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Combining the high-dose/refuge strategy and self-limiting transgenic insects in resistance management- a test in experimental mesocosms

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Running head: Transgenic insects & Bt resistance management

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Abstract

The high-dose/refuge strategy has been the primary approach for resistance management in transgenic crops engineered with *Bacillus thuringiensis* toxins. However, there are continuing pressures from growers to reduce the size of Bt toxin-free refugia, which typically suffer higher damage from pests. One complementary approach is to release male transgenic insects with a female-specific self-limiting gene. This technology can reduce population sizes and slow the evolution of resistance by introgressing susceptible genes through males. Theory predicts that it could be used to facilitate smaller refugia or reverse the evolution of resistance. In this study, we used experimental evolution with caged insect populations to investigate the compatibility of the self-limiting system and the high-dose/refuge strategy in mitigating the evolution of resistance in diamondback moth, *Plutella xylostella*. The benefits of the self-limiting system were clearer at smaller refuge size, particularly when refugia were inadequate to prevent the evolution of resistance. We found that transgenic males in caged mesocosms could suppress population size and delay resistance development with 10% refugia and 4% - 15% initial resistance allele frequency. Fitness costs in hemizygous transgenic insects are particularly important for introgressing susceptible alleles into target populations. Fitness costs of the self-limiting gene in this study (*P. xylostella* OX4139 line L) were incompletely dominant, and reduced fecundity and male mating competitiveness. The experimental evolution approach used here illustrates some of the benefits and pitfalls of combining mass-release of self-limiting insects and the high-dose/refuge strategy, but does indicate that they can be complementary.

Keywords: Cry1Ac toxin, high-dose/refuge strategy, fitness costs, resistance management, self-limiting insects

Introduction

The damage caused by invertebrate pests accounts for 10%-15% of agricultural production, costing approximately US$8 billion in the United States (Metcalf, 1996), US$17.7 billion in Brazil (Oliveira, Auad, Mendes, & Frizzas, 2014) and US$359.8 million in Australia (Murray, Clarke, & Ronning, 2013). One approach to control pests and maintain sustainable agricultural yields is through the use of biopesticides such as *Bacillus thuringiensis (Bt)*. *Bt* is extremely valuable in modern agriculture. This utility results from the insecticidal crystal (Cry) proteins that have high specificity to particular insect groups and hence low toxicity to non-target organisms (Schnepf et al., 1998). The application of these insecticidal proteins through conventional spray formulations and in transgenic crops can provide effective pest management while maintaining agro-ecosystem biodiversity (Bravo, Likitvivatanavong, Gill, & Soberon, 2011). Nineteen crops and over 60 million hectares of land have been cultivated with biotech crops expressing *Bt* toxins (James, 2014). However, despite the success of genetically modified (GM) crops, a range of pest species have developed increased levels of resistance to *Bt* biopesticides and to the Cry toxins expressed in GM crops (Gassmann, Petzold-Maxwell, Keweshan, & Dunbar, 2011; Kruger, Van Rensburg, & Van den Berg, 2011; Storer, Kubiszak, Ed King, Thompson, & Santos, 2012; Tabashnik, Gassmann, Crowder, & Carrière, 2008; Tabashnik, Van Rensburg, & Carrière, 2009; Zhang et al., 2012; Zhang et al., 2011). While current resistance management strategies have been effective in a range of species (Carrière, Crowder, & Tabashnik, 2010) there is still scope for improvement and development.

75 The cornerstone of resistance management for GM crops is the high-dose/refuge
76 strategy, an approach mandated in several countries. In the high-dose/refuge strategy,
77 one part of target pest population is exposed to high concentrations (high-doses) of
78 toxins produced by *Bt* crops, rendering resistance functionally recessive. When the
79 inheritance of resistance is recessive, only homozygous-resistant individuals (RR
80 genotype) survive on *Bt* crops. Another proportion of the pest population is maintained
81 in nearby refuges of non-*Bt* host plants, providing a reservoir of susceptible alleles (from
82 RS and SS genotypes). If the resistance allele frequency is low, homozygous-resistant
83 pests surviving on *Bt* crops will be relatively rare, while susceptible pests will be
84 abundant and readily available to mate with resistant individuals. Progeny from such
85 matings will be heterozygous for resistance alleles and phenotypically susceptible to
86 high-dose *Bt* crops, thereby hindering the evolution of resistance. Theoretical models
87 and empirical observations have shown that the high-dose/refuge strategy is an
88 effective approach to delay or prevent the development of resistance when the above
89 conditions are met (Alphey, Coleman, Bonsall, & Alphey, 2008; Alstad & Andow, 1995;
90 Caprio, Faver, & Hankins, 2004; Gould, 1998; Gryspeirt & Gregoire, 2012; Huang, Andow,
91 & Buschman, 2011; Hutchison et al., 2010; Tyutyunov, Zhadanovskaya, Bourguet, &
92 Arditi, 2008).
93
94 The high-dose/refuge strategy cannot be applied without regard to its basic
95 assumptions. Certain genetic and ecological conditions need to hold true before it can
96 be used to delay the evolution of resistance. These include: low initial resistance allele
97 frequency; effectively recessive resistance; and efficient dispersal to refugia. The latter
98 condition includes both random mating between the resistant and susceptible
99 genotypes as well as random oviposition on *Bt* crop and in refugia (Burd, Gould, Bradley,

Van Duyn, & Moar, 2003; Frutos, Rang, & Royer, 2008; Liu et al., 2001; Tellez-Rodriguez et al., 2014). Theoretical models and practical experience have shown that violation of these assumptions of the high-dose/refuge strategy can lead to rapid evolution of resistance (Alstad & Andow, 1995; Campagne et al., 2016; Caprio et al., 2004; Georghiou & Taylor, 1977; Gould, 1998; Gryspeirt & Gregoire, 2012; Hutchison et al., 2010; Tyutyunov et al., 2008). In addition, if growers fail to plant refugia then evolution of resistance to GM crops can also be rapid (Farias et al., 2014; Kruger et al., 2011; Monnerat et al., 2015; Storer et al., 2010). Thus, recent incidences of the evolution of resistance to *Bt* toxins in GM crops can largely be traced to failure of the basic assumptions, *i.e.* low doses or non-recessive resistance (Gassmann et al., 2011; Storer et al., 2012) or to the fact that farmers are not adhering to the mandatory refuge planting requirements (Tabashnik, Brevault, & Carrière, 2013).

The high-dose/refuge strategy can be made more resilient through a range of approaches. These include the use of multiple toxins ('pyramiding'), which further reduces the frequency of effective phenotypic resistance (Carrière, Crickmore, & Tabashnik, 2015; Zhao et al., 2005), and through seed mixes or 'refuge in a bag' approaches that enforce farmer compliance (Yang et al., 2014) or manipulation of the fitness costs of resistance using natural enemies or alternative plant varieties (Gassmann, Stock, Sisterson, Carrière, & Tabashnik, 2008; Raymond, Sayyed, Hails, & Wright, 2007; Raymond, Wright, & Bonsall, 2011). Alternative approaches may include the use of transgenic insects to mitigate resistance and to reduce pest population size directly.

Here, we will address experimentally whether the release of transgenic insects to

suppress insect population size is compatible with the high-dose/refuge strategy and can improve its resilience. Recent advances in genetic engineering have enabled the development of transgenic insects carrying a repressible female-specific lethal gene (Thomas, Donnelly, Wood, & Alphey, 2000). In a strategy mimicking sterile insect technique programs, the release of large numbers of transgenic males can reduce target populations, as there will be no viable offspring arising from mating of wild females and transgenic males (Alphey, Bonsall, & Alphey, 2009; Alphey, Coleman, Donnelly, & Alphey, 2007; Gentile, Rund, & Madey, 2015; Thomas et al., 2000). Since these transgenes are designed to reduce insect fitness and will decline in frequency post-release this transgenic approach has been termed 'self-limiting' (Gould, Huang, Legros, & Lloyd, 2008). In addition to suppressing pest population sizes, the mass release of self-limiting transgenic males can affect the genetic make-up of pest populations if lethality is targeted only at females, *i.e.* female-specific self-limiting transgenes. For example, alleles conferring susceptibility to insecticides carried by the transgenic population can be introgressed into the target population through the male line. Deterministic models of the mass release of self-limiting males show that this technology can be a valuable tool in slowing the evolution of resistance (Alphey et al., 2009; Alphey et al., 2007).

Given the importance of the high-dose/refuge strategy for managing the evolution of resistance in modern agriculture, a significant advance would be to understand how best to combine refugia with the use of transgenic insects bearing female-specific self-limiting genes. Theoretically, the mass release of the self-limiting males could facilitate the planting of smaller refugia while still preventing the evolution of resistance (Alphey et al., 2009; Alphey et al., 2007). With increasing release ratios of the self-limiting insects, the mass release of the genetically engineered males could even reverse

150 resistance development (Alphey et al., 2009; Alphey et al., 2007). Smaller refuge sizes
151 may be particularly attractive to farmers who are reluctant to tolerate large refugia or
152 where it is difficult to enforce compliance. The mass release of the self-limiting males
153 could also potentially help tackle issues like non-random mating between resistant and
154 susceptible individuals as a result of different development times and population
155 structure (Cerda & Wright, 2004; Liu, Tabashnik, Dennehy, Patin, & Bartlett, 1999).
156 Local mass release of the self-limiting insects might also, for example, eradicate resistant
157 populations before they become widespread.
158
159 Building on previous work on the high-dose/refuge strategy and the self-limiting insects,
160 we will investigate the interaction between the release of self-limiting transgenic insects
161 and the high-dose/refuge strategy in mitigating the evolution of resistance in model
162 experimental system using the diamondback moth (DBM), *Plutella xylostella*. DBM is a
163 well-known and widespread pest of cruciferous crops. Globally it imposes management
164 costs of US$1.3 billion - US2.3 billion, and causes yield losses estimated at US$2.7 billion
165 per annum worldwide (Furlong, Wright, & Dosdall, 2013; Zalucki et al., 2012). Control
166 failure of DBM is a major concern in agriculture, as this species has developed resistance
167 to almost every insecticide applied in the field as well as resistance to microbial *Bt*
168 sprays (Sarfraz & Keddie, 2005; Tabashnik, 1994). Diamondback moth is also a well-
169 established model for evaluating novel resistance management strategies (Raymond et
170 al., 2007; Zhao et al., 2005). Genetic markers for resistance to the *Bt* toxin Cry1Ac in our
171 resistant line have been well established (Baxter et al., 2011) and this protein can be
172 incorporated into artificial diet at doses that render resistance functionally recessive.
173 Transgenic strains of DBM with female-specific self-limiting constructs have been
174 developed (Jin et al., 2013). Evidence of population suppression by the DBM self-

limiting system has been observed in caged continuous generation studies and low numbers of released self-limiting males have been shown to slow the evolution of resistance to *Bt* in transgenic crucifers (Harvey-Samuel et al., 2015).

Using DBM populations with known frequencies of Cry1Ac-resistance alleles, we tested the compatibility of self-limiting DBM releases with the high-dose/refuge strategy in single-generation and multi-generation experiments. We investigated whether the release of Cry-susceptible self-limiting insects could slow or reverse the evolution of resistance at a range of refuge sizes, release ratios and initial frequencies of resistance. In order to compare experimental results to previous theoretical and experimental work we also characterized the fitness costs associated with transgenic constructs and resistance alleles in our experimental set-up.

Materials and methods

Experimental conditions and insect populations

All insect populations were reared at 25°C (±1°C) and 45% (±5%) relative humidity, with a 12:12 light/dark cycle. The rearing procedure of DBM followed published protocols (Martins et al., 2012). The construction of the self-limiting DBM (OX4319L, Oxitec Ltd) has also been described (Jin, Walker, Fu, Harvey-Samuel, Dafa'alla, Miles, Marubbi, Granville, Humphrey-Jones, O'Connell, et al., 2013). In brief, the self-limiting system has also been implemented in our *Bt*-susceptible line using sequences from the self-limiting gene derived from the *doublesex* (*dsx*) gene of pink bollworm (Jin, et al., 2013). Sex-alternate splicing of this *dsx* sequence allows the development of a female-specific lethal genetic system that is repressible by provision of tetracycline, or suitable

analogues, in the larval feed (Jin, et al., 2013). The OX4319L moths are denoted as genotype LL, where "L" represents the OX4319L construct insertion (Jin, et al., 2013) and are all homozygous-susceptible to Cry1Ac toxin (genotype SS).

Exogenous *B. thuringiensis* Cry1Ac was purified from *Escherichia coli* JM109 cells carrying the plasmid pGem1Ac, a gift of Dr Neil Crickmore (University of Sussex), following published protocols (Cornforth, Matthews, Brown, & Raymond, 2015). The purified Cry1Ac toxin was incorporated into artificial diet (F9221B, Frontier Agricultural Sciences) to make toxin diet, at doses (0.5 μg ml^{-1}) sufficient to cause near-recessive resistance (Supplementary Information: toxin bioassays). Our resistant population, designated VB-R, was constructed from a Cry1Ac-resistant population NO-QAGE (Baxter et al., 2005; Heckel, Gahan, Liu, & Tabashnik, 1999) and a susceptible population Vero Beach, which is the genetic background of the self-limiting population (VB, Oxitec Ltd). The VB-R population was constructed by backcrossing a hybrid population of VB and NO-QAGE into VB, and selecting for resistance to Cry1Ac for three generations. To create a Cry1Ac-susceptible population with a similar genetic background, we reared VB-R without toxin selection for five generations (before resistance became fixed); thereafter we genotyped mated pairs of males and females using the length polymorphism marker for Cry1Ac resistance (Baxter et al., 2011). Our susceptible population VB-S was then established using 20 pairs of homozygous-susceptible individuals. PCR conditions for genotyping homozygous susceptible alleles were 5 min at 95°C, 30 × (30 s at 94°C, 30 s at 63°C, 1 min at 72°C), 10 min at 72°C, using primers abcc2F (5'–GGACGTGATCCCGGTGGGCAGCG–3') and abcc2R (5'–CGTGCGGCAGCTTAGTGTAC–3'). Both the VB-R and VB-S populations were non-

transgenic (ww genotype, where "w" represents wild type or absence of the “L” construct).

Single and multiple generations, with the same basic design, investigated the impact of transgenic male release on the evolution of resistance to *Bt* toxins (Table 1, details below). Homozygous susceptible LL male pupae were introduced into resistant populations with confirmed resistance allele frequencies. Following LL male releases, resistant populations were exposed to toxin selection and refuge treatment. Population size (number of pupae) and resistant frequencies were monitored throughout the experiments.

Single-generation experiment

These experiments assessed the effect of the susceptible self-limiting DBM in resistance management at a range of refuge sizes. We hypothesized that the use of susceptible self-limiting DBM will have a greater effect on slowing the evolution of resistance at smaller refuge sizes. The single generation experiments were timed so that wild type adults and transgenic males would emerge from their pupae over the same period (24-48 hours) and compete for mates in experimental cages. The eggs produced within each replicate cage were allocated to Cry1Ac toxin diet or toxin refugia where larvae experienced selection for resistance. These experiments sought to control for any differences in development time between wildtype and transgenic insects (and between Cry1Ac resistant and susceptible insects) but otherwise allowed genetic background to affect mating behaviour.

Experiments were set up with 200 individuals of the wild type population with a 15% resistance allele frequency (R). The population was reared for at least two generations prior to selection starting and frequencies were confirmed with PCR, using methods described above. In the transgenic LL male release treatment, 200 LL male pupae were added to each replicate, so that the release ratio was 2:1 OX4319L males to wild type non-transgenic males. Here, we crossed a refuge size treatment (10% and 20% Cry1Ac toxin-free refugia) with a transgenic treatment (with and without LL male release), each replicated three times (Table 1). Refugia were based on the percentage of egg population: refugia eggs were reared separately on toxin-free diet, while remaining eggs were reared on toxin diet (0.5 μg ml^{-1}) to pupation. For every replicate, pupae survivors from both the selection diet and refuge diet were collected and pooled for bioassays in the following generation (N = 90 larvae and three Cry1Ac doses including 0.131 μg ml^{-1}, 0.262 μg ml^{-1} and 0.524 μg ml^{-1}) in order to assess for differences in resistance to Cry1Ac.

Three-generation experiments

To investigate the value of the self-limiting DBM in resistance management over multiple generations, we designed two multi-generation selection experiments with weekly releases of LL males (Table 1). Populations with 4% and 15% resistance allele initial frequencies were generated as above. After confirming the resistant frequency with PCR, we started the first experiment (15% resistance allele frequency) with two treatments (with and without LL male release) and four replicates (400 pupae) in each treatment. In the release treatment, male pupae were introduced into the experimental populations twice a week at approximately a 6:1 ratio (LL male to pupal survivors from each cage, assuming 1:1 sex ratio in cage survivors) for 12 weeks.

Eggs were collected every two days with 10% of the eggs placed onto toxin-free refuge diet. The diet infestation was staggered every two days to build gradually a continuous population with overlapping generations. Thus genotype differences in development time or mating success are allowed to influence results, adding more realism than in single generation experiments.

The experimental populations were bio-assayed every generation to measure the proportion of homozygous-resistant (RR) individuals in the population. Survival data – the numbers of pupae surviving the selection diet and refuge diet – were collected weekly. To test whether the release of transgenic insects was capable of reversion, *i.e.* decreasing the resistance allele frequency in the face of selection, the experiment was repeated with another population with initial resistance allele frequency at 4%.

Life history and fitness cost experiments

To evaluate the fitness costs of the self-limiting gene and the resistance allele, we measured life history traits and mating competitiveness of the aforementioned *P. xylostella* populations. All males denoted as LL and Lw were homozygous-susceptible at the resistance locus (SS), and all VB-S and VB-R individuals were non-transgenic (ww). We confirmed that the VB-R population used in this experiment was fixed for resistance by PCR screening of 96 individuals. Single-pair mating of LL male × SS female, VB-S individuals (SS), VB-R individuals (RR) and SS × RR genotype were set up to measure fecundity, egg hatch rate and larval survival until pupation. Single pairs were mated in 106 pots. The number of eggs laid on cabbage juice-infused green cloths (3 cm × 3 cm) from the single-pairs was counted manually for all pots, and eggs allowed to hatch *in situ*

(Raymond et al., 2007). Twenty freshly emerged neonates from each mating pot were randomly selected to grow on artificial diet until pupation. After scoring survival, pupae developed from single-pair pots were used in mate competition experiments. In these experiments 10 non-transgenic SS males competed with the same number of LL males, RR males, or hemizygous susceptible OX4319L males (LwSS) for mating with 10 SS females. LL males were also competed with hemizygous Lw males for mating with SS females. As the self-limiting gene contains a dominant heritable, fluorescent DsRed2 protein marker (Jin, et al., 2013), pupae can be sorted using a binocular microscope with Nightsea™ light source (excitation 510-540nm) and 600nm filter. Mating success of either LL or Lw males in competition with SS males was scored based on the proportion of fluorescent male offspring. The mating success of RR males was calculated from the proportion of heterozygous-resistant progeny (RS) using PCR genotyping described above. For Lw males in competition with LL males, the proportion of non-fluorescent male offspring determined the mating success of Lw males.

Statistical analyses and experimental design

To assess the potential discriminatory power of the experiments, we simulated discrete generations of DBM classified by sex and genotype (at L/w and S/R loci), assuming a constant proportion of released LLSS males to emerging males (initial males or, after the first generation, emerging males of any genotype) and random mating. Where known, parameter values were set to match experimental protocols. These simulations were adapted from a previously published discrete-generation deterministic model of this genetic system in a generic pest insect (Alphey et al., 2009; Alphey et al., 2007) (see Supplementary Information for details). Deterministic model results indicated that the single-generation experiments were expected to be insensitive to error in allocation of

323 eggs to either toxin or refuge diet. Deterministic modelling showed that the ability to
324 discriminate between treatments over one or three generations is inferior if resistance
325 is more effective and/or if fitness costs of resistance are small. These results informed
326 and refined the experimental design.
327
328 Statistical analysis was carried out in R (http://www.r-project.com) using analysis of
329 variance and generalized linear modelling. The numbers of pupal survivors from
330 selection diet and refuge diet in the single-generation experiment were analysed with a
331 generalized linear model with Poisson errors. Survival data was analysed using a
332 generalized linear mixed model (GLMM) with Poisson errors (Venables & Ripley, 2002);
333 proportional data were analysed with GLMMs with binomial errors, mixed model
334 analyses used replicate as a random effect and nested generation, week and bioassay
335 dose within replicate. Mating success was analysed with a Chi-squared goodness of fit
336 tests, which compared the expected frequency of L and R alleles under random mating
337 with observed frequencies. All model assumptions were checked with graphical
338 analysis of error distribution assumptions.
339
340 **Results**
341 Single-generation experiment
342 We predicted that larger refuge sizes and the addition of transgenic males would slow
343 the evolution of resistance. However, given the increased population size associated
344 with larger refugia, we anticipated that the release of transgenic insects would have
345 more impact at smaller refuge sizes. After one discrete generation, at 10% refuge size,
346 one replicate in the release treatment had only 5 pupal survivors. The replicate went
347 extinct in the following generation and was excluded from bioassays, but was included

348 in the population size analysis. As predicted, the larger refuge size (20%) led to a lower
349 frequency of phenotypic resistance, *i.e.* frequency of RR genotype inferred from bioassay
350 results, compared to replicates with 10% refuge size (Fig. 1A, Likelihood ratio test =
351 10.04, $P = 0.0015$). At 10% refuge size, the addition of transgenic males also lowered
352 the proportion of phenotypic resistance compared to replicates without LL male release
353 treatment (Fig. 1A, Likelihood ratio test = 8.10, $P = 0.0044$). However, at 20% refuge
354 size, there was no significant difference between the release and non-release treatments
355 (Fig. 1A, Likelihood ratio test = 0.34, $P = 0.56$).
356
357 The release of transgenic males was also expected to suppress population size by killing
358 female progeny (Alphey et al., 2009; Alphey et al., 2007). We define total survivors as
359 the number of surviving pupae pooled from Cry1Ac-containing diet and refuge diet
360 across replicates. Given an initial R allele frequency of 15%, after one discrete
361 generation, neither refuge size ($F_{1,10} = 0.025$, $P = 0.88$) or the release of transgenic males
362 ($F_{1,9} = 0.0008$, $P = 0.98$) had an impact on the total survivors (Fig. 1B).
363
364 The single generation design is less realistic and has less power than the multiple
365 generation experiment below. In addition to controlling for differences in development
366 time, self-limiting alleles cannot build up over time in the targeted populations.
367 However, these experiments were informative in terms of illustrating the parameter
368 values (resistance frequency, refuge size, release ratios) over which we might see effects
369 of transgenic insects on evolution of resistance.
370
371
372

Three-generation experiments

While the single-generation experiment showed an effect on resistance frequency at lower refuge size, it was weaker than that predicted by theory. Here we hypothesized that a higher release ratio of transgenic males in a continuous generation experiment should produce a more robust impact on both population size and resistance frequency since transgene frequencies are expected to increase over time in target populations under continuous release. In the first multi-generation experiment, initial conditions were: initial resistance allele frequency of 15%, and a 10% refuge size, and a release ratio of 6:1 transgenic: wild-type males. Under these conditions, the release of transgenic males significantly reduced phenotypic resistance compared to controls without release (Fig. 2A, treatment * generation interaction, Likelihood ratio test = 11.94, $P < 0.001$; treatment * generation^2 interaction, Likelihood ratio test = 3.99, $P = 0.046$). Model comparison showed that a GLMM model with quadratic interaction between treatment and week (AIC = 359.07) had greater explanatory power than a model with a linear interaction (AIC = 442.74) (Chi-squared test = 89.67, $df = 3$, $P <<< 0.001$).

In addition to phenotypic resistance reduction, we also observed population size suppression (Fig. 2B, treatment * week interaction, Likelihood ratio test = 6.28, $P = 0.012$; treatment * week^2 interaction, Likelihood ratio test = 6.19, $P = 0.013$). Model comparison showed that the effects of time were non-linear; adding week as a quadratic term improved model fitting (treatment*week^2 interaction, AIC = 355.09, Chi-squared test = 42.61, $df = 3$, $P <<< 0.001$) relative to a simple linear analysis (AIC = 385.62). Note that larval populations on toxins and refugia diets crashed in week 10, due to undiagnosed issues in the insectary; populations rebounded in week 11 since adults and

eggs in each replicate were unaffected by this additional mortality. We also estimated the selective advantage of resistance in experiments from the proportion of insects that survived on Cry1Ac-containing diet relative to total pupal survivors. If there is no effective resistance then this value should be 0; while if resistance is at fixation this value should be equal to (1- refuge size), or 0.9 with a 10% refuge. Over the course of the experiment, the proportion of Cry1Ac survivors increased in both released populations and controls (Fig. 2C, Likelihood ratio test = 4.06, $P = 0.044$). We also observed an increase of Cry1Ac survivors at first and later (after week 10) a decrease of Cry1Ac survivors in populations treated with transgenic males (Fig. 2C, treatment * week interaction, Likelihood ratio test = 33.49, $P <<< 0.001$; treatment * week^2 interaction, Likelihood ratio test = 31.85, $P <<< 0.001$). A quadratic interaction between treatment and week (AIC = 645.24) had greater explanatory power than a linear interaction (AIC = 678.39) (Chi-square test = 37.15, $df = 2$, $P <<< 0.001$).

Following the success of the first experiment, we tested whether we could drive reversion (decrease in frequency) of resistance in populations initiated with 4% R allele frequency. In this experiment, population sizes across all treatments and replicates (5~158 pupae) were lower than in the experiment with 15% initial R allele frequency (115~1516 pupae), a consequence of the lower mean reproductive ability associated with reduced phenotypic resistance. Particularly after two generations, all four replicates with weekly release of LL males had zero survivors from Cry1Ac diet and a very low number of survivors from refuge diet (5~72 pupae). Nevertheless, we found support for reduction of population sizes and frequency of resistance alleles in treatments with release of transgenic males.

The release of transgenic males reduced population size by generation 2 (Fig. 3A, treatment * generation interaction, Likelihood ratio test = 65.51, P <<<0.001). The release of transgenic males also significantly reduced the proportion of the population surviving on Cry1Ac over the course of experiment (Fig. 3B, Likelihood ration test = 10.66, P = 0.0011). Notably, by generation 2, no insects survived on Cry1Ac diet in the transgenic release treatment. After the second generation of LL male release, population replicates did not produce enough third-instar larvae for bioassays. As a result, R allele frequency was confirmed by PCR instead of bioassay, and the experiment was terminated at the second generation. Despite the effect of the release of LL males on the Cry1Ac survivors, transgenic insects did not significantly affect the frequency of R alleles after selection, (Fig. 3C, $F_{1,6}$ = 0.85, p = 0.39); quite possibly because genetic drift/bottleneck effects in refugia confounded experimental treatments.

Life history and fitness cost experiments

We assessed the fitness cost of the self-limiting gene and the resistance allele in single-pair crosses and mate competition experiments. In the single-pair mating experiment, successful mating was defined as mating that resulted in more than 10 eggs. Only eggs from successful matings were counted and used to estimate fecundity and hatch rate as mating efficiency was assessed in competition experiments. The genotype of mating partners had a strong impact on fecundity (Fig. 4A, $F_{4, 101}$ = 5.69, P < 0.001), with highest fecundity in VB-S individuals (SS × SS) and lowest fecundity in VB-R individuals (RR × RR) (Fig. 4A). Single-pairs of LL male × SS female had an intermediate level of egg production. From the counted eggs, we estimated egg hatch rate as the percentage of successfully mated single-pairs that had eggs developed into more than 10 neonate larvae. As the self-limiting construct eliminates female progeny at larval stage (Jin, et al.,

2013), we would expect that the rate of egg hatch would be similar to that of wild type insects. However, the egg hatch rate in LL male x SS female mating was significantly lower than wild type pairs (Fig. 4B, $\chi^2 = 6.88$, $df = 1$, $P = 0.01$). Single-pairs of LL male × SS female had a significantly lower egg hatch rate than all other mating genotypes (Fig. 4B, $F_{4, 101} = 6.68$, $P <<< 0.001$). Larval survival was defined as the proportion of neonate larvae that developed into pupae in 10 days. There was no significant difference in larval survival between genotypes (Fig. 4C, $\chi^2 = 1.303$, $df = 3$, $P = 0.73$).

In the mating competition experiment, if RR males and LL males were equally as competitive as SS males for mating with SS females, we would expect half of the offspring to be RS individuals (scored by PCR) or Lw individuals (scored by red fluorescence), respectively. Contradicting our null hypothesis, both RR males (Fig. 4D, $\chi^2 = 16.58$, $df = 1$, $P < 0.001$) and LL males (Fig. 4D $\chi^2 = 591.14$, $df = 1$, $P <<< 0.001$) had lower mating success than expected. Similarly, under random mating, with competition between heterozygous Lw males and SS males a quarter of the male progeny should be fluorescent Lw individuals. Again, contradicting our null hypothesis Lw males produced fewer progeny than expected (Fig. 4D $\chi^2 = 7.79$, $df = 1$, $P = 0.0053$) indicating that the fitness costs associated with the transgene are incompletely dominant. Finally, for LL males in competition with Lw males (mating with SS females), significantly less than 75% of the male progeny were fluorescent, indicating that homozygous LL males had lower mating success than their heterozygous Lw counterparts ($\chi^2 = 209.21$, $df = 1$, $P <<< 0.001$). In a population of mixed genotypes, the hierarchy of mating success of males would be wild type (ww)> Lw > LL and SS > RR (Fig. 4D).

Discussion

Here, we have investigated the role of transgenic insect releases in mitigating levels of resistance and suppressing population growth in DBM. We found good support for population suppression and resistance reduction with the combined use of the high dose/refuge strategy and self-limiting transgenic DBM (Alphey et al., 2009; Alphey et al., 2007). The most straightforward evidence was that the transgenic DBM males were able to suppress both population size and resistance development (Fig. 2).

Here, we found effects on the evolution of resistance in this even though refuge size (10%) and the initial resistance allele frequency (15%) in this work were substantially smaller and higher, respectively, than is typical in the field (Tabashnik et al., 2008). Moreover, in comparison to conventional sterile insect technique programs, which could release typically 10, or even up to 50 sterile males to one wild type male (Dyck, Hendrichs, & Robinson, 2005; Lees, Gilles, Hendrichs, Vreysen, & Bourtzis, 2015), the release ratio of 6:1 of the self-limiting DBM is relatively modest.

Given the success of the self-limiting DBM, several factors could potentially limit the effect of the transgenic males. Our data demonstrated that the release of the self-limiting males had a greater impact on resistance frequency at smaller refuge size (Fig. 1A). Potentially, the strong effect of refuge size on slowing the evolution of resistance can mask the effect of the transgenic males at a low release ratio, rendering the effect of release undetectable (Fig. 1B). Put simply, if the refuge strategy is working well to suppress the evolution of resistance then there are limited gains to be had from the additional release of transgenic males. Notably, while resistance frequencies are low the insect population growth rate will be determined by refuge size (Teller-Rodriguez et al. 2014), so larger refugia can also mask effects on population suppression. Simulation

498 modelling indicated that very effective resistance (RR individuals have survival rates
499 that approach 100% on Cry1Ac diet in these experiments) and low fitness costs of
500 resistance could mask the effects of self-limiting transgenes at low release ratios
501 (Supplementary Information: modelling). Over multiple discrete generations, other
502 forms of fitness costs such as delayed developmental time of the self-limiting males,
503 could also limit efficacy, while continuous, overlapping insect populations might be able
504 to accommodate the other potential fitness costs (Supplementary Information:
505 modelling).
506
507 Release of insects carrying female-specific self-limiting transgenes should allow the
508 build-up of transgenic alleles over multiple generations, and we found clear evidence of
509 population suppression and resistance reduction in continuous, overlapping DBM
510 populations. Experiments initiated with 15% initial R allele frequency produced
511 consistent results in terms of population size and proportion of resistance alleles. In
512 contrast, with initial resistance alleles at 4%, transgenic males reduced population size
513 and survival on toxin diet in experiments (Fig. 3A and Fig. 3B), but we observed no
514 difference in resistance development between the release-treatment and no-release
515 populations (Fig. 3C). The combined use of refugia and transgenic release meant that
516 there was minimal survival on Cry1Ac diet, and therefore minimal selection for
517 resistance. However, cages experienced population bottlenecks, particularly in
518 replicates treated with transgenic males. Population bottlenecks can lead to increased
519 variability in allele frequencies via drift (Hartl & Clark, 1997). The bottleneck effect
520 could explain the marked variation in resistance allele frequencies in the release-
521 treatment populations.
522

Overall, transgenic males release could slow the evolution of resistance in repeated experiments, albeit at a reduced rate than that predicted by theory (Alphey et al., 2009; Alphey et al., 2007). According to the published models, a lower release ratio was associated with effective resistance management consequences when refugia are larger and R allele frequencies lower than in our experiments (Alphey et al., 2009; Alphey et al., 2007). At 10% refuge size and 10% initial R allele frequency, the release ratio of 1:1 was capable of slowing resistance development (Alphey et al., 2009; Alphey et al., 2007), but in our experiment we released five or six transgenic males to every wild-type male to achieve similar effects. As a consequence we examined whether unforeseen impacts of transgenes and resistance alleles on life history traits (not reflected in the models) might explain this discrepancy.

Homozygous-resistant individuals had reduced fitness as a result of lower fecundity and fertility (Fig. 4A and Fig. 4B). Homozygous-resistant individual males also had reduced mating success with susceptible females (Fig. 4D). In the mating competition experiment, the males and females were introduced into the mating cages as emerged adults; it is unlikely that the mating success of tested males was correlated with different development times and population structure (Liu et al., 2001). Male mating success in the studied system may be associated with reduced number of matings, as seen in previous experiments with the NOQA, the DBM line that provided the resistance alleles for our population (Groeters et al., 1993). Reduced fitness for RR individuals improves resistance management generally (Carrière & Tabashnik, 2001), but non-random mating could obstruct the effectiveness of the high dose/refuge strategy (Gould, 1998; Tabashnik et al., 2009).

548 Our results showed that the self-limiting males were less competitive than wild type
549 males in terms of accessing wild type females (Fig. 4D) and that these matings resulted
550 in fewer hatched eggs relative to wild type counterparts (Fig. 4B). The fitness of the
551 transgenic males was greatly reduced, as very few progeny were produced and survived
552 from the mating. In addition, heterozygous transgenic males, which are responsible for
553 introgressing susceptible alleles into the population at large, showed incompletely
554 dominant fitness costs associated with transgenes (Fig 4D). If transgenes reduce the
555 fitness of heterozygous males then the potential for introgression of pesticide
556 susceptibility alleles will be limited and the genetic consequences of release will
557 approximate that of "bisex-lethal" strains rather than female-specific lethal. The process
558 of building up the L allele frequency through releases over multiple generations, and its
559 consequences for population suppression, will be attenuated by high dominant fitness
560 costs. Critically, the efficacy of self-limiting transgenic insects as tools in resistance
561 management (above and beyond their use in population suppression) will be partly
562 dependent on the dominance and degree of fitness costs associated with transgenes.
563
564 These fitness costs are higher than previously described for this DBM strain, potentially
565 a result of variation in rearing conditions between laboratories (Jin et al, 2013; Harvey-
566 Samuel et al., 2014), or because of the effects of differences in genetic background of
567 non-transgenic insects arising from out-crossing wild type lines with NO-QAGE
568 (Raymond et al. 2011). Note that transfer of *P. xylostella* OX4139 line L from Oxitec to
569 laboratories at Cornell also resulted in increased fitness costs (A. Walker, unpubl. dat.),
570 which were partly ameliorated reducing the temperature under which larvae are reared.
571 We also saw weaker population suppression insects than in earlier experiments with
572 self-limiting *P. xylostella* on broccoli plants (*Brassica oleracea*) expressing Cry1Ac

573 (Harvey-Samuel et al, 2015). In contrast to that study we introduced toxin-free refugia,
574 which can substantially increase the reproductive potential of a population when
575 resistance frequencies are low. In addition, in this study experiments used artificial diet,
576 which imposes minimal mortality on early instars, whereas *B. oleracea* can cause
577 substantial mortality on neonates, rising to 70% for genotypes resistant to Cry toxins
578 (Raymond et al. 2011). Both these factors would facilitate population suppression on
579 broccoli plants.
580
581 It is difficult to assess how relevant experiments conducted in caged insect population
582 are for real-world resistance dynamics. We hope that mate competition experiments in
583 the laboratory capture sufficient naturalistic behavior to be able to reflect what might
584 happen in the field. The effects of relatively small population sizes can clearly impose
585 some limitations and create additional variability when gene frequencies are low.
586 Nevertheless, we have constructed experimental conditions that pose a very challenging
587 scenario for resistance management. Frequencies of resistance alleles were high,
588 refugia sizes were small and the release ratios low (Dyck et al. 2015). For diamondback
589 moth on artificial diet the fitness costs of resistance were relatively modest and resistant
590 insects had survival rates of up to 100% on diet containing very high levels of Cry toxins,
591 a situation that does not occur in the field, even in insect species prone to evolve
592 resistance to *Bt* toxins readily (Teller-Rodriguez et al 2014). In addition, while fitness
593 costs of transgenes in terms of mate competitiveness were higher than in previous
594 experiments with *P. xylostella* (estimated at 0.09 in this study, where equal fitness with
595 wild type = 1), they are lower than those observed for other species such as *Aedes*
596 *aegypti* (0.008-0.31) (Carvalo, McKerney, Garziera, Lacroix, Donnelly, Alphey, Malavasi,
597 Capurro, 2015) suggesting that our experiments are not unrealistic. Thus, even under

598 relatively stringent experimental conditions our results suggest that the self-limiting
599 DBM is a promising, compatible strategy with the high-dose/refuge strategy.
600
601 **Acknowledgements**
602 This work was supported by the Biotechnology and Biological Sciences Research Council
603 [grant numbers BB/L00948X/1 to MBB and NA, and BB/L00819X/1&2 to BR].
604
605 **Data sharing** Raw data for this study are available as supplementary data files.
606
607 **Literature Cited**

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817 Table 1. Overview of experimental designs

	Release ratio (transgenic males to wild-type males)	Initial resistance allele frequency	Refuge size	Experiment time
Single-generation experiment	2:1 & no-release	15%	10% & 20%	One discrete generation (2 weeks)
Three-generation experiments	6:1 & no release	15% & 4%	10%	12 weeks

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819

820 Figure 1 Efficacy of release of transgenic self-limiting insects in preventing evolution of
821 resistance to *Bt* toxin in single generation experiments. (A) Proportion of phenotypic
822 resistance (in bioassays) of populations treated with no release (black open triangles,
823 black dashed line) and release of the self-limiting DBM males (yellow solid circles,
824 yellow solid line) at 10% and 20% refuge size. (B) Mean total survivors (±SE) of
825 populations treated with no release (grey bar) and release of the self-limiting DBM
826 males (yellow bar) at 10% and 20% refuge size.

827

828 Figure 2 Efficacy of release of transgenic self-limiting insects in preventing evolution of
829 resistance to *Bt* toxin with continuous generations experiments and high (15%) initial
830 resistance allele frequency (A) Proportion of phenotypic resistance (in bioassays) of
831 populations treated without LL male release (black open triangles, black dashed line)
832 and with weekly LL male release (yellow solid circles, yellow solid line) over 3
833 generations. (B) Total survivors and (C) Proportion of toxin survivors (in cage) of
834 populations treated with non-release (black open triangles, black dashed line) and with
835 weekly LL male release (yellow solid circles, yellow solid line) over 7 weeks' time points.
836 Proportion of toxin survivors represent the ratio of homozygous resistant survivors (RR
837 pupae) from Cry1Ac selection diet to total pupae survivors pooled from selection diet
838 and refuge diet in each cage population. Experiments used a 10% refuge size.

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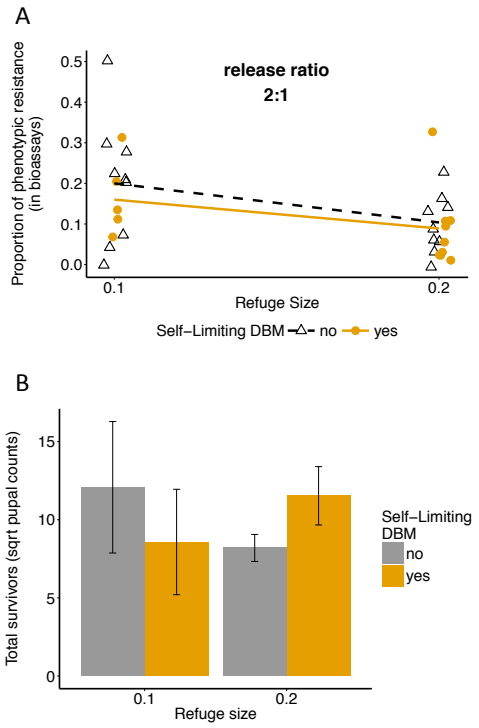
840

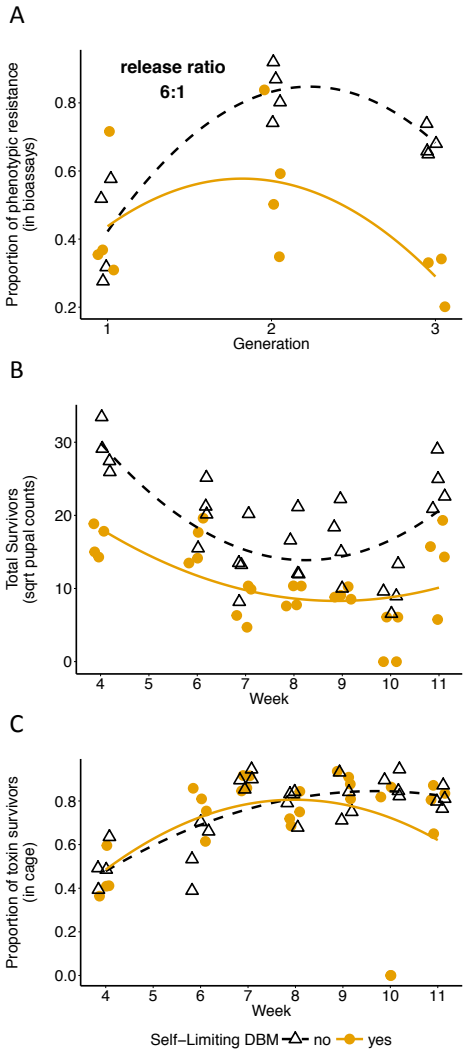
841 Figure 3 Efficacy of release of transgenic self-limiting insects in preventing evolution of
842 resistance to *Bt* toxin with continuous generations experiments and low (4%) (A) Total
843 survivors and (B) Proportion of toxin survivors (in cage) of populations treated with

non-release (black open triangles, black dashed line) and with weekly LL male release (yellow solid circles, yellow solid line) over 2 generations. Proportion of observed resistant represent the ratio of homozygous resistant survivors (RR pupae) from Cry1Ac selection diet to total pupae survivors pooled from selection diet and refuge diet in each population. (C) Resistance allele frequency of populations treated without LL male release (black open triangles) and with weekly LL male release (yellow solid circles) at the second generation. Black solid circles and error bars represent the mean resistance allele frequency (±SE) for respective treatments. Experiments used a 10% refuge size.

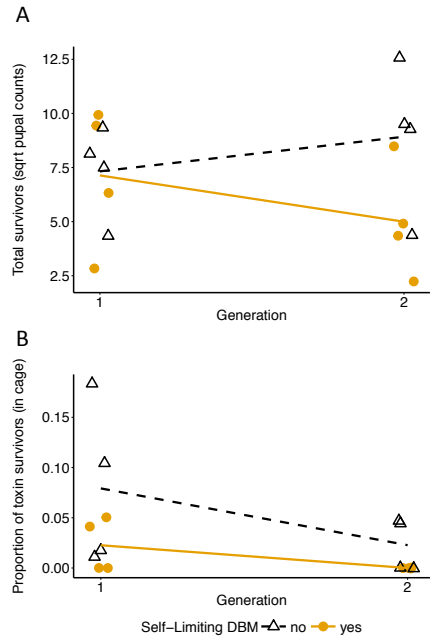
Figure 4 Fitness costs associated with self-limiting transgenes and *Bt* resistance alleles in *P. xylostella* in this study. (A) Egg production of successfully mated (> 10 eggs) single pairs of LL male × SS female, SS male × SS female, RR male × RR female, RR male × SS female and SS male × RR female. Black circles and error bars represent the mean egg production (±SE). (B) Egg hatch rate (±SE) (> 10 larvae emerged) of successfully mated single pairs. (C) Larvae survival of larvae genotype Lw, SS, RR and RS. Black circles and error bars represent the mean larvae survival (±SE) for respective genotypes. (D) Mean mating success (±SE) of RR males (vs SS males – in competition with SS males), LL males (vs SS males), Lw males (vs SS males) and LL males (vs Lw males). Yellow bars and error bars represent the mean observed mating success (±SE), while grey bars represent the expected mating success. All males denoted as LL and Lw were homozygous susceptible (SS), and all SS and RR individuals were non-transgenic (ww).


A
release ratio
2:1
Proportion of phenotypic resistance (in bioassays)
0.5
0.4
0.3
0.2
0.1
0.0
0.1
0.2
Refuge Size
Self−Limiting DBM no yes
B
Total survivors (sqrt pupal counts)
15
10
5
0
0.1
0.2
Refuge size
Self−Limiting DBM
no
yes

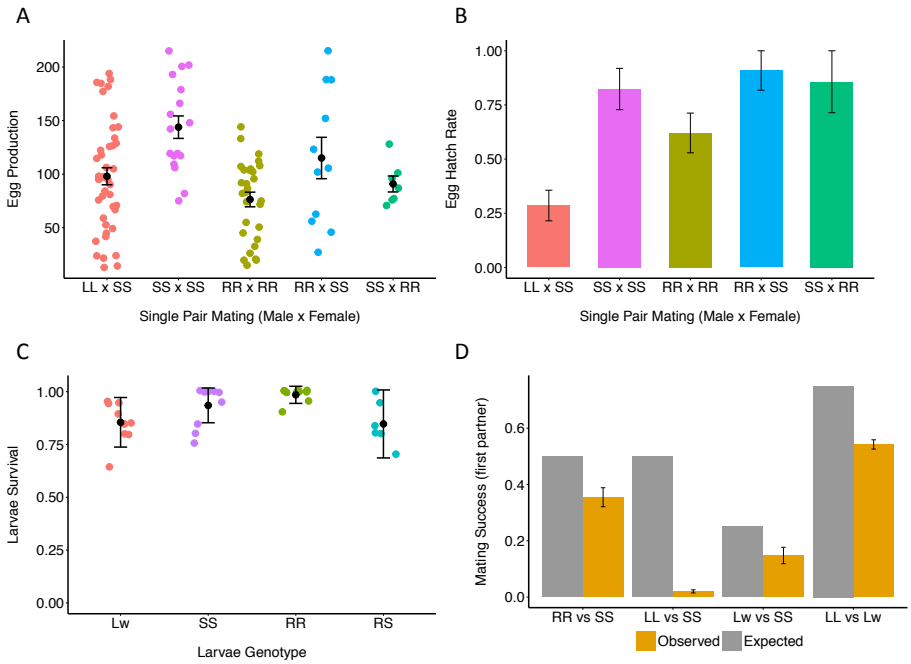
867


A
release ratio
6:1
Proportion of phenotypic resistance
(in bioassays)
0.2
0.4
0.6
0.8
1
2
3
Generation
B
Total Survivors
(sqrt pupal counts)
0
10
20
30
4
5
6
7
8
9
10
11
Week
C
Proportion of toxin survivors
(in cage)
0.0
0.2
0.4
0.6
0.8
4
5
6
7
8
9
10
11
Week
Self−Limiting DBM
no
yes

868


A
Total survivors (sqrt pupal counts)
12.5
10.0
7.5
5.0
2.5
1
2
Generation
B
Proportion of toxin survivors (in cage)
0.15
0.10
0.05
0.00
1
2
Generation
Self−Limiting DBM no yes
C
Resistant allele frequency
0.40
0.35
0.30
0.25
0.20
0.15
0.10
Control
Self−limiting DBM

869


A
Egg Production
200
150
100
50
LL x SS
SS x SS
RR x RR
RR x SS
SS x RR
Single Pair Mating (Male x Female)
B
Egg Hatch Rate
1.00
0.75
0.50
0.25
0.00
LL x SS
SS x SS
RR x RR
RR x SS
SS x RR
Single Pair Mating (Male x Female)
C
Larvae Survival
1.00
0.75
0.50
0.25
0.00
Lw
SS
RR
RS
Larvae Genotype
D
Mating Success (first partner)
0.6
0.4
0.2
0.0
RR vs SS
LL vs SS
Lw vs SS
LL vs Lw
Observed
Expected

870