there were no successful larger scale tests of this SIT approach with DBM, but it is difficult to discern how much effort was put into follow-up testing because of a lack of available research articles.

With the advent of insect genetic engineering, the British company, Oxitec, developed a strain of DBM in which the female larvae died if they did not have tetracycline in their food (Jin et al. 2013). Release of such a strain should be an improvement over use of irradiated moths, because only males would be released and heterozygous male offspring that carry one copy of the transgene would transmit the female-killing element to half of the next generation of offspring (Schliekelman and Gould 2000; Thomas et al. 2000). In a laboratory experiment, these moths had a fitness of about one half that of moths from a normal laboratory strain (Harvey-Samuel et al. 2014). This decreased fitness is substantial but not as great as expected due to radiation.

Recently, Oxitec and collaborators at Cornell University petitioned the USDA for a permit for a field cage and open field plot test of this female-lethal strain of diamondback moth. The petition was examined and approved based on an environmental assessment (Accessed 18 September 2017 at https://www.aphis.usda.gov/brs/aphisdocs/13_297102r_fonsi.pdf). The experiments themselves were delayed by Cornell University, but field cage experiments were conducted in 2015 (A. Shelton Personal Communication). In 2016 the permit for release was withdrawn by USDA because the original permitting process was flawed (Accessed 18 September 2017 at https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/sa_environmental_documents/sa_environmental_assessments/diamondback-moth-permit-withdrawal). The following year, the USDA released the final environmental assessment with a finding of no significant impact (FONSI) (Accessed 23 September 2017 at https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/brs-news-

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and-information/final_diamondback_moth). With an approved permit, open field tests with the transgenic DBM strain were planned for later in 2017 (Accessed 23 September 2017 at https://shelton.entomology.cornell.edu/diamondbackmoth/diamondback-moth-project-at-cornell-university-faq/).

One of the arguments in favor of the field test was that it would be conducted in upstate New York where the moths could not overwinter and so were expected to be biologically contained (APHIS 2014). The DBM migrates from the Southern USA to the Northern USA and to Canada in the summer, but there are no data indicating that there is return migration (Furlong, Wright, and Dosdall 2013; APHIS 2014). Because some data indicate that once in a field with host plants the DBM moves very little (Shirai and Nakamura 1994; Mo et al. 2003), the argument was made that released moths would not infest neighboring fields (APHIS 2014).

If the assumptions in the USDA APHIS environmental assessment hold, it is very possible that releases of this moth strain in the northern summer range of the DBM could suppress local population growth. A question remains of whether this approach will be appropriate for DBM in areas where they can breed all year or overwinter successfully. While there appears to be limited movement of moths when they are in suitable habitats, they are known for long distance movement at other times (Chapman et al. 2002; Fu et al. 2014), so local releases of the engineered strain could lower numbers temporarily, but this would be expected to have limited value if there was re-infestation from other areas. Furthermore, in the northern areas, the population size in the early summer is very low, making small releases of the engineered strain feasible, but in tropical and sub-tropical areas, the population size tends to remain high, making suppression difficult.

Suppression of DBM with a female-killing strain, even in areas where feasible, would require repeated release of large numbers of moths. This might be less expensive than use of insecticides when resistance to all available insecticides was a problem, but it would require community-level coordination in order to be most effective. Given that resource-poor farmers in tropical and sub-tropical areas are having the greatest problem suppressing DBM, a more efficient, cheaper means of control without associated environmental or health problems would be useful. This is where the use of a gene drive system could be of special interest.

As noted above, the DBM females lay approximately 150 eggs in their lifetimes. This suggests that they have the capacity to increase in density by about 75-fold per generation, given a 1:1 sex ratio and ideal conditions for survival. The actual rate of increase is substantially lower than that, but field experiments have found that DBM populations can triple in a 6-week period (e.g. Riggin-Bucci and Gould 1997). Burt (2003) has shown theoretically that a meiosis-based, gene-drive system with 90% efficiency could reduce survival by 80%. If the maximal rate of increase of the drive in a DBM field population was 5-fold per generation, then such a gene drive could be effective. Burt (2003) and others (Deredec, Burt, and Godfray 2008) outline additional gene drive systems that could be even more efficient and would be theoretically predicted to limit even tropical populations of DBM.

Given the generally limited movement of the DBM adults when host plants are abundant, local release of a suppressive gene-drive strain even at low frequencies could begin to have effects within one season. A suppressive gene-drive approach reverting insecticide resistance to susceptibility (Esvelt et al. 2014) may be a useful mechanism to assist in

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DBM population suppression. Insecticide (including Bt toxin) resistance has been successfully managed in several pest species, often using a combination of high-dose applications, pyramiding of active ingredients, and maintenance of untreated refuges (Ives et al. 2011). Management of insecticide resistance is only possible while the resistance allele frequencies remain low in target populations (Alstad and Andow 1995). DBM is prone to develop insecticide resistance, and most existing active ingredients are no longer useful against this pest (Grzywacz et al. 2010). In many cases, the genetic basis of resistance to these active ingredients has been determined (You et al. 2013). For some of these, it may be possible to deploy a gene-drive approach to return the resistance allele frequency to a level that would allow for management of resistance using other strategies. In the longer term, release of resistance-suppressive gene-drive strains of DBM could form an integral part of an integrative resistance management (IRM) strategy.

A major caveat to the development of gene drives against DBM is that between harvest and the next planting, when there are no host plants locally available, some DBM adults are likely to move long distances. With a gene drive system that has no minimum for release ratio needed to ensure successful drive, there would seem to be no method to contain the suppression strain to a single country, so trans-boundary issues would arise immediately. Before implementing such an approach, it may also be necessary determine if phenotypically-similar, distinct species are present, which could reduce the efficacy of the treatment. Indeed, a new diamondback moth species (*Plutella australiana*) was recently identified in Australia (Landry and Hebert 2013).

The red flour beetle: a worldwide pest of stored grains

The red flour beetle, *Tribolium castaneum*, is a global pest of stored grains and cereals. *T. castaneum* has a relatively short generation time, developing from egg to adult in approximately 4 weeks at 30°C (Sokoloff 1972, 1974, 1977). Eggs are laid in flour or grain and hatch within 3–5 days. Larvae burrow into flour or damaged kernels of grain and will feed for about 2–3 weeks until they become pupae, when they will spend 3–5 days undergoing major physical changes to develop the adult form. *T. castaneum* is a problem for agriculture because it has evolved a number of behavioral traits that help it survive and spread under adverse conditions. The first trait helps *T. castaneum* to deal with food shortages – larvae are cannibalistic and prey on eggs and pupae when food is scarce. This trait not only helps them survive periods of low-food supply, but is also thought to facilitate their adaptation to new environments (Via 1999). Second, *T. castaneum* are capable of broad dispersal. The global distribution of this beetle has long been thought to be due in large part to human conveyance. However, it was recently discovered that adults are strong flyers and actively disperse between spatially separated resources (Ridley et al. 2011). Ridley et al. (2011) also discovered that the majority of beetles caught in their pheromone traps were females. Since a single female can produce thousands of offspring in a relatively short period of time, the migration of mated females is expected to have a major impact on beetle infestations in poorer countries where grain storage is less secure (i.e. not stored in well-maintained grain elevators). Together, these traits make *T. castaneum* an especially problematic pest in developing countries.

T. castaneum and other stored grain pests have traditionally been controlled via methyl bromide fumigation of grain elevators, granaries, and flour mills. However, use of methyl

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bromide has been banned due to its impact on atmospheric ozone (Gareau 2010). Despite having a worldwide ban on methyl bromide, exemptions still exist for the use of methyl bromide as a fumigant, since alternatives are more expensive, labor intensive, and/or simply not viable for particular applications (Fields and White 2002). Since *T. castaneum* is a greater problem in poorer countries, less expensive alternatives are needed. Is traditional SIT a viable option for *T. castaneum* population control? If we consider the key factors that Knipling lists for determining the suitability of a species for SIT-based control (Knipling 1955), *T. castaneum* does fairly well. These beetles (1) are easily reared, (2) have a relatively short generation time, (3) have good dispersal, (4) readily mate, and (5) can have low initial population sizes.

Since infestations in poorer countries are frequently the result of a few beetles invading locally stored grain, and migration of mated females pose a significant problem, skewing sex ratios could be a better option. How could this be accomplished? While there is still much to learn about sex determination in *T. castaneum*, several critical genes have already been identified. The *T. castaneum doublesex* (*Tcdsx*) gene produces sex-specific transcription factors critical for proper production of gametes (Shukla and Palli 2012a). The production of these specific transcription factors produced by *Tcdsx* transcripts is controlled by the proteins encoded by the *T. castaneum transformer* (*Tctra*) and *Transformer-2* (*Tctra-2*) genes. Like *Tcdsx*, *Tctra* functions in a sex-specific way, but unlike *Tcdsx*, only the female version of *Tctra* RNA produces an active protein (Shukla and Palli 2012b). Interestingly, the female-specific TcTRA protein is necessary to maintain its own production (Shukla and Palli 2012a). TcTRA-2 is necessary for the sex-specific versions of both *Tcdsx* and *Tctra* (Shukla and Palli 2013). Because of their important roles in regulating sex determination and reproduction, each of these genes could be targeted to control pest populations. However, the most useful gene is likely to be *Tctra*. Reduced expression of this gene has little effect on males, but females are masculinized, and no female progeny are produced (Shukla and Palli 2012b). Skewing sex ratios could serve a number of purposes. SIT is more effective when only males are used (Rendon et al. 2004). Using males that can only produce male progeny would also be effective at reducing pest populations. If this male-only mutation is coupled with gene drive, then a self-maintaining population control can easily be established. Penetrance of this approach in target populations is dependent on random mating, so its success may be impacted by the high levels of pre- and post-zygotic reproductive incompatibilities present in *Tribolium* species, which can lead to insipient speciation events (Wade, Chang, and McNaughton 1995; Demuth and Wade 2007a, 2007b).

Despite its pest status, *T. castaneum* has emerged as a sophisticated model system. Since *T. castaneum* has a wealth of genetic and genomic tools, it is a logical choice for testing the feasibility and economic value of Cas9-mediated gene drive in a coleopteran. Among these tools is a sequenced and well-annotated genome (Tribolium Genome Sequencing Consortium et al. 2008), which provides the necessary sequences for Cas9 targeting. Furthermore, a number of essential genes have been identified (Lorenzen et al. 2007; Trauner et al. 2009; Ulrich et al. 2015), and their RNAi phenotypes characterized by the iBeetle Project (Ulrich et al. 2015), establishing which candidate genes are the most efficient to target for population reduction.

Interestingly, *T. castaneum* also possesses naturally occurring selfish genetic elements, known as *Medea* elements (Beeman, Friesen, and Denell 1992), which have the ability to

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drive tightly-linked genes into non-*Medea* populations. For example, a *piggyBac* transposon that contains the enhanced green fluorescent protein (EGFP) gene inserted very close to the *Medea-1* element (~1 centiMorgan away) (Lorenzen et al. 2008), has been shown to be inherited at super-Mendelian rates (5-fold higher than predicted) (Lorenzen et al. 2007). *Medea* might also provide a more stable and reliable gene-drive mechanism than Cas9-based systems, and efforts are currently underway in the Lorenzen lab to clarify this element's mode of action, and to develop its use for pest control. Moreover, the existence of these elements in natural populations gives us an unprecedented opportunity to observe the stability and persistence of gene-drive elements as they spread through wild populations, work that cannot currently be done with transgenic mechanisms.

All of this research will be important in providing safer, cheaper methods for controlling *T. castaneum* in the developing world. *T. castaneum* research will also be important in developing Cas9-mediated gene-drive systems for beetles of greater economic importance, but with fewer genetic and genomic resources. Serious agricultural pests, such as the Western corn rootworm, *Diabrotica virgifera virgifera*, currently lack the genomic tools available to *T. castaneum*, so advances in using Cas9 gene drive in *T. castaneum* will lay important groundwork for similar research in these more critical pests.

Whitefly and other vectors of plant diseases

Plant sucking insects in the order Hemiptera include some of the world's most important insect pests in agriculture. Their impact results not only from stress and damage caused by feeding, but also from the plant pathogens they transmit while probing plant tissue and feeding. Notable examples include: *Diaphorina citri*, vector of the bacterium *Candidatus* Liberibacter asiaticus which causes citrus greening disease, also known as huanglongbing (Hall et al. 2013); *Nilaparvata lugens,* one of the most important pests of rice because of its feeding damage and transmission of viruses (Bottrell and Schoenly 2012); and *Bemisia tabaci*, a species of whitefly known to feed on over 500 host plants, and to transmit many viruses from multiple genera, including *Begomavirus*, *Torradovirus*, *Ipomovirus*, and *Crinivirus* (Byrne and Bellows 1991; Luan et al. 2014).

Insecticides, and to a lesser extent biological control (parasitoids), have been used to control many species of Hemiptera. The plant-feeding habits of many of these insects make it likely that future population control strategies are likely to include the use of plant-based systemic RNA interference (RNAi) to some extent (Upadhyay et al. 2011). Basic functional genomics tools still need to be developed for many of these species to aid in identifying genes that can act as targets for plant-based gene silencing strategies. Genomic studies that elucidate the physiological genetics of Hemipterans will be important in designing and producing plants that are resistant to these insects and the diseases they carry. In particular, plant-feeding Hemiptera act as vectors of plant pathogenic viruses through a variety of mechanisms. Some insects essentially only serve as physical carriers of viruses; viruses simply attach to specialized regions of their mouthparts. In other cases, viruses infect the insect, in ways that may or may not involve viral replication, and then circulate within the insect (Whitfield, Falk, and Rotenberg 2015). The high degree of specificity involved with these virus/insect interactions involves a number of insect proteins and genes. However, in most cases, validating the function of these proteins/genes has been difficult because of the absence of genetic technologies that allow

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for rigorous testing of structure/function relationships. Consequently, there has been no confirmed identification of insect receptor proteins responsible for specific interactions with circulative viruses (Blanc, Drucker, and Uzest 2014). Thus, without a better molecular understanding of virus-host interactions it will be difficult to develop plant-resistance mechanisms or gene-drive systems that will inhibit viral transmission. Further, multiple anti-pathogen genes would need to be developed for species such as *B. tabaci* that are vectors for several viruses. For these species, a population suppression strategy is more feasible. While RNAi-based gene downregulation, through ingestion or injection, has proven highly effective in several species of Hemipterans, including *Acyrthosiphon pisum, Lygus lineolaris, Oncopeltus fasciatus, Rhodnius prolixus, N. lugens*, and *B. tabaci* (You et al. 2013), more sophisticated genetic manipulations involving both gene knockout and transgenic technologies are needed for the types of genetic experiments required to validate putative virus-receptor proteins in these insects.

Historically, Hemiptera have not been the focus of efforts to develop genetics-based population control programs like SIT. There are some species, such as *B. tabaci*, that might be amenable to gene drive-based control or eradication strategies for a number of reasons. First, *B. tabaci* is easy to rear and has life history characteristics that make the development and introduction of various genetic technologies highly feasible in the laboratory (David O'Brochta, unpublished results). *B. tabaci* is highly fecund with a short life cycle of about three weeks under appropriate temperature conditions. Its eggs are about 150–200 microns in length, are easily collected, and have a very thin, translucent chorion (outer membrane) that is easily penetrated by the glass needles used to inject genetic technologies (nucleic acids and/or proteins) into developing embryos (Crisione, O'Brochta, and Reid 2015). *B. tabaci* has a long, slow embryonic development requiring approximately nine days to complete. Blastoderm formation, the cutoff for effective injection of most genetic technologies, occurs between the sixth and ninth hours of development (David O'Brochta, unpublished results). This extended embryonic development is convenient for developing embryo microinjection protocols for preblastoderm embryos and performing the various manipulations needed to introduce gene drive or other genetic technologies into the germ-line of insects. Preblastoderm embryo microinjection is currently the only method for introducing genetic technologies into the germ-line of insects. *B. tabaci* have haplo/diploid sex determination, with haploid males developing from unfertilized eggs and females developing from fertilized eggs, making them excellent subjects for genetic studies. Haploidy simplifies a number of genetic analyses and procedures, such as mutant screens and the assembly of complex genotypes involving linked and unlinked loci, among other things. One consequence of haploidy is that Cas9-mediated gene drive would only occur in heterozygous females as the drive mechanism involves copying the Cas9 gene casette to the homologous chromosome (Esvelt et al. 2014), which cannot occur in haploids.

The second reason *B. tabaci* is amenable to gene drive-based control strategies is that, while the species is thought to have originated in Asia, it is currently widely distributed throughout the tropics and is a pest almost wherever it is found (Byrne and Bellows 1991). Because it is an introduced species in most locations, local eradication of *B. tabaci* is likely to be highly desirable and beneficial. In northern climates, it can be a significant pest in greenhouses. These contained environments might make gene drive-based population suppression and control strategies particularly feasible because the

environment is highly controlled and the establishment of the species outdoors is precluded by cold winter temperatures, providing ideal biological containment. Indeed, this setting could be an ideal situation for developing and testing gene drive systems in general, making *B. tabaci* a potential model system for exploring these technologies in meaningful ways, as containment is likely to be a concern during the early phases of development of these technologies (Akbari et al. 2015).

Concluding remarks

SIT has been very successfully used for control of a few major agricultural pest insect species. Transgenic male-only strains that carry dominant female-lethal genes offer a potentially significant improvement over traditional SIT but nevertheless may not be economical for some pest species. Insect pest population suppression via Cas9-mediated genetic drive systems could provide an economical and effective method for control. To achieve suppression, the drive would target genes that are essential for females but not males, or required for germ-cell development or reproduction in one sex. Since targeting such genes would make it difficult to rear a strain in a factory, we argue that drives should be conditional so that drive would take place in the field but not during rearing. For example, by controlling Cas9 expression with the tetracycline transactivator, Cas9 is only produced if the insect diet lacks tetracycline. Construction of modified insect strains with gene drives will benefit from ongoing genome sequencing efforts for rapid identification of gene promoters with the desired properties such as germ-line specific activity. However, a major impediment for some pest species is our current inability to deliver DNA to the germline and select for genetically modified insects.

With a drive mechanism that targets a gene essential for female development or reproduction, far fewer transgenic males would need to be released than strains that carry a conditional female-lethal gene and produce only males when raised on diet that lacks tetracycline. Although both approaches could involve disrupting normal female development, the gene drive approach is more efficient due to the non-Mendelian copying mechanism that spreads the transgene through the targeted population. Although transgenic strains carrying conditional female-lethal genes have been developed for several important agricultural insect pests (Ant et al. 2012; Schetelig and Handler 2012; Jin et al. 2013; Li et al. 2014; Concha et al. 2016), they have not yet been utilized for pest control. For these strains, multiple successive releases of transgenic males in large excess over the wild-type males (e.g. 10:1) would need to be performed to achieve population suppression (Schliekelman and Gould 2000). The need for multiple releases of relatively large numbers of insects makes such genetic control programs relatively expensive. In contrast, only a single release of transgenic males carrying a female-specific gene drive at 1:10 ratio relative to wild type would be necessary to suppress a population after a few generations. The ability to release far fewer males (100× or more) and yet achieve population reduction is a direct consequence of the copying mechanism of the Cas9-mediated gene drive. Consequently, genetic control using a gene drive system should be more economical than the release of males carrying a conditional female-lethal gene.

All of these general conclusions are based on the expectation that the gene drive mechanisms will function as expected and that the decrease or elimination of the target organism will not cause unexpected problems. Therefore, prior to any release of a strain with a

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gene drive it will be important to carry out ecological studies investigating the possible impact of pest suppression. The strains will likely express more than one guide RNA complementary to highly conserved regions of the targeted genes which is expected to decrease the potential for the target species to evolve resistance to the drive mechanism or the female-lethal/fertility gene. Nevertheless, we should anticipate the development of resistance to the gene drive mechanism at some point after the release and have backup plans in case this happens. If a gene drive suppresses a population for a number of years without complete eradication, practioners could become complacent and be caught off guard by rapid resistance evolution. To avoid such a scenario, post-release monitoring for resistance will be needed. If a second strain with a different gene drive mechanism is available, monitoring could trigger release of the second strain. Alternatively, SIT could be used to maintain local population suppression/eradication as the released sterile males would mate with all females in the area, including those that may carry resistance to the initial gene drive.

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Fred Gould is a Distinguished University Professor of Entomology and is Co-Director of the Genetic Engineering and Society Center of North Carolina State University. He studies the ecology and genetics of pests to improve food production and human and environmental health. Dr. Gould conducts research on the application of evolutionary biology and population genetics to enable sustainable use of insect resistant crops. He also does research aimed at development of strategies for using engineered insect vectors of pathogens to decrease human disease. In 2011, he was elected to the U.S. National Academy of Sciences (NAS). He recently chaired the NASEM–NRC committee on 'Genetically Engineered Crops: Experiences and Prospects'.

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Owain Edwards, research at CSIRO has focused primarily on the molecular basis of aphid-host plant interactions, leading to board membership in the International Aphid Genomics Consortium (IAGC) and strong collaborations with the Institute of Zoology (CAS Beijing), Kansas State University (USA), INRA Rennes (France) and BGI Shenzhen (China). His work on aphids has broadened to examine the molecular basis of all aphid interactions with their environment, including the genetic and epigenetic factors controlling aphid polyphenism and the molecular basis of insecticide resistance. Building on his expertise in invertebrate genomics, he leads a CSIRO research program in genetic pest control technologies. Most recently, Dr Edwards was given a leadership role in the development of CSIRO's new Future Science Platform in Synthetic Biology. Within this platform, Dr Edwards oversees projects delivering environmental outcomes including gene drives for biological control, and engineering resistance/resilience into threatened ecosystems.

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