

The collection of this information is authorized by the Plant Protection Act of 2000. The information will be used to determine eligibility to receive all types of permits. No permit will be issued until this application has been approved.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE

APPLICATIONS FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

1. NAME, ADDRESS, TELEPHONE, AND EMAIL OF APPLICANT

Name: (b)(6)
 Position:
 Organization: Cornell University/NYSAES
 Organization Unique ID:
 Address: 630 W. North St.
 (b)(6)
 Geneva, NY 14456
 County/Province:
 Township/Island:
 Day Telephone: (b)(6)
 FAX:
 Alternate:
 Email 1: (b)(6) cornell.edu
 Email 2:

2. INTRODUCTION TYPE

- Importation
- Interstate Movement
- Interstate Movement and Release
- Release

3. PERMIT TYPE

- Standard
- Permit
- Courtesy
- Permit

4. PURPOSE OF PERMIT

- Industrial Product
- Pharmaceutical Product
- Phytoremediation
- Traditional

5. CONFIDENTIAL BUSINESS INFORMATION VERIFICATION (CBI)

Does this application contain CBI? Yes No

CBI Justification:

N/A

6. REQUEST TYPE

New Amendment Renewal

Amendment/Renewal Description:

Previous Permit Number(s):

7. MEANS OF MOVEMENT

Import by air; releases manually from the ground/vehicles.

8. VARIANCE VERIFICATION

Have you previously applied for variance(s) that you wish to apply to this permit? Yes No

Variance Number(s):

If so, describe in a brief summary how the variance will be applied:

N/A

9. REGULATED ARTICLE

Scientific Name: Plutella xylostella

Common Name: Diamondback moth

Cultivar and/or Breeding Line:

OX4319L-Pxy, OX4319N-Pxy and OX4767A-Pxy

Any biological material (e.g., culture medium, or host material) accompanying the regulated Article during movement:

Artificial insect diet. This diet will be frozen at -15°C for 12 h prior to import.

Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed, and produced:

All final engineering of the transforming constructs was performed at Oxitec Ltd, in the United Kingdom.

The genes used from the donor organisms and the piggyBac-derived portions of the vectors used to build the transforming construct were cloned off-site. The recipient organism is the moth, *Plutella xylostella*, which is endemic in temperate regions around the world, including the USA. The recipient *Plutella xylostella* strain for the transformation was a wild-type strain obtained from Syngenta (public limited company), UK, which has been reared in Oxitec insectaries since 2008.

Processes, Procedures, and Safeguards Description:

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

This is a permit request for seasonal releases (April to October) of a female-lethal, genetically marked diamondback moth (maximum 100,000 moths/week), in brassica fields at the Cornell University research station, Geneva NY.

Males of the transgenic moths will be released in cultivated brassica plots and biological parameters of these moths, such as dispersal and persistence, measured using traps, for example baited with synthetic sex pheromone. The moths of the OX4319L-Pxy strain carry a stable, heritable marker the DsRed2 fluorescent protein, viewed by fluorescence microscope or detected by PCR and their female progeny die in the absence of a dietary repressor (tetracycline or suitable analogues supplied in their artificial diet). The male-selecting (female-lethal) penetrance of the strain is >99% (Jin et al. 2013). The marker provides a means of distinguishing released moths from wild moths, and female-lethality is a self-limiting trait in the wild.

All genetically modified moths will be reared in insectaries at Cornell University, Geneva NY. The facilities and their general operation have been inspected and approved through a previous importation permit (12-227-102m). Larval rearing will be conducted in quarantine using the same approved procedures as in this previous permit. Only moths homozygous for the conditional lethal transgene, reared off tetracycline, will be released. Adult moths will be transported in sealed containers, with at least two layers of containment, labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle; for each batch the number of containers and identity of member of staff supervising the release will be recorded.

The transgenic diamondback moths encode no toxic or allergen proteins. The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA-CFSAN in the USA for human safety, and they raised no objections to its use in corn plants. This involved an assessment of the amino acid sequence using bioinformatics analyses in accordance with the Guidance provided by Codex (2003), the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, as well as the toxicity of the protein using bioinformatics analysis. The amino acid sequence in OX4319L-Pxy is the same as that evaluated in the NPC. It has been further evaluated in an Environmental Assessment (EA) by the USDA (http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf), which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk. Additional EAs on another GE moth, GE pink bollworm, expressing fluorescent genes similar to DsRed2 have also been conducted (<http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm>) and concluded that it was unlikely to present any hazard to the environment.

The other protein coding region, tTAV, is regulated by sequences from the sex-determination gene, doublesex, from pink bollworm (*Pectinophora gossypiella*), that produce different splice variants in males and females: the female transcript comprises coding sequence for the tetracycline-repressible transcription factor, tTAV, which interacts with the upstream tetracycline response element, tetO (or tRE), to form a positive-feedback loop that results in insect lethality prior to adulthood. Under the control of the doublesex sex-alternate splicing, lethality is induced only in females. The tTAV amino acid sequence in OX4319L-Pxy has also been evaluated independently using the bioinformatics analyses provided by Codex (2003) for both potential allergenicity and toxicity. No homologies with known allergens or toxins were determined. This study is available on request. Tetracycline can be provided to the insect in larval artificial diet to suppress female death and permit colony rearing in the laboratory. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material. This female-specific lethal trait was previously discussed in a USDA Environmental Impact Statement published in October 2008, entitled Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs, which concluded that the use of genetically engineered fruit flies and pink bollworm in APHIS plant pest control programs were the environmentally preferred alternative (Record of Decision (Federal Register Vol 74 (87) 21314 2009)).

Reference:

Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology, 2:160-166.

10. ARTICLE SUPPLIER AND/OR DEVELOPER

Name	Location	Contact Information
(b)(6)	Oxitec Ltd 71 Milton Park Abingdon OX144RQ County: Oxford	Day Telephone: (b)(6) FAX: Email: (b)(6) xitec.com

11. PHENOTYPES/GENOTYPE

- 1) Phenotypic Designation Name:** visual marker; repressible lethality

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

Identifying Line(s):	OX4319L-Pxy, OX4319N-Pxy, OX4767A-Pxy
Construct(s):	OX4319, OX4767
Mode of Transformation:	Direct injection
Phenotype Description: A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.	The introduced genetic material in the diamondback moth comprises three protein coding regions, one for marking the insects and two for inducing death before the insect reaches adulthood (in this instance, females only). The former allows the expression of a DsRed2 fluorescent protein originally derived from a coral (<i>Discosoma</i> sp.). The transgenic diamondback moth with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenic diamondback moth can be envisioned. The non-modified diamondback moth has no fluorescent protein gene; therefore, it does not fluorescence when illuminated under the same light frequency. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

Phenotype(s)

MG - Visual marker; DsRed2 Fluorescent Protein Expression

Genotype(s)

Screenable Marker

Gene: DsRed2 **from** *Discosoma* sp. - Screenable marker gene DsRed2 from *Discosoma* spp - Allows the expression of a fluorescent protein from *Discosoma* spp. Fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5iel promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by intense illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. Expression of a fluorescent protein will therefore permit released modified moths to be distinguished from unmodified.

Vector Sequence: piggyBac (non-autonomous) **from** piggyBac from *Trichoplusia ni* (moth) - Transformation Vector from *Trichoplusia ni* (moth) - Effects germline transformation of diamondback moth from piggyBac from *Trichoplusia ni* (moth) - 3' end of piggyBac. piggyBac is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element. In the construct used for transformation of the pink bollworm, the transposase gene of the piggyBac element was irreversibly destroyed by insertion of the transgene. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding piggyBac transposase under the control of the *Drosophila melanogaster* hsp70 promoter. One of the inverted terminal repeats that flank the wild-type piggyBac transposase in piggyBac has been removed in the helper plasmid so that the helper plasmid cannot itself integrate even though it encodes for active piggyBac transposase.

Repressible lethality

Gene: tTAV **from** *Escherichia coli* (bacterium) and *Herpes simplex* (virus) - Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from *Escherichia coli* with VP16 from a type 1 herpes simplex virus. The tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (tetO) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline, it will induce expression

from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and Drosophila melanogaster with no known adverse effects. Unmodified *Plutella xylostella* do not have a tTAV activity.

Regulatory sequence: doublesex genomic region **from** Pink bollworm, *Pectinophora gossypiella* - Female-specificity is conferred using truncated sex-alternate splicing sequences from the doublesex gene of *Pectinophora gossypiella*. Sequence encoding tTAV is inserted into this splicing sequence, allowing for the expression of tTAV in a sex-specific manner, resulting in a conditional female-lethal system (Jin et al. 2013).

A full list of construct components is provided in the attached Table of genetic elements.

References:

- Berger SL, et al. 1990 Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* 61, 1199-1208.
- Damke H, et al. 1995 Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Meth Enzymol* 257, 209-220.
- Gillespie JP, et al. 1997 Biological mediators of insect immunity. *Annu Rev Entomol* 42, 611-643.
- Gong P, et al. 2005 A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat Biotechnol* 23, 453-456.
- Gossen M, et al. 1994 Inducible gene expression systems for higher eukaryotic cells. *Curr Opin Biotechnol* 5, 516-520.
- Gossen M, and Bujard H 1992 Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc Natl Acad Sci USA* 89, 5547-5551.
- Salghetti S, et al. 2001 Regulation of transcriptional activation domain function by ubiquitin. *Science* 293, 1651-1653.
- Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2:160-166.

12. INTRODUCTION

Release Site

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Research Farm North	<p>NY County: Ontario Proposed Release Start Date: 5/1/2016 Proposed Release End Date: 12/31/2017 No. of Releases: Quantity:</p> <p>up to 72/year, up to 100,000 moths/week up to 10 acres acres</p>	
Location Unique ID:	RFN1097	
Location GPS Coordinates:	(b)(4)	
Release Site History:	Managed agricultural, cropping, research. Managed agricultural land around release site.	
Critical Habitat Involved?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	

13. DESIGN PROTOCOLS

Production Design

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

The diamondback moth strains OX4319L-Pxy, OX4319N-Pxy and OX4767A-Pxy show a tetracycline-repressible female-lethal phenotype, which could serve as an insecticide-free means of controlling pest populations of diamondback moth in the field in a species-specific manner. Successful pest control will rely upon strong performance of released males, in terms of female-seeking behavior and mating competitiveness. We will seek to measure relevant performance traits in one or more mark-release-recapture field experiments. These will be followed by pest suppression trials, in which *Plutella xylostella*-infested fields will be treated with fsRIDL male *Plutella xylostella*, and the wild populations monitored and compared with those of fields not so treated.

In the mark-release-recapture experiments, we will release up to 20,000 male fsRIDL *Plutella xylostella* (per release; up to 100,000 males per week) from single or multiple points in experimental fields of up to 10 acres planted with brassicas (e.g. cabbage or broccoli). Traps (e.g. sticky traps baited with synthetic sex pheromone) will be placed in and around the field, up to 1 km from the release point, to recapture the male moths. Traps will be collected at least once per week and the recaptured moths screened for the fluorescence marker. Additional PCR screening will be conducted to validate this visual screening. Trapping will continue until no fsRIDL male moths are recaptured for 2 consecutive weeks. To permit an overlapping series of releases in each experimental field that can be independently monitored on the traps, fsRIDL male moths will be sometimes be additionally marked, for example using different-colored fluorescent powders, which are commonly used in such field experiments with insects (reviewed by Hagler & Jackson 2001 Ann Rev Entomol 46:511-543). Crop sampling, in which a proportion of the in-field plants will be collected and closely examined in the laboratory for *Plutella xylostella* larvae and pupae (wild-type and transgenic), will be conducted at regular intervals to assess mating success of the fsRIDL males. Each experimental field will be surrounded by an approximately 10m-deep border free of potential host plants. Upon completion of the experiment, the insecticide Coragen (chlorantraniliprole) will be sprayed on the plants and surrounding area to kill remaining diamondback moth larvae.

Data from these preliminary field experiments, indicating the fsRIDL male release rate required to achieve a given overflooding rate (e.g. 10:1 fsRIDL:wild males), dispersal and field longevity, will inform the release strategy of fsRIDL male diamondback moths in a suppression trial (how many fsRIDL males to release, how frequently and from how many points), requested as part of this permit application. All of the described trials will require monitoring of a wild diamondback moth population in up to six experimental fields (up to three treated with fsRIDL male moths, and up to three untreated). These fields, of up to 10 acres in size, will be planted with brassica plants (e.g. cabbage or broccoli). If the wild diamondback moth population is not present in sufficient numbers at the trial sites, the experimental fields will be artificially infested with male and female moths from a USA-derived wild-type diamondback moth strain currently maintained in the laboratory; dye-marked wild-type moths may also be used in mark-release-recapture experiments to provide a direct comparison with the GE moths. A proportion of the experimental fields will be subjected to regular releases (up to five times weekly) of fsRIDL male moths, in numbers greater than the estimated recruitment rate of wild-type male moths in the environment, to achieve an over-flooding effect of fsRIDL males on the wild male diamondback moth population. For each experiment, fsRIDL male releases will be conducted for up to the duration of a brassica crop cycle (anticipated as 3-4 months). Adult traps (e.g. sticky traps baited with synthetic sex pheromone) will be placed in and around each field to monitor the relative numbers of wild-type and fsRIDL males present, and to assess their dispersal. The populations of wild-type moths in each field, including those receiving no fsRIDL males, will be monitored using the adult traps described and periodic crop sampling. Releases will consist of up to 100,000 fsRIDL male moths per week (depending on the overflooding ratio required) over the treatment fields over the course of these suppression experiments. Trapping will continue after the last releases of fsRIDL male moths, and will continue until at least 2 weeks of zero fsRIDL recaptures. Upon completion of the experiment, the insecticide Coragen (chlorantraniliprole) will be sprayed on the plants and surrounding area (within 100 m radius of treated fields) to kill remaining diamondback moth larvae. Post-experiment pheromone trapping will continue for 2 weeks to monitor field longevity of fsRIDL moths.

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

All genetically modified male diamondback moth used in the trials will be reared as larvae on non-tetracycline artificial diet. Releases will be conducted from the ground or vehicle on Cornell University's New York State Agricultural Research Station. Releases will be conducted up to five times per week, depending on experimental requirements.

The area around the release sites (up to 1000 m radius from release site) will be monitored with traps (e.g. sticky traps baited with synthetic sex pheromone). Traps will be collected at least weekly to count the number of genetically modified moths and wild moths captured on each trap. Samples in the laboratory will be screened for presence of the DsRed2 fluorescent marker, using fluorescence microscopy, and this will be validated by PCR detection of the DNA construct in

selected samples. Some non-viable insect samples will be sent to Oxitecs labs in the UK for the PCR analysis. Prior to each field release, samples from each cohort of male fsRIDL moths will be screened for the fluorescent marker and sexed. Only male moths will be released; the effect on the crop will therefore likely be negligible: male activity is restricted to finding and mating females, feeding on nectar from flowers, and taking shelter during the day.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations:

Adult genetically modified moths will be transported in sealed containers labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle by authorized personnel.

The conditional lethality expressed by the fsRIDL construct means that female progeny from matings with Oxitec male insects die in the absence of tetracycline, and the trait is therefore unlikely to persist in the environment. Other mitigation measures include the lack of known sexually compatible relatives of *Plutella xylostella* in the USA; the piggyBac transposable element used for the transformation has no endogenous functioning transposase, rendering it non-autonomous (it cannot mobilize itself); the release area will be monitored extensively with traps to attract and collect *Plutella xylostella* moths; release fields are no larger than 10 acres; the *Plutella xylostella* can be sprayed with insecticide at any time in the case of observed adverse events; the genetically engineered *Plutella xylostella* will be securely managed and contained in production and transport; and all viable insects reared for this trial that are not required for release or additional analysis will be devitalized by freezing.

Final Disposition Method: Destruction/Devitalization Other Storage in Contained Facility

Final Disposition Description:

All unused genetically modified eggs, larvae, pupae and moths not released, or not needed in the mass-rearing, will be frozen at a minimum of $-15^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 48 h to destroy all life stages.

14. ATTACHMENTS

Attachments

Additional CBI Justification statements (3/16/2016 @ 09:08 AM)
BRS Permit 13-297-102r-a1 (3/16/2016 @ 08:43 AM)
BRS importation permit 12-227-102m (3/16/2016 @ 08:42 AM)
Cornell University field site with GPS (3/16/2016 @ 08:43 AM)
Jin et al 2013-Scientific publication- description of the technology (3/16/2016 @ 08:38 AM)
OX4319L chlortetracycline sensitivity-CBI deleted (3/16/2016 @ 08:46 AM)
OX4319L chlortetracycline sensitivity-contains CBI (3/16/2016 @ 08:45 AM)
OX4319L construct sequencing- Contains CBI (3/16/2016 @ 08:47 AM)
OX4319L construct sequencing-CBI Deleted (3/16/2016 @ 08:47 AM)
OX4319L molecular characterisation-CBI deleted (3/16/2016 @ 08:49 AM)
OX4319L molecular characterisation-Contains CBI (3/16/2016 @ 08:48 AM)
OX4319L tTAV expression levels-CBI Deleted (3/16/2016 @ 08:50 AM)
OX4319L tTAV expression levels-Contains CBI (3/16/2016 @ 08:50 AM)
Plutella OX4319L Phenotype-Genotype text for submission 16March2016 (3/16/2016 @ 09:04 AM)
Scientific publication- Harvey-Samuel et al 2014 (3/16/2016 @ 08:44 AM)
Scientific publication- Harvey-Samuel et al 2015 (3/16/2016 @ 08:45 AM)
Table of genetic elements-CBI deleted (3/16/2016 @ 08:51 AM)
Table of genetic elements-Contains CBI (3/16/2016 @ 08:51 AM)
Threatened or endangered species (3/16/2016 @ 08:51 AM)

15. ADDITIONAL INFORMATION

i) Similar introductions were previously authorised under permit number: 13-297-102r-a1 and importation under permit number 12-227-102m

ii) Data from some of the research reports submitted under permit number: 13-297-102r-a1 have now been described in the peer reviewed publications noted below:

Harvey-Samuel, T., Ant, T., Gong, H., Morrison, N.I., and Alphey, L. (2014). Population-level effects of fitness costs associated with repressible female-lethal transgene insertions in two pest insects. *Evol Appl* 7, 597-606.

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Harvey-Samuel, T., Morrison, N.I., Walker, A.S., Marubbi, T., Yao, J., Collins, H.L., Gorman, K., Davies, T.G., Alphey, N., Warner, S., et al. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biol* 13, 49.

16. COURTESY JUSTIFICATION

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(b)(6) hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON

(b)(6)

18. DATE

March 16, 2016

The collection of this information is authorized by the Plant Protection Act of 2000. The information will be used to determine eligibility to receive all types of permits. No permit will be issued until this application has been approved.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE

APPLICATIONS FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

1. NAME, ADDRESS, TELEPHONE, AND EMAIL OF APPLICANT

Name: (b)(6)
 Position:
 Organization: Cornell University/NYSAES
 Organization Unique ID:
 Address: 630 W. North St.
 (b)(6)
 Geneva, NY 14456
 County/Province:
 Township/Island:
 Day Telephone: (b)(6)
 FAX:
 Alternate:
 Email 1: (b)(6) cornell.edu
 Email 2:

2. INTRODUCTION TYPE

- Importation
- Interstate Movement
- Interstate Movement and Release
- Release

3. PERMIT TYPE

- Standard
- Permit
- Courtesy
- Permit

4. PURPOSE OF PERMIT

- Industrial Product
- Pharmaceutical Product
- Phytoremediation
- Traditional

5. CONFIDENTIAL BUSINESS INFORMATION VERIFICATION (CBI)

Does this application contain CBI? Yes No

CBI Justification:

N/A

6. REQUEST TYPE

- New Amendment Renewal

Amendment/Renewal Description:

Previous Permit Number(s):

7. MEANS OF MOVEMENT

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8. VARIANCE VERIFICATION

Have you previously applied for variance(s) that you wish to apply to this permit? Yes No

Variance Number(s):

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N/A

9. REGULATED ARTICLE

Scientific Name: Plutella xylostella

Common Name: Diamondback moth

Cultivar and/or Breeding Line:

OX4319L-Pxy

Any biological material (e.g., culture medium, or host material) accompanying the regulated Article during movement:

Artificial insect diet.

Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed, and produced:

All final engineering of the transforming constructs was performed at Oxitec Ltd, in the United Kingdom.

The genes used from the donor organisms and the piggyBac-derived portions of the vectors used to build the transforming construct were cloned off-site. The recipient organism is the moth, *Plutella xylostella*, which is endemic in temperate regions around the world, including the USA. The recipient *Plutella xylostella* strain for the transformation was a wild-type strain obtained from Syngenta (public limited company), UK, which has been reared in Oxitec insectaries since 2008.

Processes, Procedures, and Safeguards Description:

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This is a permit request for seasonal releases (April to October) of a female-lethal, genetically marked diamondback moth (maximum 30,000 moths/week), in brassica fields at the Cornell University research station, Geneva NY.

Males of the transgenic moths will be released in cultivated brassica plots and biological parameters of these moths, such as dispersal and persistence, will be measured. The moths of the OX4319L-Pxy strain carry a stable, heritable marker the DsRed2 fluorescent protein, viewed by fluorescence microscope or detected by PCR and their female progeny die in the absence of a dietary repressor (tetracycline or suitable analogues supplied in their artificial diet). The male-selecting (female-lethal) penetrance of the strain is >99% (Jin et al. 2013). The marker provides a means of distinguishing released moths from wild moths, and female-lethality is a self-limiting trait in the wild.

All genetically modified moths will be reared in insectaries at Cornell University, Geneva NY. The facilities and their general operation have been inspected and approved through a previous importation permit (12-227-102m). Larval rearing will be conducted in quarantine using the same approved procedures as in this previous permit. Only moths homozygous for the conditional lethal transgene, reared off tetracycline, will be released. Adult moths will be transported in sealed containers, with at least two layers of containment, labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle; for each batch the number of containers and identity of member of staff supervising the release will be recorded.

The transgenic diamondback moths encode no toxic or allergen proteins. The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA-CFSAN in the USA for human safety, and they raised no objections to its use in corn plants. This involved an assessment of the amino acid sequence using bioinformatics analyses in accordance with the Guidance provided by Codex (2003), the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, as well as the toxicity of the protein using bioinformatics analysis. The amino acid sequence in OX4319L-Pxy is the same as that evaluated in the NPC. It has been further evaluated in an Environmental Assessment (EA) by the USDA (http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf), which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk. Additional EAs on another GE moth, GE pink bollworm, expressing fluorescent genes similar to DsRed2 have also been conducted (<http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm>) and concluded that it was unlikely to present any hazard to the environment.

The other protein coding region, tTAV, is regulated by sequences from the sex-determination gene, doublesex, from pink bollworm (*Pectinophora gossypiella*), that produce different splice variants in males and females: the female transcript comprises coding sequence for the tetracycline-repressible transcription factor, tTAV, which interacts with the upstream tetracycline response element, tetO (or tRE), to form a positive-feedback loop that results in insect lethality prior to adulthood. Under the control of the doublesex sex-alternate splicing, lethality is induced only in females. The tTAV amino acid sequence in OX4319L-Pxy has also been evaluated independently using the bioinformatics analyses provided by Codex (2003) for both potential allergenicity and toxicity. No homologies with known allergens or toxins were determined. This study is available on request. Tetracycline can be provided to the insect in larval artificial diet to suppress female death and permit colony rearing in the laboratory. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material. This female-specific lethal trait was previously discussed in a USDA Environmental Impact Statement published in October 2008, entitled Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs, which concluded that the use of genetically engineered fruit flies and pink bollworm in APHIS plant pest control programs were the environmentally preferred alternative (Record of Decision (Federal Register Vol 74 (87) 21314 2009)).

Reference:

Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology, 2:160-166.

10. ARTICLE SUPPLIER AND/OR DEVELOPER

Name	Location	Contact Information
(b)(6)	Oxitec Ltd 71 Milton Park Abingdon OX144RQ County: Oxford	Day Telephone: 0044-1235-832393 FAX: Email: (b)(6) oxitec.com

11. PHENOTYPES/GENOTYPE

1) Phenotypic Designation Name: visual marker; repressible lethality

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Identifying Line(s):	OX4319L-Pxy
Construct(s):	OX4319
Mode of Transformation:	Direct injection
Phenotype Description: A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.	The introduced genetic material in the diamondback moth comprises three protein coding regions, one for marking the insects and two for inducing death before the insect reaches adulthood (in this instance, females only). The former allows the expression of a DsRed2 fluorescent protein originally derived from a coral (<i>Discosoma</i> sp.). The transgenic diamondback moth with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenic diamondback moth can be envisioned. The non-modified diamondback moth has no fluorescent protein gene; therefore, it does not fluorescence when illuminated under the same light frequency. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

Phenotype(s)

MG - Visual marker; DsRed2 Fluorescent Protein Expression

Genotype(s)

Screenable Marker

Gene: DsRed2 **from** *Discosoma* sp. - Screenable marker gene DsRed2 from *Discosoma* spp - Allows the expression of a fluorescent protein from *Discosoma* spp. Fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5iel promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by intense illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. Expression of a fluorescent protein will therefore permit released modified moths to be distinguished from unmodified.

Vector Sequence: piggyBac (non-autonomous) **from** piggyBac from *Trichoplusia ni* (moth) - Transformation Vector from *Trichoplusia ni* (moth) - Effects germline transformation of diamondback moth from piggyBac from *Trichoplusia ni* (moth) - 3' end of piggyBac. piggyBac is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element. In the construct used for transformation of the pink bollworm, the transposase gene of the piggyBac element was irreversibly destroyed by insertion of the transgene. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding piggyBac transposase under the control of the *Drosophila melanogaster* hsp70 promoter. One of the inverted terminal repeats that flank the wild-type piggyBac transposase in piggyBac has been removed in the helper plasmid so that the helper plasmid cannot itself integrate even though it encodes for active piggyBac transposase.

Repressible lethality

Gene: tTAV **from** *Escherichia coli* (bacterium) and *Herpes simplex* (virus) - Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from *Escherichia coli* with VP16 from a type 1 herpes simplex virus. The tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (tetO) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline, it will induce expression

from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and Drosophila melanogaster with no known adverse effects. Unmodified *Plutella xylostella* do not have a tTAV activity.

Regulatory sequence: doublesex genomic region **from** Pink bollworm, *Pectinophora gossypiella* - Female-specificity is conferred using truncated sex-alternate splicing sequences from the doublesex gene of *Pectinophora gossypiella*. Sequence encoding tTAV is inserted into this splicing sequence, allowing for the expression of tTAV in a sex-specific manner, resulting in a conditional female-lethal system (Jin et al. 2013).

A full list of construct components is provided in the attached Table of genetic elements.

References:

- Berger SL, et al. 1990 Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* 61, 1199-1208.
- Damke H, et al. 1995 Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Meth Enzymol* 257, 209-220.
- Gillespie JP, et al. 1997 Biological mediators of insect immunity. *Annu Rev Entomol* 42, 611-643.
- Gong P, et al. 2005 A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat Biotechnol* 23, 453-456.
- Gossen M, et al. 1994 Inducible gene expression systems for higher eukaryotic cells. *Curr Opin Biotechnol* 5, 516-520.
- Gossen M, and Bujard H 1992 Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc Natl Acad Sci USA* 89, 5547-5551.
- Salghetti S, et al. 2001 Regulation of transcriptional activation domain function by ubiquitin. *Science* 293, 1651-1653.
- Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2:160-166.

12. INTRODUCTION

Release Site

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Research Farm North	<p>NY County: Ontario Proposed Release Start Date: 7/1/2016 Proposed Release End Date: 12/31/2017 No. of Releases: Quantity:</p> <p>up to 72/year, up to 30,000 moths/week up to 10 acres</p>	
Location Unique ID:	RFN1097	
Location GPS Coordinates:	(b)(4)	
Release Site History:	Managed agricultural, cropping, research. Managed agricultural land around release site.	
Critical Habitat Involved?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	

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13. DESIGN PROTOCOLS

Production Design

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

The diamondback moth (*Plutella xylostella*) strain OX4319L-Pxy shows a tetracycline-repressible female-lethal phenotype, which could serve as an insecticide-free means of controlling pest populations of *Plutella xylostella* in the field in a species-specific manner. Successful pest control will rely upon strong performance of released males, in terms of female-seeking behavior and mating competitiveness. We will seek to measure relevant performance traits in one or more mark-release-recapture field experiments. We will also conduct caged trials to support information about relevant performance traits, including release rates.

Mark-release-recapture studies. In the mark-release-recapture experiments, we will release up to 10,000 male OX4319L-Pxy moths per release (up to 30,000 males per week) from a single point in an experimental field of up to 10 acres planted with brassicas (e.g. cabbage or broccoli). The anticipated release point is (b)(4) and brassicas will be planted up to a (b)(4) (b)(4) from the release point. Exact coordinates will be provided upon planting. Movement patterns of moths within the field will be assessed using sticky traps and mating stations.

Mating stations will consist of confined, wild-type females that attract males. Traps will be collected at least once per week and the recaptured moths screened for the fluorescence marker. Additional PCR screening will be conducted to validate this visual screening. Females from the mating stations will be screened for whether they mated to OX4319L-Pxy males.

Sticky traps baited with synthetic sex pheromone will be placed outside the field, up to 1 km from the release point, to capture male moths and detect the presence of any OX4319L-Pxy *Plutella xylostella*. Traps and mating stations will be collected at least once per week and moths screened for the fluorescence marker indicating they are OX4319L-Pxy *Plutella xylostella*. Additional PCR screening will be conducted to validate this visual screening.

To permit an overlapping series of releases in the experimental field, OX4319L-Pxy male moths will sometimes be additionally marked, for example using different-colored fluorescent powders, which are commonly used in such field experiments with insects (reviewed by Hagler & Jackson 2001 Ann Rev Entomol 46:511-543). Powder-marked USA-derived wild-type moths may also be used in mark-release-recapture experiments to provide a comparison with OX4319L-Pxy male *Plutella xylostella*. Each experimental field will be surrounded by an approximately 10-m-wide border free of potential host plants. Upon completion of the experiment, an insecticide (e.g. Coragen-chlorantraniliprole) will be sprayed on the plants and surrounding area to kill remaining *Plutella xylostella* larvae and adults. Pheromone-baited traps will be deployed at the trial site that will continue until no OX4319L-Pxy male moths are recaptured for 2 consecutive weeks.

Data from these field experiments will provide information on dispersal and field longevity of OX4319L-Pxy male moths.

Field cage studies. During the cage experiments, we will: conduct mating competition experiments between male moths of OX4319L-Pxy and recently-colonized wild-type male moths, for wild-type female mates; assess longevity of OX4319L-Pxy male moths and wild-type male moths in field cages; assess the reproductive rate of *Plutella xylostella* in field cages; and test suppression of field cage populations of wild-type *Plutella xylostella* through releases of OX4319L-Pxy male moths. Cages will consist of screened cages approximately 24ft x 12ft x 6ft (L x W x H) covering a metal frame that does not permit ingress or egress of *Plutella xylostella* moths.

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

All OX4319L-Pxy moths used in the trials will be reared as larvae on non-tetracycline artificial diet. Releases will be conducted from the ground or vehicle on Cornell University's New York State Agricultural Research Station. Releases will be conducted up to five times per week, depending on experimental requirements.

The area around the release sites (up to 1000 m radius from release site) will be monitored with traps (e.g. sticky traps baited with synthetic sex pheromone). Traps will be collected at least weekly to count the number of OX4319L-Pxy moths and wild moths captured on each trap. Samples in the laboratory will be screened for presence of the DsRed2 fluorescent marker, using fluorescence microscopy, and this will be validated by PCR detection of the DNA construct in selected samples. Some non-viable insect samples will be sent to Oxitecs labs in the UK for the PCR analysis. Prior to each field release, samples from each cohort of male OX4319L-Pxy moths will be screened for the fluorescent marker and sexed. Only male moths - which do not directly damage crops - will be released so the effect on the crop will therefore be negligible: male activity is restricted to finding and mating with females, imbibing liquids from plants, and taking shelter during the day.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the

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regulated article at each of the intended destinations:

Adult genetically modified moths will be transported in sealed containers labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle by authorized personnel.

The conditional lethality expressed by the construct carried by OX4319L-Pxy means that female progeny from matings with Oxitec male insects die in the absence of tetracycline, and the trait is therefore unlikely to persist in the environment. Other mitigation measures include the lack of known sexually compatible relatives of *Plutella xylostella* in the USA; the piggyBac transposable element used for the transformation has no endogenous functioning transposase, rendering it non-autonomous (it cannot mobilize itself); the release area will be monitored extensively with traps to attract and collect *Plutella xylostella* moths; the release field is no larger than 10 acres; the *Plutella xylostella* can be sprayed with insecticide at any time in the case of observed adverse events; the OX4319L-Pxy *Plutella xylostella* will be securely managed and contained in production and transport; and all viable insects reared for this trial that are not required for release or additional analysis will be devitalized by freezing.

Final Disposition Method: Destruction/Devitalization Other Storage in Contained Facility

Final Disposition Description: All unused genetically modified eggs, larvae, pupae and moths not released, or not needed in the mass-rearing, will be frozen at a minimum of $-15^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 48 h to destroy all life stages.

14. ATTACHMENTS**Attachments**

BRS Permit 13-297-102r-a1 (3/16/2016 @ 08:43 AM)
BRS importation permit 12-227-102m (3/16/2016 @ 08:42 AM)
CBI Justification statement- Amended (5/31/2016 @ 03:51 AM)
Cornell University field site with GPS (3/16/2016 @ 08:43 AM)
Jin et al 2013-Scientific publication- description of the technology (3/16/2016 @ 08:38 AM)
OX4319L chlortetracycline sensitivity-CBI deleted (3/16/2016 @ 08:46 AM)
OX4319L chlortetracycline sensitivity-contains CBI (3/16/2016 @ 08:45 AM)
OX4319L construct sequencing- Contains CBI (3/16/2016 @ 08:47 AM)
OX4319L construct sequencing-CBI Deleted (3/16/2016 @ 08:47 AM)
OX4319L molecular characterisation-CBI deleted (3/16/2016 @ 08:49 AM)
OX4319L molecular characterisation-Contains CBI (3/16/2016 @ 08:48 AM)
OX4319L tTAV expression levels-CBI Deleted (3/16/2016 @ 08:50 AM)
OX4319L tTAV expression levels-Contains CBI (3/16/2016 @ 08:50 AM)
Plutella OX4319L Phenotype-Genotype text for submission 16March2016 (3/16/2016 @ 09:04 AM)
Scientific publication- Harvey-Samuel et al 2014 (3/16/2016 @ 08:44 AM)
Scientific publication- Harvey-Samuel et al 2015 (3/16/2016 @ 08:45 AM)
Table of genetic elements-Contains CBI (3/16/2016 @ 08:51 AM)
Threatened or endangered species (3/16/2016 @ 08:51 AM)

15. ADDITIONAL INFORMATION

i) Similar introductions were previously authorised under permit number: 13-297-102r-a1 and importation under permit number 12-227-102m

ii) Data from some of the research reports submitted under permit number: 13-297-102r-a1 have now been described in the peer reviewed publications noted below:

Harvey-Samuel, T., Ant, T., Gong, H., Morrison, N.I., and Alphey, L. (2014). Population-level effects of fitness costs associated with repressible female-lethal transgene insertions in two pest insects. *Evol Appl* 7, 597-606.

Harvey-Samuel, T., Morrison, N.I., Walker, A.S., Marubbi, T., Yao, J., Collins, H.L., Gorman, K., Davies, T.G., Alphey, N., Warner, S., et al. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biol* 13, 49.

16. COURTESY JUSTIFICATION

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(b)(6) hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON

(b)(6)

18. DATE

March 16, 2016



INTERNAL RESEARCH REPORT

- 1. Title:** Investigating the expression of the tetracycline-repressible, female-specific lethal trait in the fsRIDL strain, OX4319L-Pxy, in response to different concentrations of chlortetracycline in larval feed.

- 2. Statement Of Data Confidentiality Claims:**

This document contains confidential business information which is proprietary and the publication or disclosure of which would harm the legitimate business interests of Oxitec Ltd.

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The information contained in this document may not be used by any third party including but not limited to any regulatory authority to support registration or approval of this product or any other product without the prior consent in writing of the company supplying the relevant information.

- 3. Statement Concerning Good Laboratory Practices:**

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

- 4. Authors:**

Study Coordinator (Signature): [(b)(6)]]	Study Supervisor (Signature): [(b)(6)]
Study Coordinator (Name And Position): (b)(6) Research Scientist	Study Supervisor (Name And Position): (b)(6)
Date Signed: 17 th September 2013	Date Signed: 17 th September 2013

5. Associated Personnel:

Name	Tasks
(b)(6)	Study coordination, experimental design, report writing, data collection
	Experimental design, approval
	Study Sponsor

6. Test Facility:

This research was performed at Oxitec Ltd facilities at:

71 Milton Park,
Abingdon,
Oxfordshire,
OX14 4RX,
UK

7. Objectives:

Investigate the effect of dietary chlortetracycline on the penetrance of the female-lethal trait in OX4319L-Pxy-heterozygotes.

8. Summary:

This experiment was designed to investigate at which concentration chlortetracycline is able to repress engineered female-specific lethality in OX4319L-Pxy females. There is a possibility of tetracycline availability in the wild, for instance through manure from antibiotic-treated farm animals used to fertilise crops, but this is uncommon and - when detected - concentrations found in crops are low (Hu et al., 2010; Migliore et al., 2010; Seo et al., 2010). We tested survival of male and female larvae, heterozygous for the OX4319L-Pxy transgene insertion, when reared on different chlortetracycline concentrations in artificial diet (Jin et al., 2013).

9. Introduction:

The tetracycline-repressible, female-specific mortality in fsRIDL insect strains provides a means of producing large male-only cohorts of insects and a population suppression effect in the target population: reduction of females reduces a population's reproductive potential. Efficacy relies upon high penetrance of the female-lethal trait in the field. In laboratory test crosses, in the absence of dietary tetracycline, OX4319L-Pxy heterozygotes have shown 0% survival of females (i.e. 100% penetrance of the female-lethal phenotype), and high survival of males. A theoretical concern is that environmental tetracyclines could repress the female lethal trait and allow the transgene to persist for longer in the environment. We therefore set out to establish sensitivity concentration-response relationship between dietary chlortetracycline and the penetrance of the female-lethal phenotype in OX4319L-Pxy heterozygotes. We tested OX4319L-Pxy heterozygotes, rather than homozygotes, as we anticipated that one copy of the transgene per cell is likely more susceptible to repression than is two copies, and the larvae present in the field are expected to be heterozygous for the OX4319L-Pxy insertion (the progeny of released homozygous males and wild-type females).

10. Methods

Eggs heterozygous for the OX4319L-Pxy transgene insertion were generated by establishing 10 replicated crosses with OX4319L-Pxy-homozygous males with wild-type females. Artificial diet (Bioserv beet armyworm diet, cat # F9221B) was prepared with six different concentrations of chlortetracycline: 0 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml, 10 µg/ml and 100 µg/ml. Egg collections from each cross were divided between these diets, and the hatched larvae reared to pupation. These pupae were separated by sex then incubated in Petri dishes; survival to adulthood was recorded.

11. RESULTS

As in previous test crosses, OX4319L-Pxy-heterozygous male survival is approximately equivalent to, but slightly lower than, that of wild-type, irrespective of chlortetracycline concentration (Figures 1 & 2). Mortality was well-repressed in females reared on 10 µg/ml and 100 µg/ml. As the chlortetracycline concentrations reduced to 1.0 µg/ml and 0.1 µg/ml, female survival rates dropped sharply, and below this (0.01 µg/ml and 0.0 µg/ml) only very small numbers of females survived to pupation (<2%) and none survived to adulthood.

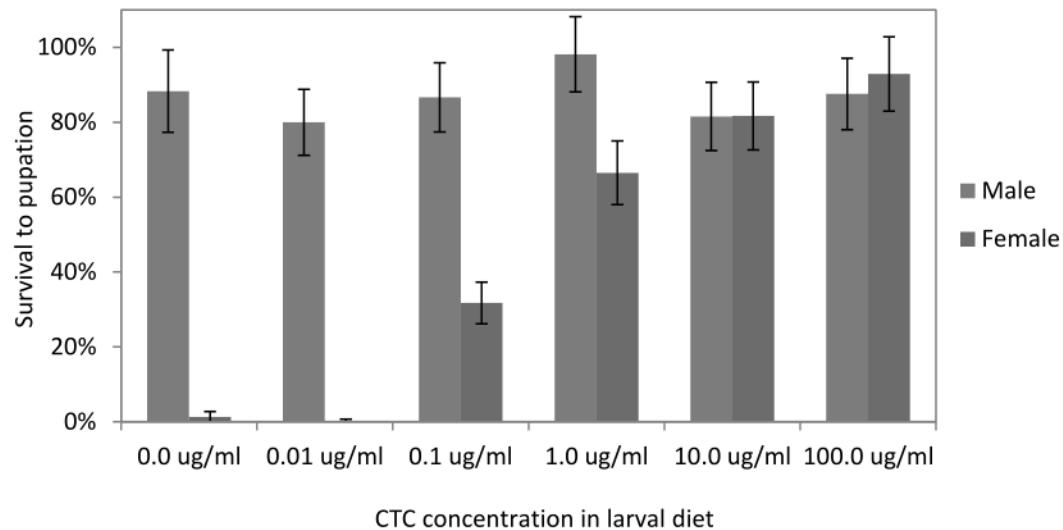


Figure 1: Survival-to-pupation of OX4319L-Pxy-heterozygous diamondback moth reared on different concentrations of chlortetracycline (CTC) in larval diet. Survival is expressed relative to that of wild-type counterparts. Error bars indicate 95% confidence intervals.

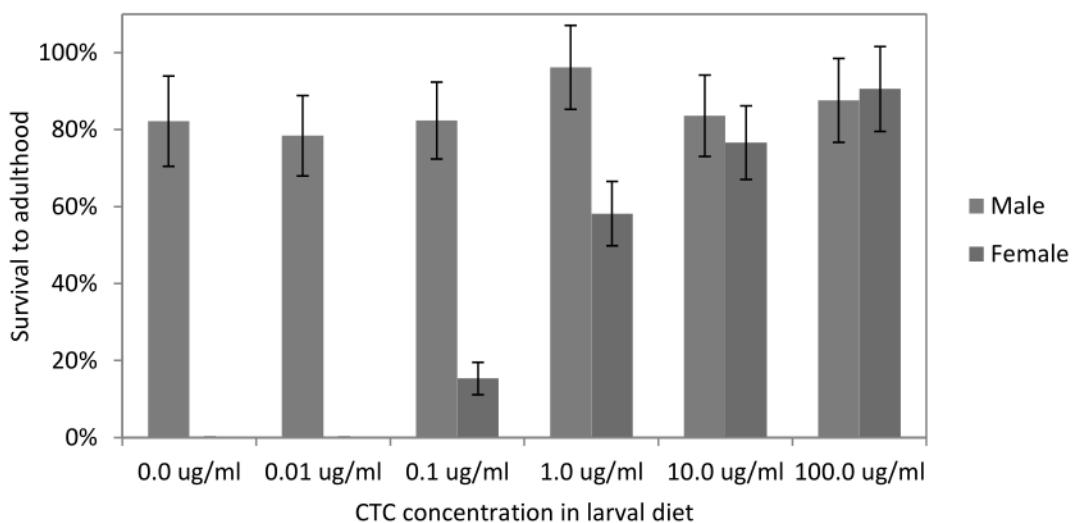


Figure 2: Survival-to-adulthood of OX4319L-Pxy-heterozygous diamondback moth reared on different concentrations of chlortetracycline (CTC) in larval diet. Survival is expressed relative to that of wild-type counterparts. Error bars indicate 95% confidence intervals.]

12. Discussion and Conclusions:

No OX4319-Pxy-heterozygous females survived to adulthood on 0.01 $\mu\text{g}/\text{ml}$ CTC, while at or above 10 $\mu\text{g}/\text{ml}$ CTC OX4319L-Pxy-heterozygous female survival to adulthood, relative to wild-type, was similar to that of males.]The level of CTC needed for survival far exceeds that which diamondback moth might be expected to encounter in the wild. For comparison, laboratory experiments growing cabbage on soil artificially contaminated with manure from CTC-fed pigs, and spiked with CTC solution, found <0.004 $\mu\text{g}/\text{ml}$ CTC in foliage (Kumar et al., 2005). These results provide evidence that fsRIDL trait-repressing concentrations of tetracycline are highly unlikely to be encountered on host plants in the environment.

13. Literature:

- Hu, X., Zhou, Q., and Luo, Y. (2010). Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environmental Pollution 158*, 2992-2998.
- Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T.H., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., et al. (2013). Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology 2*, 160-166.
- Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y., and Rosen, C.J. (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *Journal of environmental quality 34*, 2082-2085.
- Migliore, L., Godeas, F., De Filippis, S., Mantovi, P., Barchi, D., Testa, C., Rubattu, N., and Brambilla, G. (2010). Hormetic effect(s) of tetracyclines as environmental contaminant on *Zea mays*. *Environmental Pollution 158*, 129-134.
- Seo, Y., Cho, B., Kang, A., Jeong, B., and Jung, Y.-S. (2010). Antibiotic uptake by plants from soil applied with antibiotic-treated animal manure. *Korean J Soil Sci Fert 43*, 466-470.



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U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE
PERMIT UNDER 7 CFR 340
(Genetically Engineered Organisms or Products)

This permit was generated electronically via the ePermits system

Enclosed is the BRS Permit Application

PERMITTEE NAME: (b)(6)

ORGANIZATION: Cornell
University/NYSAES

ADDRESS: 630 W. North St.
(b)(6)
Geneva, NY 14456

PHONE: (b)(6)

FAX:

RELEASE:

NY

PERMIT NUMBER 13-297-102r-a1

AMENDMENT DATE: July 15, 2015

ORIGINAL EFFECTIVE DATE: November 10, 2014

EXPIRES: November 10, 2017

INTRODUCTION TYPE Release

PERMIT TYPE Standard

PURPOSE OF PERMIT Traditional

Under the conditions specified, this permit authorizes the following:

Regulated Article: Plutella xylostella

Permit Number: 13-297-102r-a1

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS

SIGNATURE OF BRS OFFICIAL

Subray Hegde

DATE

July 15, 2015

The collection of this information is authorized by the Plant Protection Act of 2000. The information will be used to determine eligibility to receive all types of permits. No permit will be issued until this application has been approved.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE

APPLICATIONS FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

1. NAME, ADDRESS, TELEPHONE, AND EMAIL OF APPLICANT

Name: (b)(6)
 Position: Professor
 Organization: Cornell University/NYSAES
 Organization Unique ID:
 Address: 630 W. North St.
 (b)(6)
 Geneva, NY 14456
 County/Province:
 Township/Island:
 Day Telephone: (b)(6)
 FAX:
 Alternate:
 Email 1: (b)(6)@cornell.edu
 Email 2:

2. INTRODUCTION TYPE

- Importation
- Interstate Movement
- Interstate Movement and Release
- Release

3. PERMIT TYPE

- Standard
- Permit
- Courtesy
- Permit

4. PURPOSE OF PERMIT

- Industrial Product
- Pharmaceutical Product
- Phytoremediation
- Traditional

5. CONFIDENTIAL BUSINESS INFORMATION VERIFICATION (CBI)

Does this application contain CBI? Yes No

CBI Justification:

N/A

6. REQUEST TYPE

- New Amendment Renewal

Amendment/Renewal Description:

The proposed amendment of the use of cages for preliminary trials is to be located in exactly the same experimental area as described in the original permit and the cage increases the confinement of the regulated article.

As it is in the same location as previously assessed in the EA (pgs 10-12) relating to that permit, we therefore understand that no new NEPA assessment is required.

Previous Permit Number(s): 13-297-102r

7. MEANS OF MOVEMENT

Import by air; releases manually from the ground/vehicles.

8. VARIANCE VERIFICATION

Have you previously applied for variance(s) that you wish to apply to this permit? Yes No

Variance Number(s):

If so, describe in a brief summary how the variance will be applied:

N/A

9. REGULATED ARTICLE

Scientific Name: Plutella xylostella

Common Name: Diamondback moth

Cultivar and/or Breeding Line:

Any biological material (e.g., culture medium, or host material) accompanying the regulated Article during movement:

Artificial insect diet. This diet will be frozen at -15°C for 12 h prior to import.

Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed, and produced:

All final engineering of the transforming constructs was performed at Oxitec Ltd, in the United Kingdom.

The genes used from the donor organisms and the piggyBac-derived portions of the vectors used to build the transforming construct were cloned off-site. The recipient organism is the moth, *Plutella xylostella*, which is endemic in temperate regions around the world, including the USA. The recipient *Plutella xylostella* strain for the transformation was a wild-type strain obtained from Syngenta (public limited company), UK, which has been reared in Oxitec insectaries since 2008.

Processes, Procedures, and Safeguards Description:

This is a permit request for 3 years of seasonal releases (April to October) of a female-lethal, genetically marked diamondback moth (maximum 100,000 moths/week), in brassica fields at the Cornell University research station, Geneva NY.

Males of the transgenic moths will be released in cultivated brassica plots and biological parameters of these moths, such as dispersal and persistence, measured using traps, for example baited with synthetic sex pheromone. The moths of the OX4319L-Pxy strain carry a stable, heritable marker the DsRed2 fluorescent protein, viewed by fluorescence microscope or detected by PCR and their female progeny die in the absence of a dietary repressor (tetracycline or suitable analogues supplied in their artificial diet). The male-selecting (female-lethal) penetrance of the strain is >99% (Jin et al. 2013). The marker provides a means of distinguishing released moths from wild moths, and female-lethality is a self-limiting trait in the wild.

All genetically modified moths will be reared in insectaries at Cornell University, Geneva NY. The facilities and their general operation have been inspected and approved through a previous importation permit (12-227-102m). Larval rearing will be conducted in quarantine using the same approved procedures as in this previous permit. Only moths homozygous for the conditional lethal transgene, reared off tetracycline, will be released. Adult moths will be transported in sealed containers, with at least two layers of containment, labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle; for each batch the number of containers and identity of member of staff supervising the release will be recorded.

The transgenic diamondback moths encode no toxic or allergen proteins. The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA-CFSAN in the USA for human safety, and they raised no objections to its use in corn plants. This involved an assessment of the amino acid sequence using bioinformatics analyses in accordance with the Guidance provided by Codex (2003), the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, as well as the toxicity of the protein using bioinformatics analysis. The amino acid sequence in OX4319L-Pxy is the same as that evaluated in the NPC. It has been further evaluated in an Environmental Assessment (EA) by the USDA (http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf), which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk. Additional EAs on another GE moth, GE pink bollworm, expressing fluorescent genes similar to DsRed2 have also been conducted (<http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm>) and concluded that it was unlikely to present any hazard to the environment.

The other protein coding region, tTAV, is regulated by sequences from the sex-determination gene, doublesex, from pink bollworm (*Pectinophora gossypiella*), that produce different splice variants in males and females: the female transcript comprises coding sequence for the tetracycline-repressible transcription factor, tTAV, which interacts with the upstream tetracycline response element, tetO (or tRE), to form a positive-feedback loop that results in insect lethality prior to adulthood.

Under the control of the doublesex sex-alternate splicing, lethality is induced only in females.

The tTAV amino acid sequence in OX4319L-Pxy has also been evaluated independently using the bioinformatics analyses provided by Codex (2003) for both potential allergenicity and toxicity. No homologies with known allergens or toxins were determined. This study is available on request.

Tetracycline can be provided to the insect in larval artificial diet to suppress female death and permit colony rearing in the laboratory. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

This female-specific lethal trait was previously discussed in a USDA Environmental Impact Statement published in October 2008, entitled Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs, which concluded that the use of genetically engineered fruit flies and pink bollworm in APHIS plant pest control programs were the environmentally preferred alternative (Record of Decision (Federal Register Vol 74 (87) 21314 2009).

Reference:

Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology, 2:160-166.

10. ARTICLE SUPPLIER AND/OR DEVELOPER

Name	Location	Contact Information
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WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

(b)(6)

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71 Milton Park
Abingdon
OX144RX
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11. PHENOTYPES/GENOTYPE

1) Phenotypic Designation Name: visual marker; repressible lethality

Identifying Line(s): OX4319L-Pxy, OX4319N-Pxy, OX4767A-Pxy

Construct(s): OX4319, OX4767

Mode of Transformation: Direct injection

Phenotype Description:

A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.

The introduced genetic material in the diamondback moth comprises three protein coding regions, one for marking the insects and two for inducing death before the insect reaches adulthood (in this instance, females only). The former allows the expression of a DsRed2 fluorescent protein originally derived from a coral (*Discosoma sp.*). The transgenic diamondback moth with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenic diamondback moth can be envisioned. The non-modified diamondback moth has no fluorescent protein gene; therefore, it does not fluorescence when illuminated under the same light frequency. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

Phenotype(s)

MG - Visual marker; DsRed2 Fluorescent Protein Expression

Genotype(s)

Screenable Marker

Gene: DsRed2 **from** *Discosoma sp.* - Screenable marker gene DsRed2 from *Discosoma spp* - Allows the expression of a fluorescent protein from *Discosoma spp*. Fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5iel promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by intense illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. Expression of a fluorescent protein will therefore permit released modified moths to be distinguished from unmodified.

Vector Sequence: piggyBac (non-autonomous) **from** piggyBac from *Trichoplusia ni* (moth) - Transformation Vector from *Trichoplusia ni* (moth) - Effects germline transformation of diamondback moth from piggyBac from *Trichoplusia ni* (moth) - 3' end of piggyBac. piggyBac is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element. In the construct used for transformation of the pink bollworm, the transposase gene of the piggyBac element was irreversibly destroyed by insertion of the transgene. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding piggyBac transposase under the control of the *Drosophila melanogaster* hsp70 promoter. One of the inverted terminal repeats that flank the wild-type piggyBac transposase in piggyBac has been removed in the helper plasmid so that the helper plasmid cannot itself integrate even though it encodes for active piggyBac transposase.

Repressible lethality

Gene: tTAV **from** Escherichia coli (bacterium) and Herpes simplex (virus) - Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from Escherichia coli with VP16 from a type 1 herpes simplex virus. The tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (tetO) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline, it will induce expression from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and Drosophila melanogaster with no known adverse effects. Unmodified Plutella xylostella do not have a tTAV activity.

Regulatory sequence: doublesex genomic region **from** Pink bollworm, Pectinophora gossypiella - Female-specificity is conferred using truncated sex-alternate splicing sequences from the doublesex gene of Pectinophora gossypiella. Sequence encoding tTAV is inserted into this splicing sequence, allowing for the expression of tTAV in a sex-specific manner, resulting in a conditional female-lethal system (Jin et al. 2013).

A full list of construct components is provided in the attached Table of genetic elements.

References:

- Berger SL, et al. 1990 Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* 61, 1199-1208.
- Damke H, et al. 1995 Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Meth Enzymol* 257, 209-220.
- Gillespie JP, et al. 1997 Biological mediators of insect immunity. *Annu Rev Entomol* 42, 611-643.
- Gong P, et al. 2005 A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat Biotechnol* 23, 453-456.
- Gossen M, et al. 1994 Inducible gene expression systems for higher eukaryotic cells. *Curr Opin Biotechnol* 5, 516-520.
- Gossen M, and Bujard H 1992 Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 89, 5547-5551.
- Salghetti S, et al. 2001 Regulation of transcriptional activation domain function by ubiquitin. *Science* 293, 1651-1653.
- Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2:160-166.

12. INTRODUCTION**Release Site**

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Research Farm North	<p>NY County: Ontario</p> <p>Proposed Release Start Date: 4/1/2014 Proposed Release End Date: 3/31/2017 No. of Releases: up to 72/year Quantity: up to 100,000 moths/wk; 10 acres acres</p>	
Location Unique ID:	RFN1097	
Location GPS Coordinates:	(b)(4)	

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Release Site History: Managed agricultural, cropping, research. Managed agricultural land around release site.

Critical Habitat Involved? Yes No

13. DESIGN PROTOCOLS

Production Design

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

The diamondback moth strains OX4319L-Pxy, OX4319N-Pxy and OX4767A-Pxy show a tetracycline-repressible female-lethal phenotype, which could serve as an insecticide-free means of controlling pest populations of diamondback moth in the field in a species-specific manner. Successful pest control will rely upon strong performance of released males, in terms of female-seeking behavior and mating competitiveness. We will seek to measure relevant performance traits in one or more mark-release-recapture field experiments. These will be followed by pest suppression trials, in which *Plutella xylostella*-infested fields will be treated with fsRIDL male *Plutella xylostella*, and the wild populations monitored and compared with those of fields not so treated.

In the mark-release-recapture experiments, we will release up to 20,000 male fsRIDL *Plutella xylostella* (per release; up to 100,000 males per week) from single or multiple points in experimental fields of up to 10 acres planted with brassicas (e.g. cabbage or broccoli). Traps (e.g. sticky traps baited with synthetic sex pheromone) will be placed in and around the field, up to 1 km from the release point, to recapture the male moths. Traps will be collected at least once per week and the recaptured moths screened for the fluorescence marker. Additional PCR screening will be conducted to validate this visual screening. Trapping will continue until no fsRIDL male moths are recaptured for 2 consecutive weeks. To permit an overlapping series of releases in each experimental field that can be independently monitored on the traps, fsRIDL male moths will be sometimes be additionally marked, for example using different-colored fluorescent powders, which are commonly used in such field experiments with insects (reviewed by Hagler & Jackson 2001 Ann Rev Entomol 46:511-543). Crop sampling, in which a proportion of the in-field plants will be collected and closely examined in the laboratory for *Plutella xylostella* larvae and pupae (wild-type and transgenic), will be conducted at regular intervals to assess mating success of the fsRIDL males. Each experimental field will be surrounded by an approximately 10m-deep border free of potential host plants. Upon completion of the experiment, the insecticide Coragen (chlorantraniliprole) will be sprayed on the plants and surrounding area to kill remaining diamondback moth larvae.

Data from these preliminary field experiments, indicating the fsRIDL male release rate required to achieve a given overflooding rate (e.g. 10:1 fsRIDL:wild males), dispersal and field longevity, will inform the release strategy of fsRIDL male diamondback moths in a suppression trial (how many fsRIDL males to release, how frequently and from how many points), requested as part of this permit application. All of the described trials will require monitoring of a wild diamondback moth population in up to six experimental fields (up to three treated with fsRIDL male moths, and up to three untreated). These fields, of up to 10 acres in size, will be planted with brassica plants (e.g. cabbage or broccoli). If the wild diamondback moth population is not present in sufficient numbers at the trial sites, the experimental fields will be artificially infested with male and female moths from a USA-derived wild-type diamondback moth strain currently maintained in the laboratory; dye-marked wild-type moths may also be used in mark-release-recapture experiments to provide a direct comparison with the GE moths. A proportion of the experimental fields will be subjected to regular releases (up to five times weekly) of fsRIDL male moths, in numbers greater than the estimated recruitment rate of wild-type male moths in the environment, to achieve an over-flooding effect of fsRIDL males on the wild male diamondback moth population. For each experiment, fsRIDL male releases will be conducted for up to the duration of a brassica crop cycle (anticipated as 3-4 months). Adult traps (e.g. sticky traps baited with synthetic sex pheromone) will be placed in and around each field to monitor the relative numbers of wild-type and fsRIDL males present, and to assess their dispersal. The populations of wild-type moths in each field, including those receiving no fsRIDL males, will be monitored using the adult traps described and periodic crop sampling. Releases will consist of up to 100,000 fsRIDL male moths per week (depending on the overflooding ratio required) over the treatment fields over the course of these suppression experiments. Trapping will continue after the last releases of fsRIDL male moths, and will continue until at least 2 weeks of zero fsRIDL recaptures. Upon completion of the experiment, the insecticide Coragen (chlorantraniliprole) will be sprayed on the plants and surrounding area (within 100 m radius of treated fields) to kill remaining diamondback moth larvae. Post-experiment pheromone trapping will continue for 2 weeks to monitor field longevity of fsRIDL moths.

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

All genetically modified male diamondback moth used in the trials will be reared as larvae on non-tetracycline artificial diet. Releases will be conducted from the ground or vehicle on Cornell University's New York State Agricultural Research Station. Releases will be conducted up to five times per week, depending on experimental requirements.

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

The area around the release sites (up to 1000 m radius from release site) will be monitored with traps (e.g. sticky traps baited with synthetic sex pheromone). Traps will be collected at least weekly to count the number of genetically modified moths and wild moths captured on each trap. Samples in the laboratory will be screened for presence of the DsRed2 fluorescent marker, using fluorescence microscopy, and this will be validated by PCR detection of the DNA construct in selected samples. Some non-viable insect samples will be sent to Oxitecs labs in the UK for the PCR analysis. Prior to each field release, samples from each cohort of male fsRIDL moths will be screened for the fluorescent marker and sexed. Only male moths will be released; the effect on the crop will therefore likely be negligible: male activity is restricted to finding and mating females, feeding on nectar from flowers, and taking shelter during the day.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations:

Adult genetically modified moths will be transported in sealed containers labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle by authorized personnel.

The conditional lethality expressed by the fsRIDL construct means that female progeny from matings with Oxitec male insects die in the absence of tetracycline, and the trait is therefore unlikely to persist in the environment. Other mitigation measures include the lack of known sexually compatible relatives of *Plutella xylostella* in the USA; the piggyBac transposable element used for the transformation has no endogenous functioning transposase, rendering it non-autonomous (it cannot mobilize itself); the release area will be monitored extensively with traps to attract and collect *Plutella xylostella* moths; release fields are no larger than 10 acres; the *Plutella xylostella* can be sprayed with insecticide at any time in the case of observed adverse events; the genetically engineered *Plutella xylostella* will be securely managed and contained in production and transport; and all viable insects reared for this trial that are not required for release or additional analysis will be devitalized by freezing.

Final Disposition Method: Destruction/Devitalization Other Storage in Contained Facility

Final Disposition Description:

This amendment does not change anything described in final disposition, as described in the original permit.

All unused genetically modified eggs, larvae, pupae and moths not released, or not needed in the mass-rearing, will be frozen at a minimum of $-15^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 48 h to destroy all life stages.

14. ATTACHMENTS

Attachments

BRS Importation permit (Exp. 9/2013) (7/6/2015 @ 01:17 PM)
Cornell University proposed field release site (7/6/2015 @ 01:17 PM)
OX4319L allele persistence report (7/6/2015 @ 01:17 PM)
OX4319L chlortetracycline sensitivity report (7/6/2015 @ 01:17 PM)
OX4319L construct sequencing report (7/6/2015 @ 01:17 PM)
OX4319L molecular characterisation report (7/6/2015 @ 01:17 PM)
OX4319L population suppression cages report (7/6/2015 @ 01:17 PM)
OX4319L resistance management report (7/6/2015 @ 01:17 PM)
Peer-reviewed publication (Jin et al. 2013) describing development/testing of RIDL DBM (7/6/2015 @ 01:17 PM)
Table of genetic elements, OX4319 and OX4767 (7/6/2015 @ 01:17 PM)
Threatened or endangered species (7/6/2015 @ 01:17 PM)
tTAV expression levels report (7/6/2015 @ 01:17 PM)

15. ADDITIONAL INFORMATION

During the cage experiments, we will: conduct mating competition experiments between male moths of the Oxitec strain (OX4319L) and recently-colonised wild-type male moths, for wild-type female mates; assess longevity of OX4319L male moths and wild-type male moths in field cages; assess the reproductive rate of diamondback moths in field cages; and test suppression of field cage populations of wild-type diamondback moth through releases of OX4319L male moths.

16. COURTESY JUSTIFICATION

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

I, (b)(6) hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON

(b)(6)

18. DATE

July 6, 2015

SUPPLEMENTAL PERMIT CONDITIONS

For Release of *Plutella xylostella*

- (1) All persons working with the genetically-engineered (GE) diamondback moth must be informed of these permit conditions. Anyone working with these insects must agree to and sign/ initial these conditions before beginning work. These signed conditions do not need to be submitted to USDA/ APHIS but must be readily accessible in the event of an inspection and presented upon request.

Note: these conditions may be copied and stored electronically for electronic signature and initialing provided that the permit number, authorized organisms and life stages, release locations, and authorization statement all appear on the document with the permit number. The residency condition does not need to be signed. Signing these conditions only indicates that the person working under this permit has read them; the permit holder is the sole responsible party under this permit.

- (2) Modifications to the containment of these organisms must be approved prior to making changes by applying for an amendment in ePermits.
- (3) This permit does not authorize movement or use of plant pathogens listed in the Public Health Security and Bioterrorism Preparedness and Response Act of 2002. If any organism listed as a Select Agent is identified from materials associated with this research, the permit holder is required to notify APHIS, Agricultural Select Agent Program (ASAP) immediately by phone at 301-851-3300, and within seven (7) days submit APHIS/CDC Form 4 (Report of Identification of a Select Agent or Toxin in a Clinical or Diagnostic Laboratory) to APHIS, ASAP; 4700 River Rd, Unit 2, Riverdale, MD 20737 (see instructions at: http://www.aphis.usda.gov/programs/ag_selectagent/index.shtml). Failure to comply with this requirement is a violation of the Agricultural Bioterrorism Protection Act of 2002.
- (4) If organisms that are not authorized in this permit are received, the permit holder must take all prudent measures to contain the organism(s) and notify the BRS Compliance Staff by contacting a compliance officer immediately (that is, within one business day) by calling 301-851-3935 or by e-mail to BRSCompliance@aphis.usda.gov. The permit holder must immediately notify the permit unit of the destruction of regulated organisms received under this permit, as above. Similarly, the permit holder must immediately notify the permit unit if facilities are destroyed or decommissioned for any reason.
- (5) The permit holder must maintain an official permanent work assignment at the address identified on this permit. If the permit holder ceases assignment/affiliation at the address identified on this permit, or personnel circumstances change in any way, then a compliance officer must be notified at the BRS Compliance Staff immediately (that is, within one business day) by either (a) email to BRSCompliance@aphis.usda.gov, (b) verbal communication at 301-851-3935, or (c) conventional mail to BRS Compliance Staff, 4700 River Road, Riverdale, Unit 91, MD 20737. The permit holder must destroy all regulated organisms prior to departure unless the permit holder either (a) requests cancellation of this permit and complies with all permit-specific termination conditions, (b) applies for and receives a permit to move the organisms to a new facility, or (c) relinquishes control of the regulated organisms to a qualified individual who obtained a permit for the continued use of these regulated organisms prior to this permit holder's departure.
- (6) All laboratories and growth chambers where this genetically engineered insect is employed will be locked with limited access by authorized personnel. In each area where a regulated genetically engineered organism is used, at least one sign must be posted stating that a regulated genetically engineered organism is being used.
- (7) Upon completion of research all engineered insects (except those retained for future research), will be disposed of by freezing at -20 for 24 hours/or autoclaving.
- (8) There is to be no further distribution of these genetically engineered insects under this permit without prior approval from State (intrastate movement) and Federal regulatory officials.
- (9) THIS AUTHORIZATION IS VALID FOR THE RELEASE INTO THE ENVIRONMENT OF THIS GENETICALLY ENGINEERED INSECT ONLY IN THE AREAS DESCRIBED IN THE APPLICATION.
- (10) THIS IS A CROP DESTRUCT TRIAL. Brassicas will be destroyed at the end of the research field trial. No plants of produce shall be used for food or feed. Upon completion of the experiment, the insecticide shall be sprayed on the plants and surrounding area within a 100 m radius of treated fields to kill remaining diamondback moth larvae.
- (11) Without prior notice and during reasonable hours authorized Plant Protection and Quarantine and State regulatory officials shall be allowed to inspect the conditions under which this genetically engineered insect is being kept.
- (12) Reporting an Unauthorized or Accidental Release

a. According to the regulation in 7 CFR § 340.4(f)(10)(i), APHIS shall be notified orally immediately upon discovery and notified in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.

- For immediate verbal notification, contact APHIS BRS Compliance Staff at (301) 851-3935 and ask to speak to a Compliance and Inspection staff member. Leave a verbal report on voicemail if the phone is not answered by a Compliance Officer.
- In addition, in the event of an emergency in which you need to speak immediately to APHIS personnel regarding the situation, you may call:

The APHIS/BRS Regional Biotechnologist assigned in the region where the field test occurs:

For Western Region, contact the Western Region Biotechnologist at (970) 494-7513

or e-mail: BRSWRBT@aphis.usda.gov

For Eastern Region, contact the Eastern Region Biotechnologist at (919) 855-7622 or e-mail: BRSERBT@aphis.usda.gov Or

The APHIS State Plant Health Director for the state where the unauthorized release occurred. The list of APHIS State Plant Health Directors is available at: http://www.aphis.usda.gov/services/report_pest_disease/report_pest_disease.shtml.

or

<http://pest.ceris.purdue.edu/stateselect.html>

b. Written notification should be sent by one of the following means:

By e-mail:
BRSCompliance@aphis.usda.gov

By mail:
Biotechnology Regulatory Services (BRS)

Permit Number: 13-297-102r-a1

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS	
SIGNATURE OF BRS OFFICIAL Subray Hegde	DATE July 15, 2015



United States
Department of
Agriculture



Animal and
Plant Health
Inspection Service

Biotechnology
Regulatory
Services

4700 River Road, Unit 147
Riverdale, Maryland
20737-1236

Regulatory Operations Program
USDA/APHIS
4700 River Rd. Unit 91
Riverdale, MD 20737

Additional instructions for reporting compliance incidents may be found at http://www.aphis.usda.gov/biotechnology/compliance_incident.shtml

- (13) No person shall move a regulated article interstate unless the number of the limited permit appears on the outside of the shipping container.

Permit Number: 13-297-102r-a1

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS

SIGNATURE OF BRS OFFICIAL

Subray Hegde

DATE

July 15, 2015

Standard Permit Conditions for the Introduction of a Regulated Article

(7 CFR 340.4 (f))

Permit Conditions: A person who is issued a permit and his/her employees or agents shall comply with the following conditions , and any supplemental conditions which shall be listed on the permit, as deemed by the Deputy Administrator to be necessary to prevent the dissemination and establishment of plant pests:

- (1) The regulated article shall be maintained and disposed of (when necessary) in a manner so as to prevent the dissemination and establishment of plant pests.
- (2) All packaging material, shipping containers, and any other material accompanying the regulated article shall be treated or disposed of in such a manner as to prevent the dissemination and establishment of plant pests.
- (3) The regulated article shall be kept separate from other organisms, except as specifically allowed in the permit.
- (4) The regulated article shall be maintained only in areas and premises specified in the permit.
- (5) An inspector shall be allowed access, during regular business hours, to the place where the regulated article is located and to any records relating to the introduction of a regulated article.
- (6) The regulated article shall, when possible, be kept identified with a label showing the name of the regulated article, and the date of importation.
- (7) The regulated article shall be subject to the application of measures determined by the Administrator to be necessary to prevent the accidental or unauthorized release of the regulated article.
- (8) The regulated article shall be subject to the application of remedial measures (including disposal) determined by the administrator to be necessary to prevent the spread of plant pests.
- (9) A person who has been issued a permit shall submit to APHIS a field test report within 6 months after the termination of the field test. A field test report shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment.
- (10) APHIS shall be notified within the time periods and manner specified below, in the event of the following occurrences:
 - (i) Orally notified immediately upon discovery and notify in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article;
 - (ii) In writing as soon as possible but not later than within 5 working days if the regulated article or associated host organism is found to have characteristics substantially different from those listed in the application for a permit or suffers any unusual occurrence (excessive mortality or morbidity, or unanticipated effect on non-target organisms).
- (11) A permittee or his/her agent and any person who seeks to import a regulated article into the United States shall:
 - (i) Import or offer the regulated article for entry only through any USDA plant inspection station listed in 7 CFR 319.37-14;
 - (ii) Notify APHIS promptly upon arrival of any regulated article at a port of entry, of its arrival by such means as a manifest, customs entry document, commercial invoice, waybill, a broker's document, or a notice form provided for such purpose; and
 - (iii) Mark and identify the regulated article in accordance with 7 CFR 340.7.

Rev. January 1, 2010

Permit Number: 13-297-102r-a1

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS	
SIGNATURE OF BRS OFFICIAL Subray Hegde	DATE July 15, 2015

Any regulated article introduced not in compliance with the requirements of 7 CFR 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. Any regulated article introduced not in compliance with the requirements of 7 Code of Federal Regulation Part 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act (7 U.S.C. 7701-7772).

Permit Number: 13-297-102r-a1

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SIGNATURE OF BRS OFFICIAL

Subray Hegde

DATE

July 15, 2015



Animal and
Plant Health
Inspection Service

Biotechnology
Regulatory
Services

4700 River Road, Unit 147
Riverdale, Maryland
20737-1236

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE
PERMIT UNDER 7 CFR 340
(Genetically Engineered Organisms or Products)

This permit was generated electronically via the ePermits system

Enclosed is the BRS Permit Application

PERMITTEE NAME: (b)(6)

ORGANIZATION: Cornell/NYSAES

ADDRESS: 614 W. North St.
Geneva, NY 14456

PHONE: (b)(6)

FAX:

DESTINATION: NY

PERMIT NUMBER 12-227-102m

DATE ISSUED: September 12, 2012

EFFECTIVE: September 15, 2012

EXPIRES: September 15, 2013

INTRODUCTION TYPE Importation

PERMIT TYPE Standard

PURPOSE OF PERMIT Traditional

Under the conditions specified, this permit authorizes the following:

Regulated Article: Plutella xylostella

Permit Number: 12-227-102m

THIS PERMIT HAS BEEN APPROVED ELECTRONICALLY BY THE FOLLOWING BRS OFFICIAL VIA EPERMITS

SIGNATURE OF BRS OFFICIAL

Steven M. Bennett

DATE

September 12, 2012

The collection of this information is authorized by the Plant Protection Act of 2000. The information will be used to determine eligibility to receive all types of permits. No permit will be issued until this application has been approved.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE

APPLICATIONS FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

1. NAME, ADDRESS, TELEPHONE, AND EMAIL OF APPLICANT

Name: (b)(6)
 Position: Professor of Entomology
 Organization: Cornell/NYSAES
 Organization Unique ID:
 Address: 614 W. North St.
 Geneva, NY 14456
 County/Province:
 Township/Island:
 Day Telephone: (b)(6)
 FAX:
 Alternate:
 Email 1: (b)(6) cornell.edu
 Email 2:

2. INTRODUCTION TYPE

- Importation
- Interstate Movement
- Interstate Movement and Release
- Release

3. PERMIT TYPE

- Standard
- Permit
- Courtesy
- Permit

4. PURPOSE OF PERMIT

- Industrial Product
- Pharmaceutical Product
- Phytoremediation
- Traditional

5. CONFIDENTIAL BUSINESS INFORMATION VERIFICATION (CBI)

Does this application contain CBI? Yes No

CBI Justification:

N/A

6. REQUEST TYPE

- New Amendment Renewal Variance Amendment, Renewal and/or Variance

Amendment/Renewal Description:

Previous Permit Number(s):

7. MEANS OF MOVEMENT

Express carrier or in baggage or carry-on luggage

8. VARIANCE VERIFICATION

Have you previously applied for variance(s) that you wish to apply to this permit? Yes No

Variance Number(s):

If so, describe in a brief summary how the variance will be applied:

N/A

9. REGULATED ARTICLE

Scientific Name: Plutella xylostella

Common Name: Diamondback moth

Cultivar and/or Breeding Line:

Phenotypic designation name: fsRIDL

Any biological material (e.g., culture medium, or host material) accompanying the regulated Article during movement:

Artificial insect diet

Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed, and produced:

All final engineering of the transforming constructs was performed at Oxitec Ltd, in the United Kingdom.

The genes used form the donor organisms and the piggyBac-derived portions of the vectors used to build the transforming construct were cloned off-site. The recipient organism is the moth, *Plutella xylostella*, which is endemic in temperate regions around the world, including the USA.

The recipient *Plutella xylostella* strain for the transformation was a wild-type strain obtained from Syngenta plc, UK, which has been reared in Oxitec insectaries since 2008.

Processes, Procedures, and Safeguards Description:

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

10. ARTICLE SUPPLIER AND/OR DEVELOPER

<u>Name</u>	<u>Location</u>	<u>Contact Information</u>
(b)(6)	Oxitec Ltd 71 Milton Park Oxford OX14 4RX United Kingdom County: Oxfordshire	Day Telephone: (b)(6) FAX: (b)(6) Email: (b)(6)

11. PHENOTYPES/GENOTYPE**1) Phenotypic Designation Name:** fsRIDL**Identifying Line(s):****Construct(s):** fsRIDL**Mode of Transformation:** Direct injection**Phenotype Description:**

A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.

Genetically modified *Plutella xylostella* were produced using the transposable element piggyBac, isolated from the cabbage looper moth, *Trichoplusia ni*. These transposons insert in single or multiple copies in the moth genome. Ie1/Hr5 specific promoters derived from *Autographa californica* nuclear polyhedrosis virus (AcMNPV) are used to drive the expression of the fluorescent proteins DsRed2, which enables easy visualization of the transgenes.

Phenotype(s)

MG - Pigment composition altered

OO - Sterile

Genotype(s)

Sterile

Gene: Screenable marker Gene **from** *Discosoma* spp - Allows the expression of a fluorescent protein from *Discosoma* spp. this allows the expression of a fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5iel promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. The unmodified moths are not strongly fluorescent. Expression of a fluorescent protein will therefore permit all other modified moths to be distinguished from unmodified.

Enhancer: Enhancer **from** *Escherichia coli* - Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from *Escherichia coli* with VP16 from a type 1 herpes simplex virus the tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (tetO) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline it will induce expression from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and *D. melanogaster* with no known adverse effects. Unmodified *Plutella xylostella* do not have a tTAV activity.

Gene: Female-specificity **from** *Escherichia coli* - tTAV is inserted into genomic regions containing specific sequences enabling the alternative splicing allowing for the expression of tTAV in a sex-specific manner, resulting in a female-lethal system.

12. INTRODUCTION**Point of Origin**

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Oxitec Ltd	United Kingdom	

Destination

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Cornell University/NYSAES - (b)(4)	(b)(4) 630 W. North St. Geneva, NY 14456 County: Ontario Proposed Start Date: 9/14/2012 Proposed End Date: 9/13/2013 Quantity: 5000 Individual Adult, eggs, larvae or pupae Inspected by BRS or PPQ? Yes Previous Permit No.: 63937	

13. DESIGN PROTOCOLS**Production Design**

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

N/A

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

Shipment of no more than 5,000 *Plutella xylostella* eggs/larvae/pupae from the United Kingdom to Cornell University will involve packaging the moths into shatter-resistant plastic containers with a further two layers of containment, which will be closed and sealed with tape. The box will be labelled as to its contents, origin, destination and contact telephone numbers.

Upon arrival at the insectaries at Cornell University the shipment of moths will be opened and maintained (b)(4) (b)(4)

At this time there are no plans for release, dissemination, distribution, open release- field trials or sale of these insects. The received insects will be used to populate a laboratory colony that will be used for research purposes within the laboratory and trials in fully contained glasshouses with restricted access.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the regulated article at each of the intended destinations:

Transgenic diamondback moths at Cornell University will be housed (b)(4)

(b)(4) (b)(4) and is entered through a secure door within a laboratory room whose doors are kept shut. The laboratory has no windows. Access to the (b)(4) is by elevator and stairs. (b)(4) (b)(4)

Within these greenhouses, cages (1.8 m long x 0.9 m wide x 1.7 m high) constructed of nylon netting are hung and insects and plants are placed into cages. The mesh of the cages does not permit adults or larvae to move through them. Access into the cages is through sleeves sewn into the cages.

Even if there are escapes from the cages and through the greenhouse, it has been well documented that diamondback moth is incapable of surviving the winter in our area. Diamondback moth colonizes cabbage in western New York from the shipment of infested transplants from southern areas or greenhouses, or migrates here on weather patterns.

Diamondback moth in the rearing room and glasshouses:

Diamondback moths will be reared according to our established protocols (<http://web.entomology.cornell.edu/shelton/diamondback-moth/>)

In the laboratory, moths will be reared at 25°C with a natural photoperiod. Eggs are laid on aluminium foil that has been dipped into cabbage juice and larvae and pupae are kept in plastic rearing containers. Pupae are transferred to cages for subsequent egg collection. In caged glasshouse experiments, diamondback moth will be reared on cabbage or broccoli throughout their life-cycle, with some samples moved to the rearing room to be reared as described above. All

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adults are kept in sturdy screened, tightly closed cages within the laboratory or glasshouse. All surplus eggs, larvae, pupae or adults, and associated food material, are killed by freezing at -20oC for at least 12 hours.

Shipment of diamondback moth from the United Kingdom to Cornell University will involve placing the moths into shatter-resistant plastic containers with a further two layers of containment, which will be closed and sealed with tape. The box will be labelled as to its contents, origin, destination and contact telephone numbers. That sealed container will be shipped via TNT or another designated carrier or occasionally carried by a traveller.

Final disposition:

Transgenic diamondback moths will be reared until such time as experiments pertaining to the specific transgenes under investigation are finished. At such time all remaining eggs, larvae, pupae and adults will be frozen at -20oC for 12 hours.

Final Disposition Method: Destruction/Devitalization Other Storage in Contained Facility

Final Disposition Description:

Transgenic diamondback moths will be reared until such time as experiments pertaining to the specific transgenes under investigation are finished. At such time all remaining eggs, larvae, pupae and adults will be frozen at -20oC for 12 hours.

14. ATTACHMENTS

15. ADDITIONAL INFORMATION

Insects will be shipped in a shatter-resistant plastic containers with a further two layers of containment, which will be closed and sealed with tape. The box will be labelled as to its contents, origin, destination and contact telephone numbers.

16. COURTESY JUSTIFICATION

(b)(6) hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON

(b)(6)

18. DATE

August 14, 2012

**SUPPLEMENTAL PERMIT CONDITIONS
For Movement of *Plutella xylostella***

- (1) The *Plutella xylostella* eggs, larvae, or pupae are to be shipped in containers as specified in 7 CFR Part 340.8(4), for insects, mites, and related organisms or as stated in the permit application.
- (2) This authorization is strictly for rearing and research in a controlled laboratory environment.
- (3) All laboratories and growth chambers where this genetically engineered insect is employed will be locked with limited access by authorized personnel. In each area where a regulated genetically engineered organism is used, at least one sign must be posted on the door or wall stating that a regulated genetically engineered organism is being used.
- (4) Upon completion of research all engineered insects (except those retained for future research), will be disposed of by freezing at -20 for 24 hours/or autoclaving for a minimum of 20 minutes.
- (5) This authorization for movement under permit, is valid for execution, for a period of 1 year.
- (6) There is to be no further distribution of these genetically engineered insects under this permit without prior approval from State (intrastate movement) and Federal regulatory officials (interstate movement).
- (7) **THIS AUTHORIZATION IS NOT VALID FOR THE RELEASE INTO THE ENVIRONMENT OF THIS GENETICALLY ENGINEERED INSECT.**

All necessary precautions must be taken to prevent escape of these genetically engineered insects.

- (8) Without prior notice and during reasonable hours authorized Plant Protection and Quarantine and State regulatory officials shall be allowed to inspect the conditions under which this genetically engineered insect is being kept.
- (9) Reporting an Unauthorized or Accidental Release

1. According to the regulation in 7 CFR § 340.4(f)(10)(i), APHIS shall be notified orally immediately upon discovery and notified in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article.

- For immediate verbal notification, contact APHIS BRS Compliance Staff at (301) 851-3935 and ask to speak to a Compliance and Inspection staff member. Leave a verbal report on voicemail if the phone is not answered by a Compliance Officer.
- In addition, in the event of an emergency in which you need to speak immediately to APHIS personnel regarding the situation, you may call:

The APHIS/BRS Regional Biotechnologist assigned in the region where the field test occurs:

For Western Region, contact the Western Region Biotechnologist at (970) 494-7513

or e-mail: BRSWRBT@aphis.usda.gov

For Eastern Region, contact the Eastern Region Biotechnologist at (919) 855-7622 or e-mail: BRSERBT@aphis.usda.gov

Or

The APHIS State Plant Health Director for the state where the unauthorized release occurred. The list of APHIS State Plant Health Directors is available at:
http://www.aphis.usda.gov/services/report_pest_disease/report_pest_disease.shtml.

or

<http://pest.ceris.purdue.edu/stateselect.html>

2. Written notification should be sent by one of the following means:

By e-mail:
BRSCopliance@aphis.usda.gov

By mail:
Biotechnology Regulatory Services (BRS)
Regulatory Operations Program
USDA/APHIS
4700 River Rd. Unit 91
Riverdale, MD 20737

3. Additional instructions for reporting compliance incidents may be found at http://www.aphis.usda.gov/biotechnology/compliance_incident.shtml

- (10) No person shall move a regulated article interstate unless the number of the limited permit appears on the outside of the shipping container.

Permit Number: 12-227-102m

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SIGNATURE OF BRS OFFICIAL

Steven M. Bennett

DATE

September 12, 2012

Standard Permit Conditions for the Introduction of a Regulated Article

(7 CFR 340.4 (f))

Permit Conditions: A person who is issued a permit and his/her employees or agents shall comply with the following conditions , and any supplemental conditions which shall be listed on the permit, as deemed by the Deputy Administrator to be necessary to prevent the dissemination and establishment of plant pests:

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- (9) A person who has been issued a permit shall submit to APHIS a field test report within 6 months after the termination of the field test. A field test report shall include the APHIS reference number, methods of observation, resulting data, and analysis regarding all deleterious effects on plants, nontarget organisms, or the environment.
- (10) APHIS shall be notified within the time periods and manner specified below, in the event of the following occurrences:
 - (i) Orally notified immediately upon discovery and notify in writing within 24 hours in the event of any accidental or unauthorized release of the regulated article;
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- (11) A permittee or his/her agent and any person who seeks to import a regulated article into the United States shall:
 - (i) Import or offer the regulated article for entry only through any USDA plant inspection station listed in 7 CFR 319.37-14;
 - (ii) Notify APHIS promptly upon arrival of any regulated article at a port of entry, of its arrival by such means as a manifest, customs entry document, commercial invoice, waybill, a broker's document, or a notice form provided for such purpose; and
 - (iii) Mark and identify the regulated article in accordance with 7 CFR 340.7.

Rev. January 1, 2010

Permit Number: 12-227-102m

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SIGNATURE OF BRS OFFICIAL Steven M. Bennett	DATE September 12, 2012



United States
Department of
Agriculture



Animal and
Plant Health
Inspection Service

Biotechnology
Regulatory
Services

4700 River Road, Unit 147
Riverdale, Maryland
20737-1236

Any regulated article introduced not in compliance with the requirements of 7 CFR 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. Any regulated article introduced not in compliance with the requirements of 7 Code of Federal Regulation Part 340 or any standard or supplemental permit conditions, shall be subject to the immediate application of such remedial measures or safeguards as an inspector determines necessary, to prevent the introduction of such plant pests. The responsible party may be subject to fines or penalties as authorized by the Plant Protection Act (7 U.S.C. 7701-7772).

Permit Number: 12-227-102m

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SIGNATURE OF BRS OFFICIAL

Steven M. Bennett

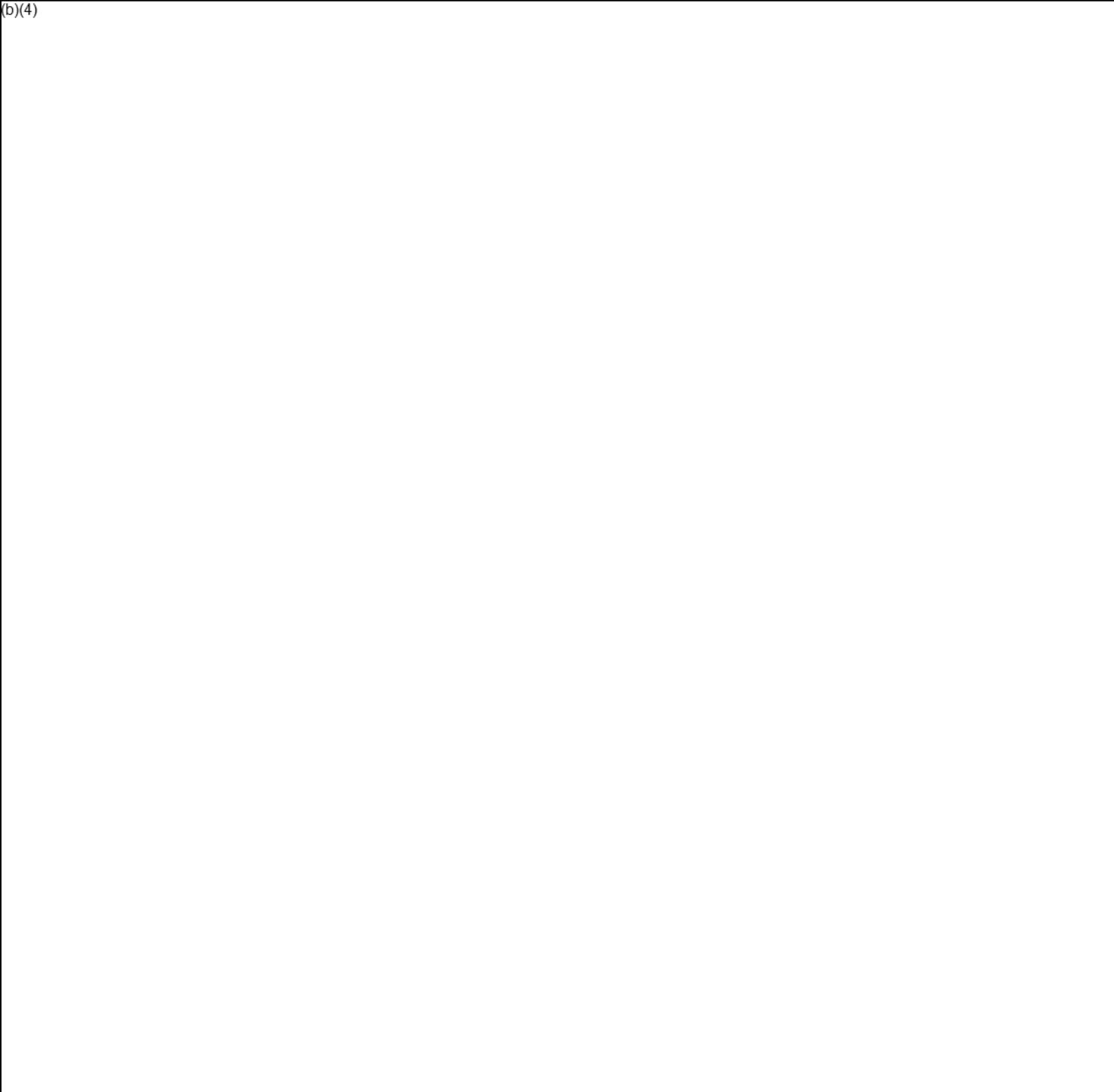
DATE

September 12, 2012

Location of experimental site, boundaries marked as red

Satellite image indicating location of proposed field releases, at Cornell University/
NYSAES, Geneva NY 14456

(b)(4)



Longitude/latitude of marked boundary points

(b)(4)



Engineered Female-Specific Lethality for Control of Pest Lepidoptera

Li Jin,^{†,‡,§} Adam S. Walker,^{†,§} Guoliang Fu,^{†,‡,§} Timothy Harvey-Samuel,^{†,‡,§} Tarig Dafa’alla,[†] Andrea Miles,[†] Thea Marubbi,[†] Deborah Granville,[†] Nerys Humphrey-Jones,[†] Sinead O’Connell,[†] Neil I. Morrison,[†] and Luke Alphey*,^{†,‡}

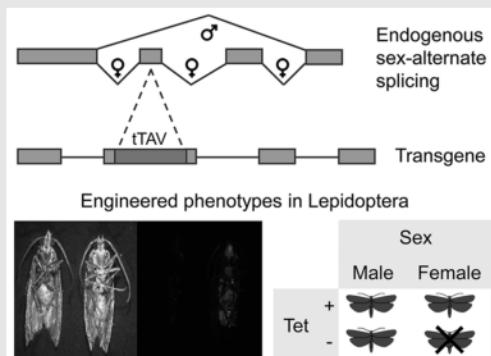
[†]Oxitec Ltd, 71 Milton Park, Oxford OX14 4RX, U.K.

[‡]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.

Supporting Information

ABSTRACT: The sterile insect technique (SIT) is a pest control strategy involving the mass release of radiation-sterilized insects, which reduce the target population through nonviable matings. In Lepidoptera, SIT could be more broadly applicable if the deleterious effects of sterilization by irradiation could be avoided. Moreover, male-only release can improve the efficacy of SIT. Adequate methods of male-only production in Lepidoptera are currently lacking, in contrast to some Diptera. We describe a synthetic genetic system that allows male-only moth production for SIT and also replaces radiation sterilization with inherited female-specific lethality. We sequenced and characterized the *doublesex* (*dsx*) gene from the pink bollworm (*Pectinophora gossypiella*). Sex-alternate splicing from *dsx* was used to develop a conditional lethal genetic sexing system in two pest moths: the diamondback moth (*Plutella xylostella*) and pink bollworm. This system shows promise for enhancing existing pink bollworm SIT, as well as broadening SIT-type control to diamondback moth and other Lepidoptera.

KEYWORDS: sterile insect technique, SIT, RIDL, transgenic, insect, doublesex, moth



Pest insects are a major constraint to global food production, and lepidopteran species rank among the most destructive pests of agriculture, stored products, and forests.¹ Their control is primarily through use of synthetic insecticides. However, off-target ecological impacts, human health concerns, and pest resistance continue to drive the need for alternatives, such as sterile insect technique (SIT).^{2,3} Owing to their economic importance, wide prevalence, and the non-phytophagous nature of their adult stages, Lepidoptera are considered highly appropriate for SIT-type control. Despite this, SIT has only been implemented successfully against a few pest moths, notably pink bollworm, a major cotton pest; codling moth (*Cydia pomonella*), a pest of apples and pears; and painted apple moth (*Orgyia anartoides*), a polyphagous forestry and horticulture pest. Broadening SIT application to other Lepidoptera is primarily constrained by three factors: the lack of mass-rearing systems for some pest species, the lack of sexing systems to ensure efficient male-only release,⁴ and by the high radiation doses required to sterilize moths^{4,5} resulting in reduced field competitiveness. The last two of these can potentially be addressed by genetic methods.

Single-sex male release not only reduces production costs but also increases the efficiency of released insects through reduced assortative mating.^{6,7} In SIT for the Mediterranean fruit fly (medfly, *Ceratitis capitata*), male-only production through the use of temperature-sensitive lethal (*tsl*) sexing strains⁸ has been a major factor in the ongoing success of these programs.^{6,7}

Equivalent sexing systems in lepidopterans are limited to those with sex-linked visible markers and balanced-lethal genetic sexing strains,^{9,10} generated through mutagenesis and chromosomal translocation. Neither system is suitable for mass rearing or available in SIT target species.¹¹ Pink bollworm and codling moth SIT programs have therefore used bisex release,^{4,12} while that for painted apple moth relied on sorting males by hand.¹³

Recent advances in recombinant DNA techniques have opened alternative avenues for insect control methods and sexing systems. A technique called release of insects carrying a dominant lethal (RIDL) has been developed in medfly,^{14,15} pink bollworm,¹⁶ and mosquitoes.^{17–19} RIDL relies on the inheritance of an engineered lethal gene to replace the radiation-induced lethal mutations of conventional SIT. In order to propagate RIDL strains, the lethality needs to be conditional; this has been achieved through use of the “tet-off” gene expression system.²⁰ Indeed, the tTA transactivator of the tet-off system has also been used as a lethal effector; high levels of tTAV expression can be obtained, through a positive feedback circuit, that are lethal to insects.^{14,15,18} The system is repressed in the presence of tetracycline, or suitable analogues, which are readily supplied in larval diet. For medfly, a female-specific variant was developed, called fsRIDL, which exploits the sex-alternate splicing of the sex determination gene,

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transformer (*tra*), to regulate tetracycline-repressible, female-only lethality.¹⁵

On a practical level, such strains enable automated production of male-only adults. After release, mating between fsRIDL males and wild females produces only male progeny, as daughters die in the absence of tetracycline. Inundative release of fsRIDL males over a period of time would thereby reduce the number of females in, and consequently the reproductive potential of, the wild pest population.²¹ Furthermore, if combined with a tightly linked fluorescent protein marker,²² genetic sexing, autocidal population control, and field monitoring can be made available in one construct.

We set out to develop fsRIDL in moths. A key feature of synthetic biology is the development of standard designs and parts. This in turn should allow designs and constructs developed in one species to be transferred successfully to other species. Though we successfully demonstrated function of a *tra*-based alternative splicing module between medfly and *Drosophila melanogaster*, *tra* homologues are only known in higher Diptera. *dsx*, another gene showing sex-specific alternative splicing, is conserved much more widely, including in Lepidoptera.^{23,24} We therefore cloned and sequenced the alternatively spliced region of the pink bollworm homologue of *dsx* (*Pgdsx*). We used these sequences to engineer the fsRIDL phenotype (Figure 1a) in two lepidopteran species, pink bollworm and diamondback moth (*Plutella xylostella*), the world's major pest of cruciferous crops.

■ RESULTS AND DISCUSSION

Characterization of the sex-alternate splicing of *Pgdsx* revealed that there are at least three different forms of female transcript and one male transcript (Figure 1b). An alignment of moth *dsx* intron sequences including the silkworm *Bombyx mori*, pink bollworm, and codling moth (*Cydia pomonella*) revealed unexpected conserved sequence blocks adjacent to the second female-specific exon (E3, Supplementary Figure S1). We tested various minigene constructs, with or without this conserved intronic sequence. Constructs including this intron showed correct sex-specific splicing, whereas the constructs without did not (data not shown). On the basis of these results, we made *Pgdsx* minigene constructs with tTAV sequence inserted into the female-specific exon E3, but retaining I3f and E4 (Figure 1c). All endogenous 5' ATG sequences were mutated to prevent premature translation start.

In four strains of pink bollworm transformed with one construct, OX4135, no females survived to adulthood in the absence of chlortetracycline (CTC, a tetracycline analogue) (Figure 2a). However, this was also the case on CTC, apart from OX4135A-Pgy, of which 6% of females survived relative to wild-type females. In addition, there were no male transgenic survivors off CTC. On CTC, there was no obvious reduction in male survival in OX4135A-Pgy, but in OX4135B-Pgy, OX4135C-Pgy, and OX4135D-Pgy male survival was 4–60% of that of wild-type males. These results indicated that the repressive effect of CTC and the fidelity of the sex-alternate splicing were insufficient to adequately repress expression of tTAV and VP16.

With the aim of reducing the indiscriminate lethal effect we generated strains with another construct, OX4319 (Figure 1c). Relative to OX4135, the number of *tetO* repeats was reduced from 21 to seven, and the *hsp70*-VP16 cassette was removed, leaving only tTAV as a lethal effector gene. Three OX4319-transformed strains of pink bollworm were subjected to on/off-

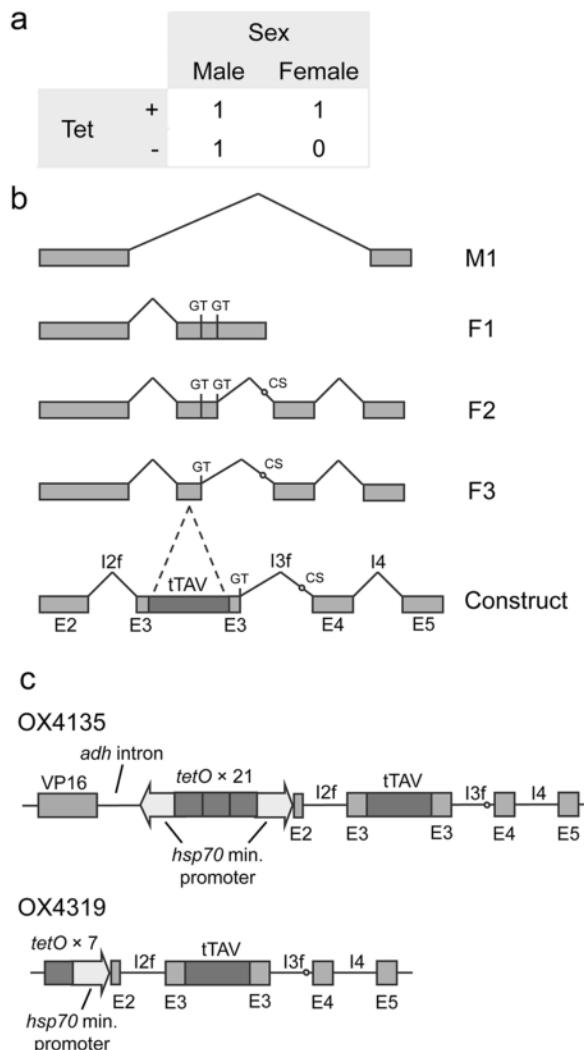


Figure 1. Splicing of pink bollworm *doublesex* (*Pgdsx*) and its incorporation in constructs for transformation of pink bollworm and diamondback moth. The transgenic construct provides a genetic circuit that responds to inputs of sex and presence of tetracycline in the larval diet and gives an output of death when activated. This is illustrated in panel a, as a truth table where an output value of 1 represents a viable condition and 0 a lethal condition. This corresponds to expression of the lethal effector in the lethal condition, so expression of the lethal effector is the inverse of the life/death phenotypic output. (b) Sex-specific alternative splicing of *Pgdsx* showing four exons; the middle two are female specific. *Pgdsx* is spliced in females to produce three transcripts: F1, F2, and F3. Transcript F1 contains one common exon and one extended second exon, which ends with poly(A). Transcripts F2 and F3 differ by using different splice donor sites (marked by GT) for intron 2. Males produce only one transcript, M1. The construct was built by inserting tTAV into exon 2 of a minigene. Following nomenclature of the *Bombyx mori* *doublesex* splicing region (ref 23), we labeled that of *Pgdsx* as follows: exons, E2, E3, E4, and E5; introns, I2f, I3f, and I4. "CS" indicates approximate location of conserved sequence (AGTGAC/T) adjacent to the second female-specific exon. Not drawn to scale. (c) Schematic diagrams of two constructs, OX4135 and OX4319, carrying a *Pgdsx* minigene, used to transform and generate sexing strains in pink bollworm (OX4135 and OX4319) and diamondback moth (OX4319 only). Both constructs additionally comprise a fluorescent marker (*Hs5ie1-DsRed2*) and *piggyBac* sequences for genomic insertion.

CTC test crosses (Figure 2b). In the absence of CTC, all strains showed high female mortality (89–100%) and low male

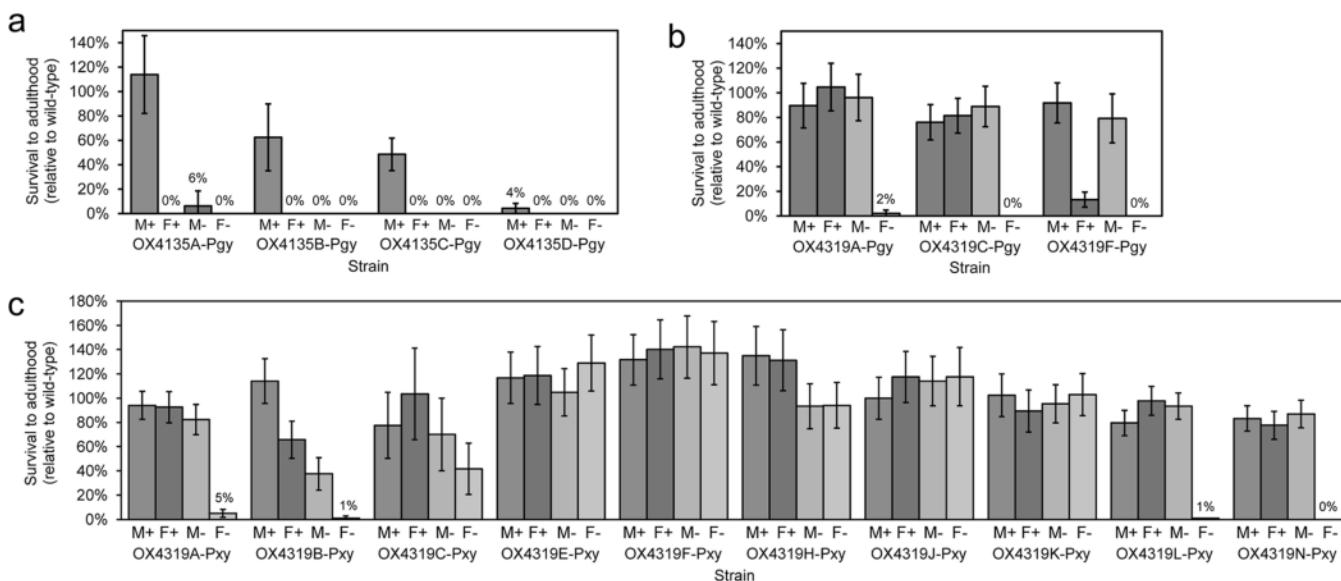


Figure 2. Tests for CTC-repressible, female-specific lethality in transformed pink bollworm and diamondback moth strains. (a) Survival to adulthood of transgenic strains of pink bollworm heterozygous for OX4135 insertions, reared on and off chlortetracycline (CTC). (b) Survival to adulthood of transgenic strains of pink bollworm heterozygous for OX4319 insertions, reared on and off CTC. (c) Survival to adulthood of transgenic strains of diamondback moth heterozygous for OX4319 insertions, reared on and off CTC. Survival is expressed relative to that of wild-type counterparts. “M+”, male survival on CTC; “F+”, female survival on CTC; “M-”, male survival off CTC; and “F-”, female survival off CTC. Error bars indicate 95% confidence intervals.

mortality (4–21%), prior to adulthood. In the presence of CTC, male survival was similar to that of wild-type males; however, female lethality was efficiently suppressed in only two lines: OX4319A-Pgy and OX4319C-Pgy; on-CTC female mortality in OX4319F-Pgy was 87%. This variability between strains is likely a result of transgene position effect. In all three lines, expression of the DsRed2 marker was visible in late embryos, larvae, pupae, and adults (Figure 3).

Early female death is preferable as this reduces diet consumption in the pre-release generation and minimizes the

damage to crops by female larvae that may occur in the field. Most off-CTC OX4319 pink bollworm females appeared to die as early larvae with survival to pupation of OX4319A-Pgy and OX4319C-Pgy only 8% and 3% relative to wild-type, respectively.

A key rationale for our modular, synthetic biology design philosophy was the prospect of efficient transfer of designs, constructs, and modules between insect species. To test the *dsx*-based sex-specific CTC-switchable positive-feedback genetic circuit and components in another lepidopteran, the OX4319 construct was used to generate 10 transgenic strains of the diamondback moth. Off-CTC rearing of transgene-heterozygous larvae showed highly variable survival to adulthood (Figure 2c). In seven lines, female survival to adulthood was moderate or similar to wild-type counterparts. However, three lines, OX4319A-Pxy, OX4319L-Pxy, and OX4319N-Pxy, showed low or zero survival to adulthood of females but near-wild-type survival of males. Moreover, provision of CTC in the diet largely repressed female mortality in these three lines. Expression of the DsRed2 marker was similar to that seen in pink bollworm, but brighter in diamondback moth adults, which appear to have a thinner and less opaque cuticle (Figure 3).

Of the diamondback moth strains showing female-specific mortality off CTC, only OX4319A-Pxy females exhibited substantial survival to pupation (17% relative to wild-type females). Off-CTC female survival to pupation was low in OX4319B-Pxy, OX4319L-Pxy, and OX4319N-Pxy (3%, 9%, and 0%, respectively). Few dead or dying late-instar larvae were observed, indicating that death occurs primarily during early larval stages.

Splicing of the *Pgdsx* mini-gene was analyzed in the transformed pink bollworm and diamondback moth strains carrying OX4319. Amplifying across the entire alternatively spliced region, only correctly spliced male and female transcripts were detected (Figure 4). Nucleotide sequencing

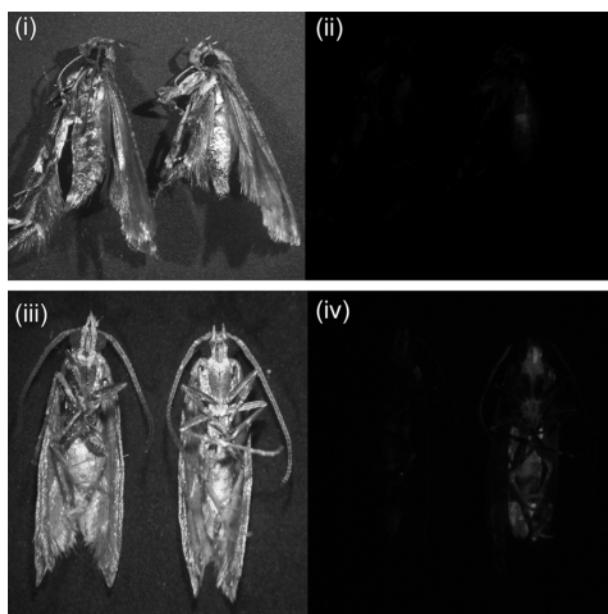


Figure 3. DsRed2 marker in pink bollworm (i, ii) and diamondback moth (iii, iv) strains transformed with OX4319. Images are in white light (i and iii) or under red fluorescent protein excitation filters.

of these transcripts from OX4319 in pink bollworm and diamondback moth strains showed that intron splicing matched that of *Pgdsx*.

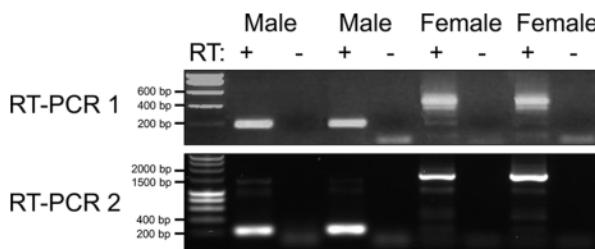


Figure 4. Gel images showing reverse transcription PCR analysis of sex-alternate splicing of endogenous *Pgdsx* and a *Pgdsx* minigene (samples from two males and two females in each). RT-PCR 1: amplified cDNA from wild-type pink bollworm using the primer pair targeting the common exons for male and female (spanning all introns shown), the correctly spliced male (217 bp) and female (486 bp) transcripts in the corresponding male and female were detected. RT-PCR 2: same primers (OX4319, in transformed diamondback moth), but with larger fragment in females (1529 bp) due to the presence of tTAV.

The strains that showed tightly controlled, early female-specific lethality, OX4319L-Pxy and OX4319N-Pxy, were established as colonies homozygous for their respective transgene insertions.

To assess potential relative field performance, we conducted laboratory tests of longevity of wild-type, OX4319L-Pxy, and OX4319N-Pxy male moths reared off CTC (Figure 5). Wild-

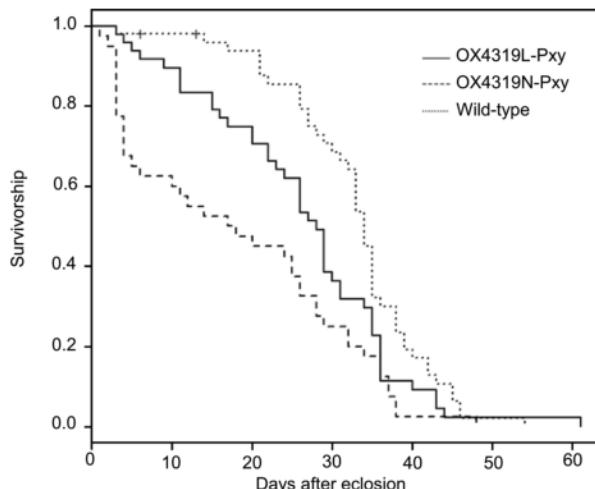


Figure 5. Kaplan-Meier object showing longevity of wild-type, OX4319L-Pxy, and OX4319N-Pxy male adult diamondback moths. OX4319 strains were transgene-homozygous.

type males showed the highest longevity ($31.7 \text{ days} \pm 1.43 \text{ se}$), although not significantly different to that of OX4319L-Pxy ($25.9 \text{ days} \pm 1.73$). OX4319N-Pxy male longevity, however, was significantly lower ($18.3 \text{ days} \pm 2.22$) than that of wild-type males.

Male mating competitiveness of OX4319L-Pxy was assessed in laboratory cages. In competition with wild-type males, OX4319L-Pxy achieved a relative sterility index (RSI)²⁵ of 0.41 (an RSI of 0.5 indicates equal mating competitiveness). Although significantly lower than that of competing wild-type

males ($\chi^2 = 6.40$, df = 1, $P = 0.01$), this result represents a promising level of performance. In irradiated medfly, an RSI of 0.2 is considered the minimum acceptable competitiveness for a strain to be effective in the SIT.²⁶ Mated diamondback moth females do not become fully refractory to remating.²⁷ In subsequent tests, therefore, we sought to include factors such as sperm competition and mating competitiveness over time in the assessment of male mating performance. OX4319L-Pxy males and wild-type males were kept with wild-type females for the duration of the females' reproductive lives. Of the resultant offspring, 37% were transgenic. That this is similar to the proportion observed mating in 1- to 3-h trials indicates that OX4319L-Pxy males perform well in other aspects of their reproductive biology such as remating over time and postcopulatory effects.

We investigated female-specific lethality at different CTC concentrations (Figure 6). No OX4319-Pxy-heterozygous

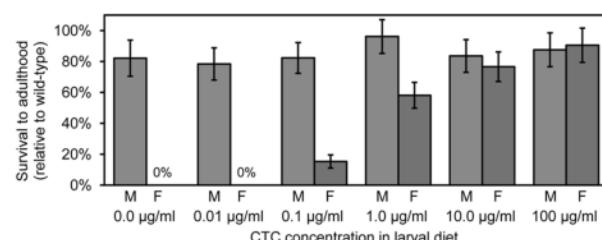


Figure 6. Survival to adulthood of OX4319L-Pxy-heterozygous diamondback moth reared on different concentrations of CTC in larval diet. Survival is expressed relative to that of wild-type counterparts. "M", male survival; "F", female survival. Error bars indicate 95% confidence intervals.

females survived to adulthood at CTC concentrations up to $0.01 \mu\text{g}/\text{mL}$, while at or above $10 \mu\text{g}/\text{mL}$ CTC OX4319L-Pxy-heterozygous female survival to adulthood, relative to wild-type, was similar to that of males. The level of CTC needed for survival far exceeds that which diamondback moth might be expected to encounter in the wild. For comparison, laboratory experiments growing cabbage on soil artificially contaminated with manure from CTC-fed pigs, and spiked with CTC solution, found $<0.004 \mu\text{g}/\text{mL}$ CTC in foliage.²⁸

The low probability that partly permissive conditions for fsRIDL female survival are encountered in the wild greatly reduces the potential for resistance to the trait to develop in a wild pest population. Evolutionary trajectories of hypothetical resistance alleles been modeled,²⁹ but such alleles have yet to be observed. Perhaps more plausible is that behavioral resistance traits are selected for, which reduce the probability of mating between wild and released insects. The same applies to SIT, and over several decades such resistance has been detected only rarely.³⁰

The strains described here demonstrate the function of a sex-alternate splicing module in multiple transgenic strains of two species of moth from different families (Yponomeutidae and Gelechiidae) and the ability to use this within a genetic circuit giving switchable sex-specific lethality.

Different insertions of the same construct showed considerable variation in phenotype. This "position effect" can be useful in generating variation from which one with optimal properties can be selected; however, it also means we are yet some way from truly predictable "plug-and-play" synthetic biology in more complex chassis' such as pest insects. In addition, relative to the best microbial systems we have only a

very limited set of molecular components and limited characterization of them. Nonetheless, we have shown that a modular design, specific components, and even a specific construct can be reused across a significant phylogenetic range. This provides support for the design philosophy and the systematic design and implementation of genetic circuits in multiple higher organism pest species.

The strains of pink bollworm and diamondback moth that have shown the most tightly controlled fsRIDL phenotype may themselves be useful pest control tools. In addition to the direct pest population control benefit of fsRIDL, previous modeling studies have indicated potentially large beneficial effects for pesticide resistance management.^{31,32} This could be of significant benefit in both cotton and brassica cultivation, where such resistance is a major problem. fsRIDL, with its species specificity and pesticide-free activity, could form an important and widespread part of future integrated pest management strategies in agriculture.

METHODS

Insect Genetics, Rearing, and Transformation. The wild-type strain of pink bollworm, called “APHIS”, used for transformation and as a wild-type comparator, is derived from the USDA mass-rearing facility in Arizona, USA. The equivalent diamondback moth strain has been maintained in the laboratory for >10 years and originates in Vero Beach, Florida, USA. Microinjection of eggs was used to transform both species. Pink bollworm were transformed and reared following methods described by Simmons et al.²² For diamondback moth, the methods of Martins et al.³³ were followed. For both species, artificial diet contained 100 µg/mL CTC. This was used for the on-tetracycline test crosses; for off-tetracycline tests, CTC was withheld from the diet. For test crosses, transgene-heterozygous males were mated with wild-type females and the resulting eggs split to the two diet types.

Amplification and Cloning of *Pgdsx* and Transcripts. To amplify *Pgdsx*, degenerate primers were designed based on conserved regions aligned from all available insect *dsx* protein sequences. cDNA fragments flanking the sex-specific splicing region were amplified from pink bollworm male and female cDNA and were sequenced to confirm their identity. The following degenerate primers were used to amplify *Pgdsx* gene (Supplementary Table S2): Dsx1, Dsx2, Dsx3, Dsx4r, and DsxSr.

Visible PCR bands were gel-purified and cloned into TOPO vector (Invitrogen, Carlsbad, California). Clones were sequenced and the *dsx* gene identity confirmed.

From the transcript sequences, we designed primers to extend the coding sequences into the intron sequences and to amplify the 3' ends using 3' RACE. The splicing structures and variant poly(A)-ending sequence were established following amplification cDNA/intron and sequencing analysis. The following primers were used to determine the splicing structure and 3' RACE (Supplementary Table S2): Pbwdx1, Pbwdx2, Pbwdx3, and Pbwdx4.

3' RACE was performed using 3' kit (Clontech, Mountain View, California) according to the manufacturer's instruction.

Primers used to amplify intron flanking sequences are shown in Supporting Information, Table S3.

Plasmid Construction. Construct OX4135 was made by inserting tTAV coding sequence into the female-specific exon 2 of a minigene construct. The minigene construct contains four ligated fragments derived from four exons and flanking introns.

OX4135's background gene structure was derived from a plasmid with *tetO*×21-VP16 and, sharing the *tetO*×21 and in inverse orientation, *tetO*×21-*Pgdsx*-tTAV. tTAV sequence has its own starting Kozak sequence, and all endogenous ATG sites of *Pgdsx* upstream of tTAV were mutated to prevent possible mis-starting.

Construct OX4319 was derived from OX4135 by deleting the *tetO*×14-VP16, leaving *tetO*×7-*Pgdsx*-tTAV.

Laboratory Experiments with Insects. All experiments were conducted in a temperature-controlled room (25 °C) with a 16:8 light:dark cycle.

Diamondback Moth Longevity Test. OX4319L-Pxy and OX4319N-Pxy strains were reared in the absence of CTC to produce males only. Fifty of these males from each transgenic strain and 50 males from their wild-type genetic background (reared off CTC and sexed by hand) were individually placed in randomized Petri dishes (9 mm) with a sugar water source, which was replenished every 2 days. Dates of eclosion and death for each individual were recorded.

Diamondback Moth Mating Tests. Relative sterility index (RSI): Wild-type and OX4319L-Pxy male moths were produced as above. Adults were collected within 24 h of eclosion, and equal numbers from each strain were placed within a 1 m × 1 m × 1.5 m insect cage (Bugdorm, Taiwan) along with a cabbage extract-treated Parafilm piece to act as a mating stimulant. At the onset of scotophase, 5 h later, a number of female wild-type adults equal to half the total number of males were released into the cage. Cages were checked every 15 min, and mating pairs were isolated and removed. The genotype of the male within each mating pair was determined through fluorescence microscopy. Six replicates were performed with a total of 220 mating pairs observed.

Lifetime relative competitiveness: Wild-type and OX4319L-Pxy male moths were produced as above. Once eclosed, 20 males from each strain were placed in a 30 cm × 30 cm × 30 cm insect cage along with a cabbage extract-treated Parafilm piece and sugar water source. Five hours later, 20 wild-type females were introduced. Parafilm and sugar water were replaced every 2 days until egg-laying ceased. Eggs were reared on artificial diet with CTC (100 µg/mL). Once each collection had pupated, individuals were scored for fluorescence, and these raw data were corrected for heterozygote mortality. Proportions of each genotype (OX4319L-Pxy heterozygotes and wild-type) were calculated, and an RSI equivalent was generated. Four replicates were performed.

Molecular Analysis of Transformed Strains. Transcripts were analyzed by RT-PCR, using SuperScript III One-Step RT-PCR System (Invitrogen), according to the manufacturer's instructions, and using the following oligonucleotide primers (Supplementary Table S4): 3816RTEX1, RTPBWMEXONR, RTPBWEXON1, and RTCOTETR1.

All major products were sequenced, and the electropherogram and sequence data were analyzed using Vector NTi (Invitrogen).

RT-PCR experiments were performed to relatively quantify the female-spliced transcript in male. RNA was reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). qPCR reactions were performed using the DyNAamo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). Primers were 3816RTEX1, RTCOTETR1, pbwdx3, and RTPBWexon2R. Amplification conditions were as follows (Supplementary Table S4): 50 °C, 2 min; 95 °C, 15

min; $40 \times (94^\circ\text{C}, 10\text{ s}; 58^\circ\text{C}, 30\text{ s}; 72^\circ\text{C}, 30\text{ s}; 80^\circ\text{C} \text{ for } 2\text{ s})$, Data Acquisition at 72 and 80°C .

Statistics. Calculation of 95% confidence intervals shown in figures for mortality ratios (standardized ratio, SR) was conducted using the following formula: $\text{CI} = \text{SR} \pm (1.96 \times \text{SE})$; where $\text{SE} = (\text{SR}/\text{square root of number of observed events})^{34}$. For comparison of strains' mating performance, Chi-square analyses were performed in Excel using a likelihood framework. Longevity of OX4319 diamondback moth strains relative to wild-type was analyzed in R (version 2.12.0) using survival analysis (ANCOVA) and Weibull errors.

■ ASSOCIATED CONTENT

● Supporting Information

Tables comprising nucleotide sequences of pink bollworm *doublesex* and primers. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare the following competing financial interest(s): Authors affiliated with Oxitec Ltd are staff or students of Oxitec and have employment, studentship support, and/or equity interest in Oxitec. Oxitec and the University of Oxford own intellectual property related to the subject matter of this study.

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■ ABBREVIATIONS

SIT, sterile insect technique; RIDL, release of insects carrying a dominant lethal; CTC, chlortetracycline

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OXITEC

INTERNAL RESEARCH REPORT

1. **Title:** Investigating the expression of the tetracycline-repressible, female-specific lethal trait in the fsRIDL strain, OX4319L-Pxy, in response to different concentrations of chlortetracycline in larval feed.

- 2. Statement Of Data Confidentiality Claims:**

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The information contained in this document may not be published or disclosed to any third party without the prior consent in writing of the company supplying the relevant information.

The information contained in this document may not be used by any third party including but not limited to any regulatory authority to support registration or approval of this product or any other product without the prior consent in writing of the company supplying the relevant information.

- 3. Statement Concerning Good Laboratory Practices:**

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

- 4. Authors:**

Study Coordinator (Signature): []	Study Supervisor (Signature): []
Study Coordinator (Name And Position): [] Research Scientist	Study Supervisor (Name And Position): [] [(b)(6)]

Date Signed: 17 th September 2013	Date Signed: 17 th September 2013
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5. Associated Personnel:

Name	Tasks
[]	Study coordination, experimental design, report writing, data collection
[]	Experimental design, approval
[]	Study Sponsor

6. Test Facility:

This research was performed at Oxitec Ltd facilities at:

71 Milton Park,
Abingdon,
Oxfordshire,
OX14 4RX,
UK

7. Objectives:

Investigate the effect of dietary chlortetracycline on the penetrance of the female-lethal trait in OX4319L-Pxy-heterozygotes.

8. Summary:

This experiment was designed to investigate at which concentration chlortetracycline is able to repress engineered female-specific lethality in OX4319L-Pxy females. There is a possibility of tetracycline availability in the wild, for instance through manure from antibiotic-treated farm animals used to fertilise crops, but this is uncommon and - when detected - concentrations found in crops are low (Hu et al., 2010; Migliore et al., 2010; Seo et al., 2010). We tested survival of male and female larvae, heterozygous for the OX4319L-Pxy transgene insertion, when reared on different chlortetracycline concentrations in artificial diet (Jin et al., 2013).

9. Introduction:

The tetracycline-repressible, female-specific mortality in fsRIDL insect strains provides a means of producing large male-only cohorts of insects and a population suppression effect in the target population: reduction of females reduces a population's reproductive potential. Efficacy relies upon high penetrance of the female-lethal trait in the field. In laboratory test crosses, in the absence of dietary tetracycline, OX4319L-Pxy heterozygotes have shown 0% survival of females (i.e. 100% penetrance of the female-lethal phenotype), and high survival of males. A theoretical concern is that environmental tetracyclines could repress the female lethal trait and allow the transgene to persist for longer in the environment. We therefore set out to establish sensitivity concentration-response relationship between dietary chlortetracycline and the penetrance of the female-lethal phenotype in OX4319L-Pxy

heterozygotes. We tested OX4319L-Pxy heterozygotes, rather than homozygotes, as we anticipated that one copy of the transgene per cell is likely more susceptible to repression than is two copies, and the larvae present in the field are expected to be heterozygous for the OX4319L-Pxy insertion (the progeny of released homozygous males and wild-type females).

10. Methods

Eggs heterozygous for the OX4319L-Pxy transgene insertion were generated by establishing 10 replicated crosses with OX4319L-Pxy-homozygous males with wild-type females. Artificial diet (Bioserv beet armyworm diet, cat # F9221B) was prepared with six different concentrations of chlortetracycline: 0 µg/ml, 0.01 µg/ml, 0.1 µg/ml, 1.0 µg/ml, 10 µg/ml and 100 µg/ml. Egg collections from each cross were divided between these diets, and the hatched larvae reared to pupation. These pupae were separated by sex then incubated in Petri dishes; survival to adulthood was recorded.

11. RESULTS

As in previous test crosses, OX4319L-Pxy-heterozygous male survival is approximately equivalent to, but slightly lower than, that of wild-type, irrespective of chlortetracycline concentration (Figures 1 & 2). Mortality was well-repressed in females reared on 10 µg/ml and 100 µg/ml. As the chlortetracycline concentrations reduced to 1.0 µg/ml and 0.1 µg/ml, female survival rates dropped sharply, and below this (0.01 µg/ml and 0.0 µg/ml) only very small numbers of females survived to pupation (<2%) and none survived to adulthood.

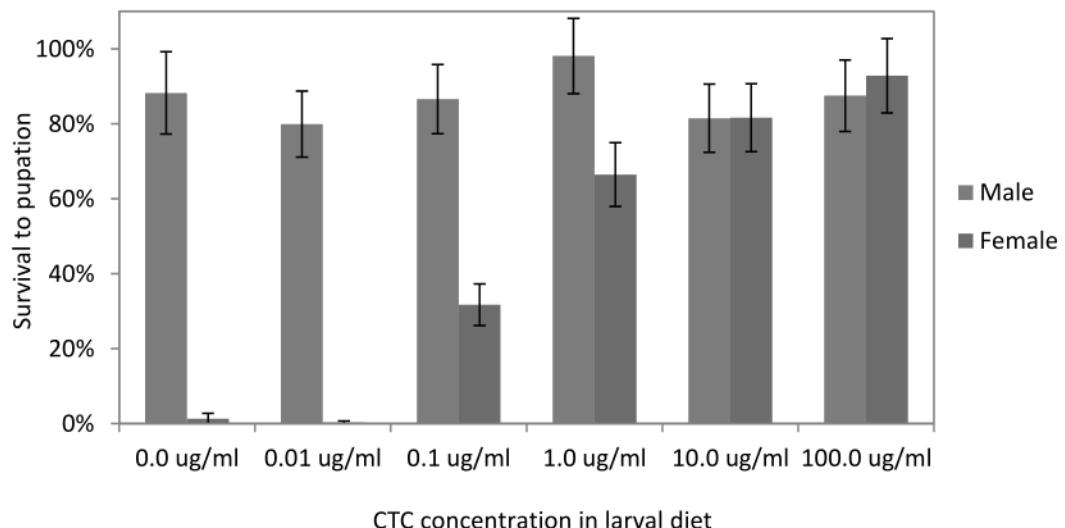


Figure 1: Survival-to-pupation of OX4319L-Pxy-heterozygous diamondback moth reared on different concentrations of chlortetracycline (CTC) in larval diet. Survival is expressed relative to that of wild-type counterparts. Error bars indicate 95% confidence intervals.

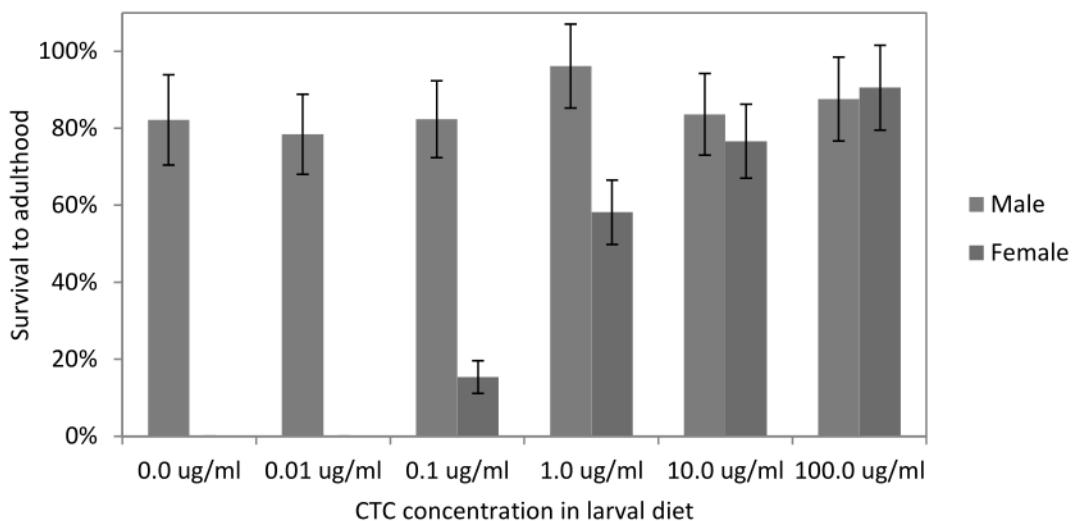


Figure 2: Survival-to-adulthood of OX4319L-Pxy-heterozygous diamondback moth reared on different concentrations of chlortetracycline (CTC) in larval diet. Survival is expressed relative to that of wild-type counterparts. Error bars indicate 95% confidence intervals.]

12. Discussion and Conclusions:

No OX4319-Pxy-heterozygous females survived to adulthood on 0.01 µg/ml CTC, while at or above 10 µg/ml CTC OX4319L-Pxy-heterozygous female survival to adulthood, relative to wild-type, was similar to that of males.]The level of CTC needed for survival far exceeds that which diamondback moth might be expected to encounter in the wild. For comparison, laboratory experiments growing cabbage on soil artificially contaminated with manure from CTC-fed pigs, and spiked with CTC solution, found <0.004 µg/ml CTC in foliage (Kumar et al., 2005). These results provide evidence that fsRIDL trait-repressing concentrations of tetracycline are highly unlikely to be encountered on host plants in the environment.

13. Literature:

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- Kumar, K., Gupta, S.C., Baidoo, S.K., Chander, Y., and Rosen, C.J. (2005). Antibiotic uptake by plants from soil fertilized with animal manure. *Journal of environmental quality* 34, 2082-2085.
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INTERNAL RESEARCH REPORT

1. Title: Sequencing the inserted OX4319 construct in the transformed line of diamondback moth, OX4319L-Pxy.

2. Statement Of Data Confidentiality Claims:

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4. Authors:

Study Coordinator (Signature): [(b)(6)] Study Coordinator (Name And Position): (b)(6) Research Scientist	Study Supervisor (Signature): [(b)(6)] Study Supervisor (Name And Position): (b)(6)
Date Signed: 17 th September 2013	Date Signed: 17 th September 2013

5. Associated Personnel:

Name	Tasks
(b)(6)	Study coordination
	Experimental design, report writing, data collection and analysis
	Data collection and analysis
	Experimental design, approval
	Study Sponsor

6. Test Facility:

This research was performed in Oxitec's laboratories at:

Oxitec Ltd,
71 Milton Park,
Abingdon,
Oxon OX14 4RX,
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7. Objectives:

To determine (b)(4)

(b)(4)

8. Summary:

(b)(4)

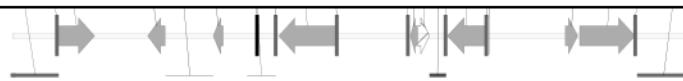
9. Introduction:

(b)(4)

(b)(4)

10. Methods and Results

(b)(4)



(b)(4)

PCR fragment	Forward primer	Reverse primer
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(b)(4)

(b)(4)

(b)(4)

PCR fragment	Primer	Region sequenced
(b)(4)		

(b)(4)

(b)(4)

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(b)(4)

(b)(4)

Raw data:

All the experimental results are included in Oxitec laboratory book OX-149.

The sequencing raw data obtained from GATC Biotech are saved and available on request.

11. Discussion and Conclusions:

The (b)(4)

(b)(4)



INTERNAL RESEARCH REPORT

- 1. Title:** Sequencing the inserted OX4319 construct in the transformed line of diamondback moth, OX4319L-Pxy.

- 2. Statement Of Data Confidentiality Claims:**

CBI Deleted

- 3. Statement Concerning Good Laboratory Practices:**

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[]	Experimental design, report writing, data collection and analysis
[]	Data collection and analysis
[]	Experimental design, approval
[]	Study Sponsor

6. Test Facility:

This research was performed in Oxitec's laboratories at:

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UK

7. Objectives:

To determine the sequence of the OX4319-derived inserted DNA in the diamondback moth strain, OX4319L-Pxy, and by comparing with the design sequence, determine the nature and origin of any nucleotide sequence changes.

8. Summary:

The sequence of the inserted transgene in OX4319L-Pxy was determined and showed 100% concordance with that of the design sequence and the OX4319 plasmid.

9. Introduction:

Extensive characterisation of a transgenic insect strain is a critical part of the product development process, as it allows for accurate, reliable, and easily analysed, quality control for strain maintenance, in tandem with various measureable phenotypic parameters. We set out to determine the sequence of the integrated construct in the OX4319L-Pxy diamondback moth strain, OX4319L-Pxy.

10. Methods and Results

[PAGES 2-7 HAVE BEEN CBI DELETED]

Raw data:

All the experimental results are included in Oxitec laboratory book OX-149.

The sequencing raw data obtained from GATC Biotech are saved and available on request.

11. Discussion and Conclusions:

The nucleotide sequence of the OX4319 construct has been determined, and confirmed as designed. The assembled sequence showed 100% concordance with both the design sequence of the OX4319 transposon and the previously determined flanking sequence.



INTERNAL RESEARCH REPORT

1. **Title:** Characterisation of the transgene insertion in the fsRIDL strain of diamondback moth, OX4319L-Pxy.

2. **Statement Of Data Confidentiality Claims:**

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3. **Statement Concerning Good Laboratory Practices:**

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Study Coordinator (Name And Position): [] Research Scientist	Study Supervisor (Name And Position): [] (b)(6)
Date Signed: 17 th September 2013	Date Signed: 17 th September 2013

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ted Personnel:

Name	Tasks
[]	Study coordination, report writing
[]	Data collection
[]	Data collection
[]	Data collection
[]	Experimental design, approval
[]	Study Sponsor

6. Test Facility:

This research was performed at Oxitec Ltd facilities at:

71 Milton Park,
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UK

7. Objectives:

To determine the genomic DNA sequence flanking the transgene insertion in the fsRIDL strain of diamondback moth, OX4319L-Pxy, and to quantify the number of transgene insertions carried by the strain.

8. Summary:

The OX4319L-Pxy carries a DsRed2 fluorescent protein marker, which enables workers to track the presence of the transgene for research and quality control purposes. The marker will also be used to monitor the strain in the field. More strain-specific information is required, however, to be able to verify strain identity and purity, and to confirm that the strain carries only this characterised transgene insertion. We therefore determined the nucleotide sequence of the flanking genomic region of the transgene insertion, and carried out Southern blot analysis, which indicated the presence of a single transgene insertion.

9. Introduction:

Insertion of a piggyBac transposable element is specific to the sequence TTAA, but there are many such sequences in an arthropod genome, therefore the transgene inserts at any of a large number of potential sites, giving pseudo-random insertion location. Furthermore, given the very large number of potential insertion sites it is highly unlikely that two independent insertions would insert at the same site. The genomic insertion site of a given transgenic strain therefore provides an event-specific identifier, particularly when combined with knowledge of the sequence of the inserted DNA. For instance, transgene insertion-homozygous colonies are typically generated using PCRs specific for the adjacent sequence (usually termed “flanking sequence”) – a PCR reaction using suitable primers binding genomic DNA either side of the insertion will amplify a characteristic band from a wild type (non-insertion) allele at the insertion site but not from homozygotes, for which both alleles have a large insertion between the two primers. [

]

10. Methods

Amplifying and sequencing DNA from the transgene insertion site:

[Pages 3-5 CBI Deleted]

12. Discussion and Conclusions:

The OX4319L-Pxy insertion site has been sequenced, providing a means of identifying the strain by PCR and distinguishing it from other transgenic strains and wild-type strains of diamondback moth: a vital quality control tool. Southern blot analysis showed single bands for each lane which, taken together with Mendelian inheritance of the transgenic phenotype, indicates that the strain carries a single transgene insertion.

13. Literature:

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual; third edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).



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INTERNAL RESEARCH REPORT

- 1. Title:** Characterisation of the transgene insertion in the fsRIDL strain of diamondback moth, OX4319L-Pxy.
- 2. Statement Of Data Confidentiality Claims:**

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Date Signed: 17 th September 2013	Date Signed: 17 th September 2013

5. Associated Personnel:

Name	Tasks
(b)(6)	Study coordination, report writing
	Data collection
	Data collection
	Data collection
	Experimental design, approval
	Study Sponsor

6. Test Facility:

This research was performed at Oxitec Ltd facilities at:

71 Milton Park,
Abingdon,
Oxfordshire,
OX14 4RX,
UK

7. Objectives:

To determine the genomic DNA sequence flanking the transgene insertion in the fsRIDL strain of diamondback moth, OX4319L-Pxy, and to quantify the number of transgene insertions carried by the strain.

8. Summary:

The OX4319L-Pxy carries a DsRed2 fluorescent protein marker, which enables workers to track the presence of the transgene for research and quality control purposes. The marker will also be used to monitor the strain in the field. More strain-specific information is required, however, to be able to verify strain identity and purity, and to confirm that the strain carries only this characterised transgene insertion. We therefore determined the nucleotide sequence of the flanking genomic region of the transgene insertion, and carried out Southern blot analysis, which indicated the presence of a single transgene insertion.

9. Introduction:

Insertion of a piggyBac transposable element is specific to the sequence TTAA, but there are many such sequences in an arthropod genome, therefore the transgene inserts at any of a large number of potential sites, giving pseudo-random insertion location. Furthermore, given the very large number of potential insertion sites it is highly unlikely that two independent insertions would insert at the same site. The genomic insertion site of a given transgenic strain therefore provides an event-specific identifier, particularly when combined with knowledge of the sequence of the inserted DNA. For instance, transgene insertion-homozygous colonies are typically generated using PCRs specific for the adjacent sequence (usually termed "flanking sequence") – a PCR reaction using suitable primers binding genomic DNA either side of the insertion will amplify a characteristic band from a wild type (non-insertion) allele at the insertion site but not from homozygotes, for which both alleles have a large insertion between the two primers

(b)(4)

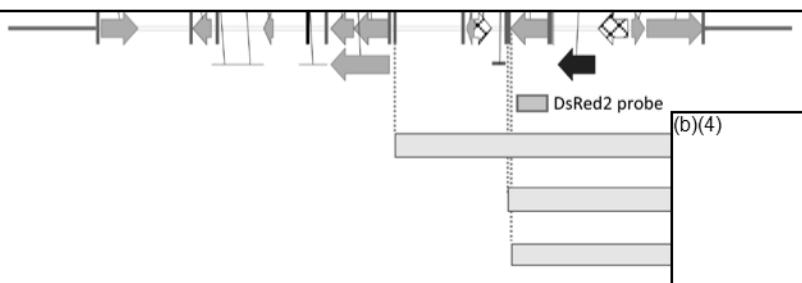
10. Methods

Amplifying and sequencing DNA from the transgene insertion site:

(b)(4)

(b)(4)

(b)(4)



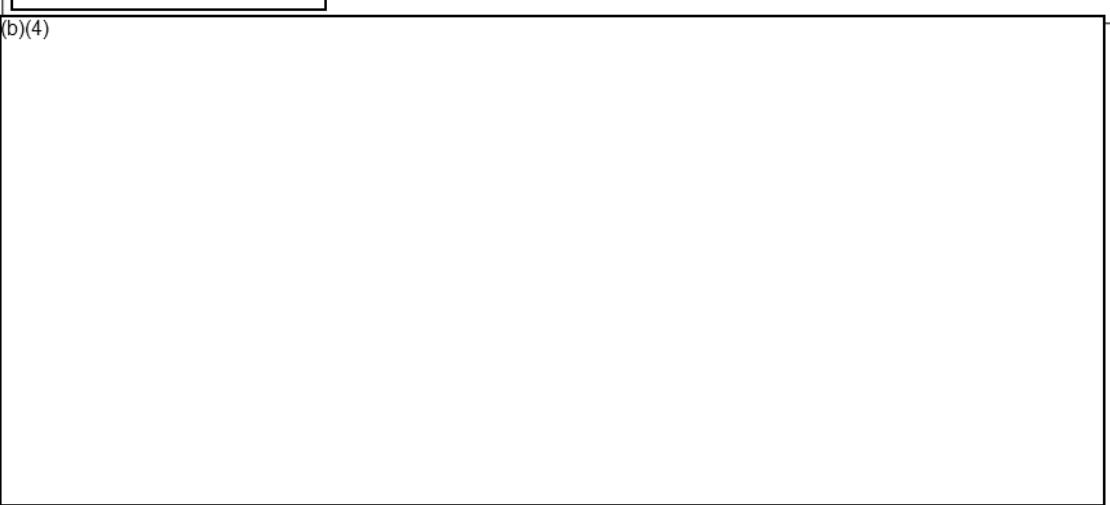
11. RESULTS

(b)(4)

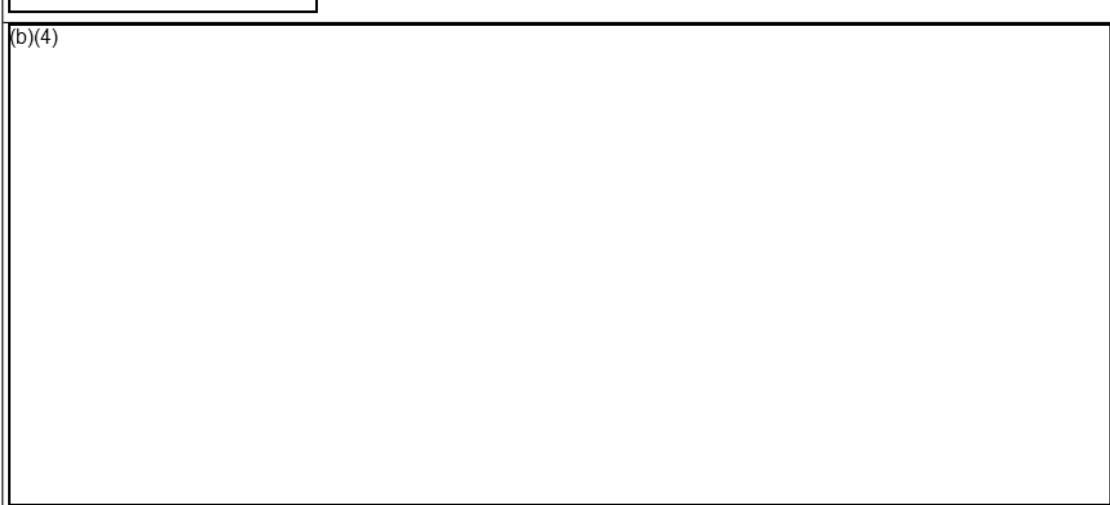
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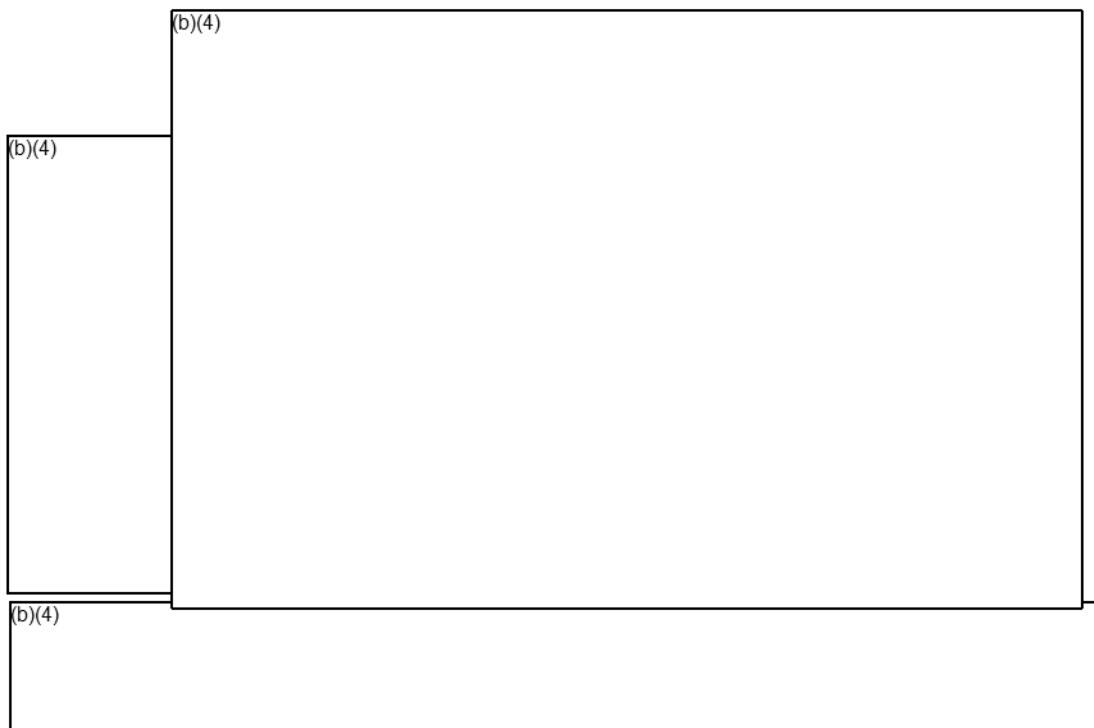


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12. Discussion and Conclusions:

The OX4319L-Pxy insertion site has been sequenced, providing a means of identifying the strain by PCR and distinguishing it from other transgenic strains and wild-type strains of diamondback moth: a vital quality control tool. Southern blot analysis showed single bands for each lane which, taken together with Mendelian inheritance of the transgenic phenotype, indicates that the strain carries a single transgene insertion.

13. Literature:

Sambrook, J., and Russell, D. (2001). Molecular Cloning: A Laboratory Manual; third edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

**OXITEC****INTERNAL RESEARCH REPORT**

- 1. Title:** Quantifying tTAV expression in different life stages of the fsRIDL diamondback moth, OX4319L-Pxy.

- 2. Statement Of Data Confidentiality Claims:**

CBI Deleted

- 3. Statement Concerning Good Laboratory Practices:**

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

- 4. Authors:**

Study Coordinator (Signature): []	Study Supervisor (Signature): []
Study Coordinator (Name And Position): [] Research Scientist	Study Supervisor (Name And Position): [] (b)(6)
Date Signed: 17 th September 2013	Date Signed: 17 th September 2013

- 5. Associated Personnel:**

Name	Tasks
[]	Study coordination, experimental design, report writing
[]	Experimental design
[]	Experimental design, data collection, data analysis
[]	Data collection

[]	Experimental design, approval
(b)(6)	Study Sponsor

6. Test Facility:

This research was performed at Oxitec laboratories at:

Oxitec Ltd,
71 Milton Park,
Abingdon,
Oxon OX14 4RX,
UK

7. Objectives:

Compare tTAV expression levels in different life stages of males and females of the fsRIDL diamondback moth, OX4319L-Pxy, homozygous and heterozygous for the transgene insertion, with and without the dietary antidote.

8. Summary:

The fsRIDL strain of diamondback moth, OX4319L-Pxy, shows tetracycline-repressible, female-specific lethality in larvae (Jin et al., 2013). This experiment sought to demonstrate that mortality correlates with over-expression of the protein, tTAV, as expected based on the design of the construct. [

]

9. Introduction:

The tetracycline-repressible, female-specific mortality in fsRIDL insect strains provides a means of producing large male-only cohorts of insects and a population suppression effect following release into a target population: pre-reproduction mortality of females reduces a population's reproductive potential.

The experiment described here set out to compare the expression levels of tTAV in different life stages of males and females of the fsRIDL diamondback moth, OX4319L-Pxy, homozygous and heterozygous for the transgene insertion, with and without the dietary antidote.

[

]

10. Methods

[PAGES 3-6 CBI deleted]

13. Literature:

Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T.H., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., *et al.* (2013). Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology 2, 160-166.



INTERNAL RESEARCH REPORT

1. Title: Quantifying tTAV expression in different life stages of the fsRIDL diamondback moth, OX4319L-Pxy.

2. Statement Of Data Confidentiality Claims:

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3. Statement Concerning Good Laboratory Practices:

This study was not conducted in compliance with the relevant provisions of Good Laboratory Practices (ENV/MC/CHEM(98)17). However, the study was conducted according to accepted scientific methods and the raw data and study records have been retained.

4. Authors:

Study Coordinator (Signature): [(b)(6)] Study Coordinator (Name And Position): (b)(6) Research Scientist	Study Supervisor (Signature): [(b)(6)] Study Supervisor (Name And Position): (b)(6)
Date Signed: 17 th September 2013	Date Signed: 17 th September 2013

5. Associated Personnel:

Name	Tasks
(b)(6)	Study coordination, experimental design, report writing
	Experimental design
	Experimental design, data collection, data analysis
	Data collection
	Experimental design, approval
	Study Sponsor

6. Test Facility:

This research was performed at Oxitec laboratories at:
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 UK

7. Objectives:

Compare tTAV expression levels in different life stages of males and females of the fsRIDL diamondback moth, OX4319L-Pxy, homozygous and heterozygous for the transgene insertion, with and without the dietary antidote.

8. Summary:

The fsRIDL strain of diamondback moth, OX4319L-Pxy, shows tetracycline-repressible, female-specific lethality in larvae (Jin et al., 2013). This experiment sought to demonstrate that mortality correlates with over-expression of the protein, tTAV, as expected based on the design of the construct. (b)(4)

(b)(4)

(b)(4)

9. Introduction:

The tetracycline-repressible, female-specific mortality in fsRIDL insect strains provides a means of producing large male-only cohorts of insects and a population suppression effect following release into a target population: pre-reproduction mortality of females reduces a population's reproductive potential.

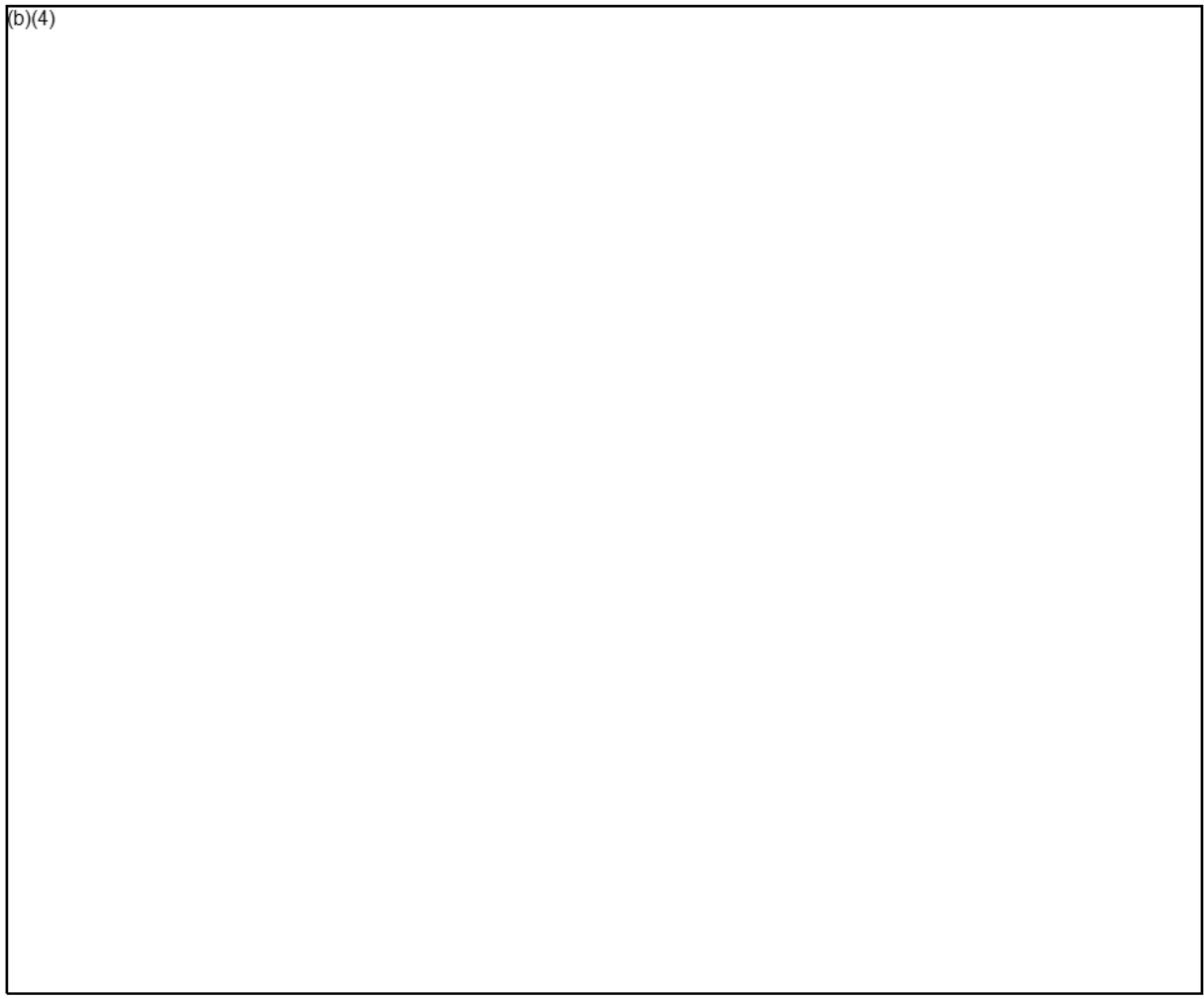
The experiment described here set out to compare the expression levels of tTAV in different life stages of males and females of the fsRIDL diamondback moth, OX4319L-Pxy, homozygous and heterozygous for the transgene insertion, with and without the dietary antidote.

(b)(4)

10. Methods

(b)(4)

(b)(4)



(b)(4)



(b)(4)

11. RESULTS

(b)(4)

(b)(4)

(b)(4)

(b)(4)

(b)(4)

(b)(4)

12. Discussion and Conclusions:

(b)(4)

13. Literature:

Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T.H., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., *et al.* (2013). Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology 2, 160-166.

Plutella OX4319L Phenotype-Genotype text for BRS submission 16March2016

Phenotype(s)

MG – Visual marker; DsRed2 Fluorescent Protein Expression

Repressible lethality

Phenotype Description:

The introduced genetic material in the diamondback moth comprises three protein coding regions, one for marking the insects and two for inducing death before the insect reaches adulthood (in this instance, females only). The former allows the expression of a DsRed2 fluorescent protein originally derived from a coral (*Discosoma* sp.). The transgenic diamondback moth with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenic diamondback moth can be envisioned. The non-modified diamondback moth has no fluorescent protein gene; therefore, it does not fluorescence when illuminated under the same light frequency. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

Genotype(s)

Screenable Marker

Construct component type: Gene

Name: DsRed2

Donor: *Discosoma* sp (coral)

Detailed description: Screenable marker gene DsRed2 from *Discosoma* spp - Allows the expression of a fluorescent protein from *Discosoma* spp. Fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5ie1 promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by intense illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. Expression of a fluorescent protein will therefore permit released modified moths to be distinguished from unmodified.

Construct component type: Vector sequence

Name: piggyBac (non-autonomous)

Donor: piggyBac from *Trichoplusia ni* (moth)

Detailed description: Transformation Vector from *Trichoplusia ni* (moth) - Effects germline transformation of diamondback moth from piggyBac from *Trichoplusia ni* (moth) - 3' end of piggyBac. piggyBac is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element.

In the construct used for transformation of the pink bollworm, the transposase gene of the piggyBac element was irreversibly destroyed by insertion of the transgene. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding piggyBac transposase under the control of the *Drosophila melanogaster* *hsp70* promoter. One of the inverted terminal repeats that flank the wild-type piggyBac transposase in piggyBac has been removed in the helper plasmid so that the helper plasmid cannot itself integrate even though it encodes for active piggyBac transposase.

Repressible lethality

Construct component type: Gene

Name: tTAV

Donor: *Escherichia coli* (bacterium) and Herpes simplex (virus)

Detailed description: Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from *Escherichia coli* with VP16 from a type 1 herpes simplex virus. The tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (*tetO*) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline, it will induce expression from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and *Drosophila melanogaster* with no known adverse effects. Unmodified *Plutella xylostella* do not have a tTAV activity.

Construct component type: Regulatory sequence

Name: doublesex genomic region

Donor: Pink bollworm, *Pectinophora gossypiella*

Detailed description: Female-specificity is conferred using truncated sex-alternate splicing sequences from the *doublesex* gene of *Pectinophora gossypiella*. Sequence encoding tTAV is inserted into this splicing sequence, allowing for the expression of tTAV in a sex-specific manner, resulting in a conditional female-lethal system (Jin et al. 2013).

A full list of construct components is provided in the 'Table of genetic elements'.

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Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2:160-166.

ORIGINAL ARTICLE

Population-level effects of fitness costs associated with repressible female-lethal transgene insertions in two pest insects

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Keywords

fitness costs, genetic engineering, insect, integrated pest management, release of insects carrying a dominant lethal, transgenic

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Abstract

Genetic control strategies offer great potential for the sustainable and effective control of insect pests. These strategies involve the field release of transgenic insects with the aim of introducing engineered alleles into wild populations, either permanently or transiently. Their efficacy can therefore be reduced if transgene-associated fitness costs reduce the relative performance of released insects. We describe a method of measuring the fitness costs associated with transgenes by analyzing their evolutionary trajectories when placed in competition with wild-type alleles in replicated cage populations. Using this method, we estimated lifetime fitness costs associated with two repressible female-lethal transgenes in the diamondback moth and olive fly as being acceptable for field suppression programs. Furthermore, using these estimates of genotype-level fitness costs, we were able to project longer-term evolutionary trajectories for the transgenes investigated. Results from these projections demonstrate that although transgene-associated fitness costs will ultimately cause these transgenes to become extinct, even when engineered lethality is repressed, they may persist for varying periods of time before doing so. This implies that tetracycline-mediated transgene field persistence in these strains is unlikely and suggests that realistic estimates of transgene-associated fitness costs may be useful in trialing ‘uncoupled’ gene drive system components in the field.

Introduction

Genetic engineering has enabled the development of new methods for the sustainable control of insect pests. Such genetic pest management strategies use the mating behavior of a pest species to introduce novel heritable traits into wild target populations. To date, such traits include lethal phenotypes and decreased vector competence (or refractoriness to infection) (Sinkins and Gould 2006; Alphey et al. 2013; Alphey 2014). The success of such an approach depends on the field performance of the engineered insects, especially in terms of finding and mating with wild counterparts. Estimation of fitness costs associated with transgenic strains, particularly those affecting mating ability, has therefore become an area of considerable research interest (Catteruccia et al. 2003; Irvin et al. 2004; Moreira et al. 2004; Marrelli et al. 2006, 2007; Lambrechts et al. 2008;

Scolari et al. 2008; White et al. 2010; Harris et al. 2011, 2012; Massonnet-Brunee et al. 2013; Paton et al. 2013).

One genetic pest management approach, called release of insects carrying a dominant lethal (RIDL), involves the RIDL transgene that renders progeny nonviable in the field (Thomas et al. 2000). Transgene-induced lethality is made conditional through the use of the ‘tet-off’ gene expression system (Gossen and Bujard 1992), which is repressed by provision of tetracycline (TC), or suitable analogs, to the insects – usually as a supplement in the larval diet (‘on-tet’). Unlike the classical autocidal control approach, the sterile insect technique (SIT) RIDL does not require sterilization by irradiation, which can have negative impacts on sexual competitiveness and field survival (Shelly et al. 1994; Lance et al. 2000; Alphey et al. 2010).

A female-specific variant of RIDL (fsRIDL) limits engineered lethality to females, allowing male-only production

and mortality of female progeny in the field (Schliekelman and Gould 2000; Fu et al. 2010; Black et al. 2011; Ant et al. 2012; Jin et al. 2013). Male-only releases can offer improved per-male efficiency (McInnis et al. 1994; Rendón et al. 2000, 2004) and reduced female-specific damage in the field, such as oviposition damage by fruit flies or biting by mosquitoes. In addition, field survival of fsRIDL male heterozygotes may provide an insecticide resistance management strategy through introgression of susceptibility alleles into wild populations (Alphey et al. 2007, 2009).

In the fsRIDL strains investigated here – OX4319L-Pxy diamondback moth (*Plutella xylostella* L.) (Jin et al. 2013) and OX3097D-Bol olive fruit fly (*Bactrocera oleae* Gmelin) (Ant et al. 2012) – engineered lethality is limited to females through the use of sex-alternate splicing sequences from sex determination genes: in OX4319L-Pxy *doublesex* from the pink bollworm, *Pectinophora gossypiella*; in OX3097D-Bol *transformer* from Mediterranean fruit fly (Medfly, *Ceratitis capitata* Wiedemann). In OX4319-Pxy, insertion of the tTAV transactivator coding sequence from the tet-off system into the female-specific exon of a *dsx* minigene results in tTAV transcription in females only. In OX3097D-Bol, the tTAV insertion in an exon of a *tra* minigene is in frame with female transcripts and out of frame with those of males. As engineered TC-repressible lethality in these strains is dependent on the expression of tTAV, these minigenes limit this phenotype to females. These strains have undergone successful glasshouse cage trials and show potential for application in the field. The diamondback moth and olive fly are the primary insect pests of their respective host crops, brassicas and olives, and cause significant economic damage. Current strategies for their control are primarily reliant on synthetic chemical insecticides, which has led to resistant pest populations, suppression of natural enemies, and the subsequent breakdown of control (Daane and Johnson 2010; Furlong et al. 2013). As such, development of novel, more sustainable, control measures is required.

In transgenic organisms, fitness can be negatively affected by several factors. These include expression of the transgene sequence, insertional mutagenic effects of the transgene insertion, and inbreeding depression, genetic drift or selection related to laboratory adaptation and rearing (Cooley et al. 1988; Bellen et al. 1989; Horn et al. 2002; Uchino et al. 2008; Ahrens and Devlin 2011). In RIDL insects, expression of the transgene sequence comprises the intended expression and off-target expression. For fsRIDL constructs, expression is intended to be lethal to females reared in the absence of the antidote TC ('off-tet'), while off-target expression might lead to negative effects on males. Similarly, females may be negatively affected by transgene expression even in the presence of TC, if expression of the transgene is not repressed below a harmful level.

As several of these effects are specific to the particular transgene insertion, fitness costs may vary between different insertion lines carrying the same transgene but at different chromosomal loci (Lyman et al. 1996; Scolari et al. 2008; Ant et al. 2012; Jin et al. 2013; Yonemura et al. 2013). Previous studies of fitness costs in RIDL/fsRIDL insects have primarily focused on specific behavioral characteristics of homozygous adult males, such as copulation success, induction of female remating refractoriness, longevity, or flight performance, as these are key to the effectiveness of released males (Morrison et al. 2009; Bargielowski et al. 2011a,b, 2012; Ant et al. 2012; Labbé et al. 2012; Jin et al. 2013). Here, however, we chose to assess the cumulative effects of fitness costs over the course of the life cycle and over multiple generations by tracking the evolution of fsRIDL allele frequencies over time. This approach allowed us to estimate whole-life-cycle fitness costs and provided parameter estimates useful in modeling the dynamics of transgene insertion alleles under a variety of scenarios.

We present results from multi-generational laboratory-cage studies measuring the time evolution of fsRIDL alleles in mixed wild-type/transgenic populations of two key pest insect species (diamondback moth and olive fly) reared under either permissive or restrictive conditions. Mass rearing of fsRIDL insects would be conducted under permissive conditions (with TC), whereas released insects and their progeny would face restrictive conditions (no TC). Fitness costs under permissive conditions therefore affect ease and efficiency of rearing and also inform consideration of the likely fate of any hypothetical wild-type allele that somehow entered such a population. Fitness costs under restrictive conditions inform models of the rate of loss of the transgene from a wild population, were releases to cease. Under permissive conditions, no significant fitness costs were found to be associated with OX3097D-Bol, whereas significant selection against the OX4319L-Pxy transgene insertion was evident. Consistent with the predictions of a stochastic simulation model based on the expected female-killing effect of the transgene under such circumstances, both transgenes disappeared rapidly from experimental populations under restrictive conditions.

Materials and methods

Experimental conditions and insect rearing

Insects were reared at 25°C, with a 16:8 light/dark cycle. Rearing of diamondback moth and olive fly followed procedures described by Martins et al. (2012) and Ant et al. (2012), respectively. In addition to TC-repressible female-specific lethality, both transgenic strains express a DsRed2 fluorescent protein marker, visible in larvae, pupae, and adults under appropriate filters. OX3097D-Bol was

generated using the 'Demokritos' strain (Greece) and was later outcrossed for five generations to the 'Argov' strain (Israel). Argov was also used as the wild-type strain in this study. OX4319L-Pxy was generated using a diamondback moth colony originating in Vero Beach, FL, USA, which was also used as the wild-type strain in this study. All wild-type strains used have been reared in captivity for >5 years.

The starting frequencies of the transgenic allele in permissive and restrictive rearing experiments were chosen to reflect those expected in extreme examples of two scenarios: (i) wild-type contamination in a mass-reared colony (≥ 0.5) and (ii) after cessation of inundative releases of fsRIDL insects into the field (0.25). In each experimental generation, the adult insects were housed for 1 week in a $30 \times 30 \times 30$ cm netted cage (Bugdorm, Taichung, Taiwan). Prior to introduction of these insects into the cages, they were sexed and screened as pupae for fluorescence and maintained as separate cohorts until eclosion. When all adults had eclosed, cohorts from each population were placed in their respective cages, with males being introduced first and females 2 h later. Two egg collections were made from each cage during this period, placed on diet and resulting pupae used to found the next generation. As egg collections were taken within approximately the first week after adult eclosion, these experiments did not seek to measure adult fitness costs which manifest after this point. In a mass-rearing setting, adults are rarely kept beyond the first week, when reproductive productivity is highest, and in the wild, mean adult life spans are expected to be <5 days (Furlong et al. 1995).

Permissive condition experiments

Transgene-permissive conditions were created by providing chlortetracycline (CTC) (for OX4319L-Pxy) and TC (for OX3097D-Bol) in larval diet and adult sugar water (10%) to a final concentration of 100 µg/mL. Olive fly adults were maintained on a yeast–sugar diet without TC. Initial populations with known frequencies of the transgene – OX4319L-Pxy, 0.75; and OX3097D-Bol, 0.5 – were established by crossing transgene-heterozygous males with homozygous females, and transgene-heterozygous males and females, respectively. For diamondback moth, 200 of the resulting progeny from each cage were selected at random as pupae to found the following generation. For olive fly, all pupae surviving in each pot were screened for the DsRed2 marker and the transgenic/wild type ratio calculated for each replicate. The number of transgenics returned to the cage was then made proportional to this ratio, with 200 pupae being selected in total. After egg collections, each cage was frozen and dead adults collected. Of these dead adults, 96 were randomly selected and their

gDNA extracted using the following method. Decapitated bodies were placed in individual wells of a 96-well PCR plate, each containing 75 µL of 100 mM NaOH. Plates were heated to 99°C for 30 min in a PCR block, and then, 15 µL of a second solution [250 mM Tris-HCl (pH 8.0) and 0.04% Phenol Red] was added to each well. Samples were genotyped by PCR for presence of the transgene and of the corresponding no-insertion wild-type allele using reactions analogous to those described by Walters et al. (2012) [diamondback moth: 2 min at 94°C, 2 × (10 s at 95°C, 1 min at 62°C, 2 min at 72°C), 26 × (10 s at 95°C, 30 s at 62°C, 30 s at 72°C), and 5 min at 72°C; olive fly: 2 min at 94°C, 35 × (30 s at 94°C, 30 s at 58°C, 50 s at 72°C), and 7 min at 72°C]. One pair of primers was used to amplify sequence spanning the 5' terminus of the transgene insertion [in diamondback moth 'OX4319L-Pxy F2' (sequence available on request) with 'PB5-out' (5'-CTCTGGACGTCACTTCAC TTACGTG-3'); and in olive fly 'OF3097Dforward7' (5'-CTTACATATAAGCAGTGCCTCACATG-3') with 'Pb1' (5'-GGCGACTGAGATGTCCTAAATGCAC-3')], and another pair was used to amplify sequence spanning the corresponding wild-type locus [in diamondback moth 'OX4319L-Pxy F2' with 'OX4319L-Pxy R1' (sequence available on request), and in olive fly 'OF3097Dforward7' with 'OF3097D3'reverse4' (5'-CCTGCGTTGGAGATGACGAA ATC-3')]. Genotyping results provided estimates of the number of individuals from each of the three genotypes (R/R, R/– and –/–), which was used to calculate generational transgene and wild-type allele frequencies. The experiment was run for 10 generations, with three replicated populations for diamondback moth and two for olive fly.

Permissive conditions analysis

Under neutral conditions, it is assumed that, having reached Hardy–Weinberg equilibrium, allele and genotype frequencies will remain relatively stable, albeit subject to genetic drift. To test for significant trends in frequency change (a nonstationary process potentially resulting from selection), Mann–Kendall tests were performed. Where these implied that selection was occurring, two further analyses were performed. To test whether significant allele frequency trends could be statistically attributed to selection (rather than drift), a frequency increment test (FIT) was performed on mean generational transgene allele frequencies (Feder et al. 2013). The fitness of individual genotypes was also analyzed by comparing corrected rate of increase (CRI) parameters using a Kruskal–Wallis rank sum test and subsequent *post hoc* testing using Tukey's contrasts. CRI parameters compare observed and expected (under Hardy–Weinberg) genotype frequencies, with the difference between these values indicating the direction and magnitude of selection against this genotype. The mean of

these parameters provided an estimated rate of change for each genotype per generation. This allowed for the calculation of mean genotype-specific fitness values (W) relative to homozygous wild type. As our best estimates of the fitness costs associate with these transgenes, these values were subsequently used to predict longer-term fsRIDL allele frequency evolution for diamondback moth and olive fly populations. Trajectories were calculated using a recursion model (Hamilton 2009) with initial simulated population genotype frequencies analogous to those used in experimental cages. The model assumes an infinite population size, random mating, constant selection, and no migration. For calculating times to extinction of modeled fsRIDL alleles, a hypothetical population size of 200 individuals was used.

Restrictive conditions experiments

Transgene restrictive conditions were created by rearing larvae and adults without access to TC sources. For both OX4319L-Pxy and OX3097D-Bol, three replicate populations were analyzed with initial transgene frequencies of 0.25, established by crossing transgene-heterozygous males with wild-type females; this represents the maximum starting allele frequency for a female-lethal transgene in the absence of artificial releases of homozygotes. Replicate populations were observed until the transgene was no longer detected and for one further generation to confirm extinction of the transgene. After pupation, 200 insects from each replicate were randomly selected, then scored for the fluorescent marker and sexed (olive fly as adults). Once eclosed, these adults were placed in new cages to found the next generation. Due to the high level of penetrance of the female-lethal trait in OX4319L-Pxy and OX3097D-Bol under restrictive conditions (>99%, Ant et al. 2012; Jin et al. 2013), and the relatively low initial transgene frequency, it was assumed that all transgenic individuals observed from Generation 2 onwards were heterozygous for the fsRIDL transgene.

Restrictive conditions analysis

Under restrictive conditions, we hypothesize that the trajectory of fsRIDL transgene frequency change will be most prominently directed by the dominant lethality in females, and the directional selection that this confers. Theoretically, this effect will result in a 50% reduction in fsRIDL allele frequency in each generation relative to the previous. However, this trajectory may be influenced by population-level stochastic effects as well as hypothetical transgene-associated fitness costs. We developed a discrete-generation stochastic model to simulate the potential trajectories of fsRIDL allele decay under situations analogous to our

experimental populations (population size of 200, initial fsRIDL allele frequency of 0.25, restrictive conditions). Our model allows for random variation in the sex ratios of the 200 individuals selected each generation. As we assume that only males can carry the fsRIDL allele, this also creates variance in the number of individuals inheriting the fsRIDL allele each generation. No fitness costs other than those imparted by female-specific lethality were included, and thus, modeled trajectories represent the potential distribution of trajectories given stochastic variation in population sex ratio and subsequent fsRIDL allele inheritance alone. This was achieved by first estimating the probability that a male in a given generation (t) was transgenic $p(t)$, where the ratio of the number of transgenic males in the previous generation $M(t-1)$ and the number of total males in the previous generation $N(t-1)$ is halved, representing the halving of the allele frequency each generation due to female-specific lethality.

$$p(t) = 0.5 \times \frac{M(t-1)}{N(t-1)} \quad (1)$$

This probability was then used to calculate the fsRIDL allele frequency in generation t $\gamma(t)$. Here $N(t)$, the number of males in generation t was generated using a Binomial (200, 0.5) distribution. The product of $N(t)$ and $p(t)$ was then divided by 200 to represent the proportion of transgenic males in the total population and halved to give the allele frequency (as transgenics are assumed to be heterozygotes).

$$\gamma(t) = 0.5 \times \frac{N(t) \times p(t)}{200} \quad (2)$$

In Generation 1, fsRIDL allele frequency and sex ratio (male/female) were set at 0.25 and 0.5, respectively, to match the known starting conditions in the experimental populations. Two hundred and fifty independent populations were simulated and allowed to persist until fsRIDL allele extinction. Modeled results were compared to empirical data collected for each species in order to assess whether observed fsRIDL allele frequency decay fell within predicted variation in trajectories, given the assumptions of the model. Subsequently, the mean fsRIDL allele frequency reduction per generation was calculated for both the model and experimental data sets and compared using a Welch two-sample t -test. All statistical analysis was performed in R (v. 3.0.02) (R Core Team, 2013). Modeling was performed in Matlab.

Results

Selection on the fsRIDL transgene under permissive conditions

In diamondback moth, OX4319L-Pxy transgene allele frequency declined by 63.3%, a trend which was significantly

non-neutral ($\tau = -0.956, P < 0.01$) (Fig. 1A). Frequency increment testing showed a significant departure by the transgene allele frequency from a null, neutral drift distribution ($t_{\text{FITT}} = 2.32, \alpha = 0.05$). The homozygous wild-type genotype (−/−) showed the highest average increase in frequency, from 0.07 (± 0.02 SE) in Generation 2 (the first generation expected to represent genotypes at Hardy–Weinberg equilibrium) to 0.47 (± 0.02 SE) in Generation 10 (Fig. 2A). Nonstationary trends were suggested for −/− ($\tau = 0.944, P < 0.01$) and homozygous transgenic (R/R) ($\tau = -0.889, P < 0.01$) genotype trajectories, but not for the heterozygous genotype (R−) ($\tau = 0.056, P > 0.1$). Relative fitness values for the R− and R/R genotypes were calculated to be $W_{R-} = 0.736 \pm 0.07$ SE and $W_{R/R} = 0.477 \pm 0.20$ SE, respectively (Fig. 2B), with significant differences in CRI calculated between R/R and R−, −/− genotypes (Stat = −4.968, $P < 0.01$; Stat = −3.509, $P < 0.01$) but not between R− and −/− genotypes (Stat = −2.068, $P > 0.1$).

In OX3097D-Bol olive flies, transgene allele frequency declined by 15.5%, a significant non-neutral trend ($\tau = -0.664, P = 0.012$) (Fig. 1B). However, no significant departure from a neutral drift distribution was observed for mean transgene allele frequencies at $\alpha = 0.05$, although a significant difference was observed at $\alpha = 0.15$ ($t_{\text{FITT}} = 1.018$). Partitioning this behavior to the genotype level, the −/− genotype showed the greatest mean increase, rising from an initial frequency of 0.25 to an average of 0.37 (± 0.05 SE) after 10 generations (Fig. 2A), a trend which was significantly non-neutral ($\tau = 0.584, P = 0.024$). However, trajectories of R− and R/R genotype frequencies showed no significant trend ($\tau = -0.477, P = 0.071$;

$\tau = -0.576, P = 0.057$). Relative fitness values of $W_{R-} = 0.974 \pm 0.07$ SE and $W_{R/R} = 0.975 \pm 0.05$ SE were calculated (Fig. 2B); however, genotype was not found to significantly explain differences in CRI values ($KW^2 = 0.730, P > 0.1$).

Relative fitness parameters estimated from these empirical data were used to predict changes in genotype frequency over time (Fig. 2C). In all projected genotype trajectories, selection led to the eventual fixation of the wild-type allele and the concomitant loss of the transgene. For OX4319L-Pxy, comparatively high transgene-associated fitness costs led to a predicted wild-type allele fixation – where the modeled number of transgenic individuals falls to <1 – within 30 generations (initial wild-type allele frequency = 0.25), while the estimation of much lower transgene-associated fitness costs for OX3097D-Bol resulted in predicted wild-type allele fixation after approximately 200 generations (initial wild-type allele frequency = 0.5).

Selection on the fsRIDL transgene under restrictive conditions

Given an initial transgene allele frequency of 0.25 in a closed population of 200 individuals under restrictive conditions, our stochastic model predicted fsRIDL transgene extinction within nine generations in approximately 95% of iterations, with a mean and maximum number of generations until allele loss of 6.5 (± 1.8 SD) and 15, respectively (Fig. 3). fsRIDL allele frequency decay in experimental populations fell well within the variation predicted by this stochastic model. Mean number of generations until disappearance of the fsRIDL allele in diamondback moth and

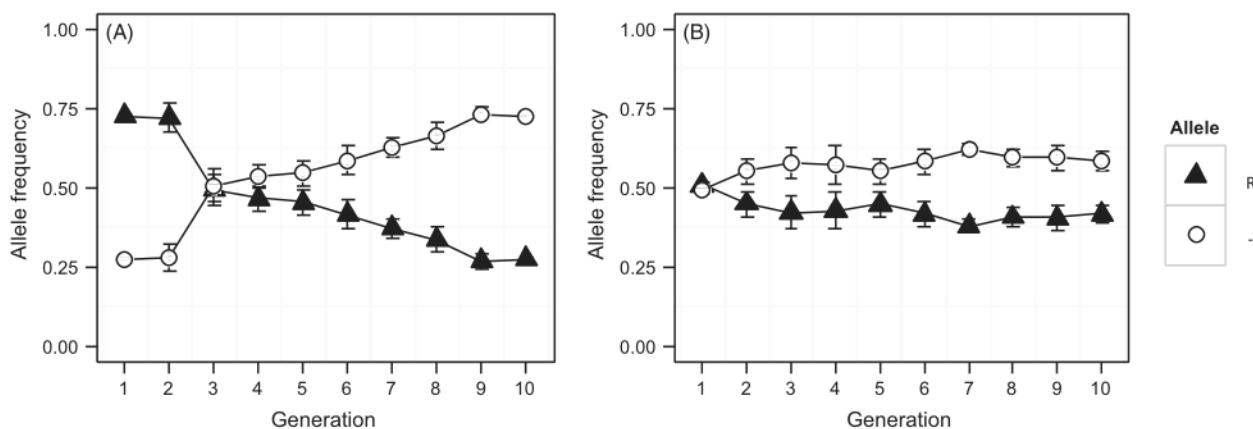


Figure 1 Mean transgene and wild-type allele frequencies (\pm SE) tracked over 10 generations in three and two mixed populations of diamondback moth (A) and olive fruit fly (B) containing the fsRIDL transgene insertions OX4319L-Pxy and OX3097D-Bol, respectively. Allele frequencies were estimated by genotyping 96 randomly chosen adults per population, per generation, for both the transgene insertion and the corresponding no-insertion wild-type allele. Triangles and circles represent the mean frequencies of the transgene (R) and wild-type (−) allele recorded in each generation, respectively.

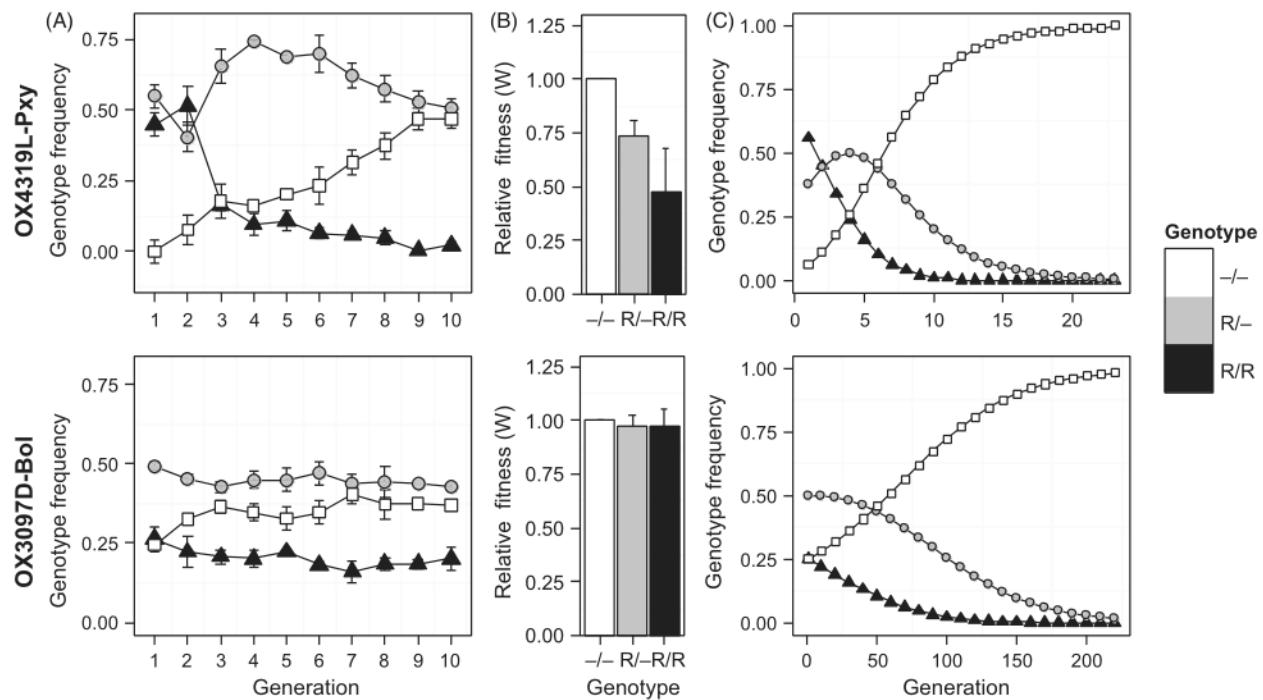


Figure 2 (A) Mean frequencies (\pm SE) of transgenic homozygous (R/R, triangles), heterozygous (R $^-$, circles), and wild type (-/-, squares) in two and three mixed-genotype populations of diamondback moth (upper panel) and olive fly (lower panel), respectively. Diamondback moth populations were established with 100 homozygous transgenic male and 100 heterozygous transgenic female insects. Olive fly populations were established with 100 heterozygous transgenic male and 100 heterozygous transgenic female insects. Experimental populations were observed for 10 generations. Engineered female lethality was suppressed throughout the experiment by provision of dietary tetracycline, and population size was maintained at 200 adults in each generation. (B) Relative fitness values for -/-, R $^-$ and R/R genotypes (\pm SE) of OX4319L-Pxy (upper panel) and OX3097D-Bol (lower panel) calculated from corrected rate of increase parameters with values relative to the -/- genotype. Relative fitness values were $W_{R/R} = 0.477$, $W_{R^-} = 0.736$, and $W_{-/-} = 1$ for diamondback moth and $W_{R/R} = 0.975$, $W_{R^-} = 0.974$, and $W_{-/-} = 1$ for olive fly. (C) Results of a deterministic population genetics model illustrating theoretical genotype trajectories in mixed-genotype populations of diamondback moth (upper panel) and olive fly (lower panel) using the experimentally derived mean estimated relative fitness values from (B). Note that model outputs represent more generations than the cage experiment, to illustrate longer-term trajectories.

olive fly populations was 6.0 (± 0.58 SE) and 8.0 (± 1.16 SE), respectively, with respective maximum number of generations until allele extinction of 7 and 11. On average, OX4319L-Pxy allele frequency decreased in each generation by 50% (± 6.0 SE), while OX3097D-Bol frequency decreased by 45% (± 4.9 SE). Modeled mean fsRIDL allele frequency fell by 47% (± 7.2 SE) per generation and did not significantly differ from either experimental estimates (OX4319L-Pxy: $t = -0.260$, $P > 0.1$; OX3097D-Bol: $t = 0.211$, $P > 0.1$).

Discussion

Under permissive conditions, we measured significant negative transgene allele frequency trends over the experimental period in both fsRIDL constructs in their respective host strains (Fig. 1). In diamondback moth populations, these frequency changes could be attributed to selection against the transgene. However, in olive fly, transgene allele

frequency changes could not be significantly differentiated from neutral drift (at $\alpha = 0.05$, although differences at $\alpha = 0.15$ showed significance). As our experimental population size (N) was substantially larger ($>20\times$) than the number of generations observed, however, we assume that genetic drift (which changes allele frequencies over periods of approximately N generations) is unlikely to have significantly affected our results in this experiment (Illingworth et al. 2012). If transgene-associated fitness costs are very low (as may be the case in OX3097D-Bol), increased replication (number of observed generations) relative to that used here may therefore be required to differentiate selection from neutral drift.

In OX4319L-Pxy, negative transgene allele frequency trends were primarily driven by selection against R/R individuals. This was the only genotype to show both significant negative frequency trends and CRI values significantly different from -/- (Fig. 2A,B). In OX3097D-Bol, neither transgenic genotype showed a significant frequency trend

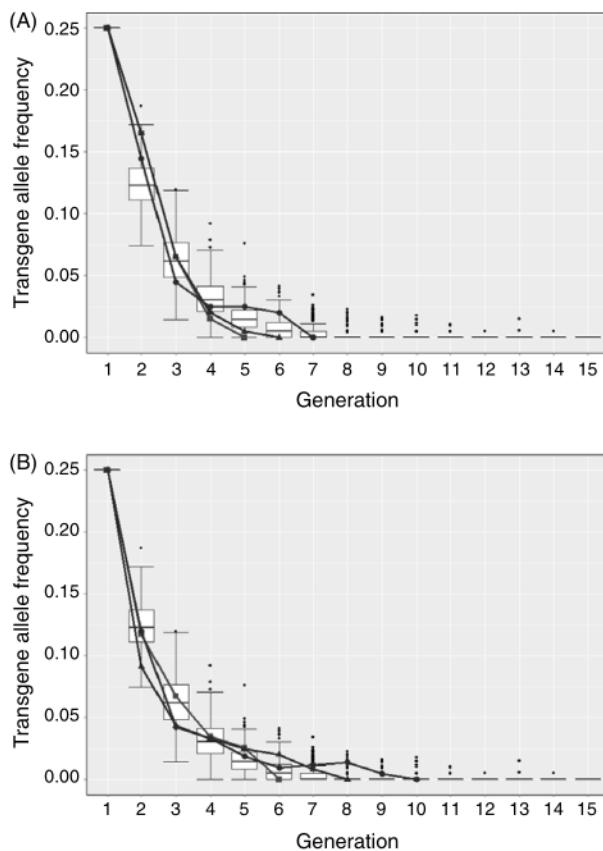


Figure 3 Boxplots showing results from 250 iterations of a stochastic model simulating engineered female-specific selection on a fsRIDL allele in a panmictic, closed population of constant size over 15 discrete generations. We consider fsRIDL allele frequency with a starting population of 200 individuals and an initial fsRIDL allele frequency of 0.25 ($f = 0.25$) propagating in the absence of the transgene repressor (under restrictive conditions). Horizontal bold lines represent generational medians; upper and lower box lines represent first and third quartiles, respectively; outer horizontal lines represent 1.5 \times the interquartile range; and dots represent data points over 1.5 \times above or below the first and third quartiles, respectively. Overlaid onto the boxplots are lines (red, blue and green) showing allele frequency changes from three replicates of caged experiments tracking fsRIDL allele frequencies in mixed populations of (A) diamondback moth and (B) olive fly reared under analogous conditions to those used in the model (initial fsRIDL allele frequency of 0.25, restrictive conditions).

(although interestingly a significant positive trend was observed for the $-/-$ genotype) nor was genotype found to significantly explain variation in CRI values (Fig. 2A,B). If very low fitness costs are associated with this strain, further replication may be required to elucidate their presence. These findings are consistent with those from previous studies of these strains exploring different measurements of relative mating competitiveness when in competition with nontransgenic counterparts. For OX4319L-Pxy, previous laboratory-cage assays of mating initiation and progeny

share (thus including postcopulatory selection) indicated small fitness costs associated with transgene-homozygous males relative to males of their wild-type background strain (Jin et al. 2013). On the other hand, glasshouse-based studies using homozygous OX3097D-Bol males and wild-type olive flies suggested the absence of transgene-related fitness costs to mating behavior. These results were further supported in experiments showing strong mating and remating competitiveness of OX3097D-Bol males when compared with field-collected wild insects and the successful suppression of caged wild-type olive fly populations in a simulated release scenario (Ant et al. 2012).

As this experiment took into account a wider range of factors than previous studies on these lines (in terms of behaviors and sexes analyzed), a lower estimate of overall fitness might have been anticipated. In particular, fitness costs for a female-lethal strain, even under permissive conditions, might be significantly higher in females than in males. In fact, the estimates of relative R/R fitness in this study (OX3097D-Bol = 0.975, OX4319L-Pxy = 0.477) (Fig. 2B) did not differ greatly from estimates of relative mating competitiveness and progeny share calculated previously for homozygous males from these strains [OX3097D-Bol = 1.083 (T. Ant, M. Koukido and L. Alphey, unpublished manuscript), OX4319L-Pxy = 0.580] (Ant et al. 2012; Jin et al. 2013). As these estimates of relative fitness are well above minimum recommendations for an efficient autocidal release program (FAO/IAEA/USDA 2003), these results reinforce the potential of these strains to be employed in such a control strategy.

Beyond their potential impacts on suppression efficacy, the transgene-associated fitness costs present in these two fsRIDL strains have a number of implications at the population level. Using our best estimates of the fitness costs associated with these lines (derived from this study), our population modeling suggests that even under conditions where engineered lethality is suppressed, competition between transgenic and wild-type alleles will result in gradual increase in frequency of the wild-type (noninsertion) allele (Fig. 2C). However, the relatively low fitness cost of the transgene, especially for OX3097D, suggests that a wild-type allele somehow entering a mass-rearing colony would spread only slowly. This is a significant improvement over classical translocation-based sexing strains, for example the medfly *tsl* strains, which have severe fitness costs due to aneuploidy in offspring (Fisher 2000). We assume that the primary cause of these transgene-associated fitness costs, where present, is insertional mutagenesis and/or incomplete female specificity of the fsRIDL phenotype. The contributions of other components of the constructs, however, such as the DsRed2 fluorescent protein marker, cannot be ruled out. However, the differing rates of selection against the two constructs, both of which include the DsRed2 gene

under the control of the same promoter/enhancer, indicate that detected fitness costs are likely not primarily due to the marker.

Although no significant fitness costs associated with R⁺ were evident in either strain under permissive conditions, we expected that fitness costs would be more marked under restrictive conditions. We compared fsRIDL allele evolution between modeled populations in which the sole selection force on fsRIDL insects was engineered female-lethality, and empirical data collected under analogous conditions. In both species, experimental fsRIDL allele decay fell well within that predicted by our stochastic model (Fig. 3). Average generational fsRIDL allele frequency reductions in both lines did not differ significantly from those predicted by our stochastic model. These results indicate that fitness costs to heterozygote males (as carriers of fsRIDL alleles under restrictive conditions), if present, have little effect on allele frequency evolution under these conditions. As predicted, both transgenes went extinct in all experimental populations within a small number of generations, indicating that transgene persistence in the field would be relatively transient in the absence of ongoing releases of additional transgenics, even from a high starting allele frequency. Even if some individuals had access to permissive conditions, that is, high levels of TC, during larval development – implausible in the field for these insects (Kumar et al. 2005; Hu et al. 2010; Seo et al. 2010) – most would likely not, and the fitness cost of the transgene under both restrictive and permissive conditions would ensure rapid disappearance of the transgene from the population. It is likely that the dynamics of these fsRIDL transgenes in a postrelease field population will be influenced to some degree by aspects of the local genetic background. In general, the local genetic background is expected to be better adapted to local conditions than the genetic background of a strain that has been reared in captivity for many generations. Linkage with maladaptive background alleles might lead to an initial reduction in transgene frequency that is slightly more rapid than seen in our experiments using similar backgrounds for wild-type and transgenic strains. This could be addressed by comparison against wild-caught or more recently colonized strains in realistic environmental settings, perhaps in small-scale release experiments.

Our data show that protein-coding transgenes can impose a low fitness cost. Fitness costs due to transgene expression would be expected to be dominant or codominant, so it is particularly striking that the observed fitness costs were substantially recessive. This is somewhat surprising for a conditional lethal transgene and suggests that basal expression of the lethal effector is relatively harmless. In contrast, taking a similar multi-generational approach, Paton et al. (2013) identified strong selection against a synthetic malaria-refractory

transgene (EVida3) in the mosquito *Anopheles gambiae*, resulting in extinction of the allele in all replicate populations by Generation 10. Significant fitness costs to the larval stages of these transgenic mosquito strains were identified, possibly due to unintended background transgene expression. The much longer predicted persistence times of our lethal transgenes under permissive conditions suggests that it is possible to design constructs and generate transgene insertion sites where such background fitness costs can be minimized. This is encouraging both for the rearing and use of these strains, and also for the development of ‘self-sustaining’ strains and strategies in which, unlike fsRIDL, the transgene is intended to persist in the environment for many generations or indefinitely after releases cease. In particular, it suggests that it may be possible to deploy such transgenes effectively, at least on trial scales, by inundative release. Even though the transgenes would eventually disappear (due to associated fitness costs), and so the approach is self-limiting, the transgene may persist in closed populations at a useful frequency for many generations (Gould et al. 2008; Rasgon 2009). This would allow effector genes and molecules to be tested in the field without requiring coupling to gene drive systems, which are more challenging from both technical and regulatory perspectives. The findings of this study support the further development, testing and use of genetic control methods and, in particular, of the fsRIDL strains and systems examined.

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Conflict of interests

All authors of this publication are, or were, employees or sponsored students of Oxitec Ltd, which therefore provided salary or stipend and other support for the research program. Also, all these employees have shares or share options in Oxitec Ltd. This does not alter the authors' adherence to all *Evolutionary Applications* policies on sharing data and materials.

Data archiving statement

Data for this study are available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.40gg7>.

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RESEARCH ARTICLE

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Pest control and resistance management through release of insects carrying a male-selecting transgene

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Abstract

Background: Development and evaluation of new insect pest management tools is critical for overcoming over-reliance upon, and growing resistance to, synthetic, biological and plant-expressed insecticides. For transgenic crops expressing insecticidal proteins from the bacterium *Bacillus thuringiensis* ('Bt crops') emergence of resistance is slowed by maintaining a proportion of the crop as non-*Bt* varieties, which produce pest insects unselected for resistance. While this strategy has been largely successful, multiple cases of *Bt* resistance have now been reported. One new approach to pest management is the use of genetically engineered insects to suppress populations of their own species. Models suggest that released insects carrying male-selecting (MS) transgenes would be effective agents of direct, species-specific pest management by preventing survival of female progeny, and simultaneously provide an alternative insecticide resistance management strategy by introgression of susceptibility alleles into target populations. We developed a MS strain of the diamondback moth, *Plutella xylostella*, a serious global pest of crucifers. MS-strain larvae are reared as normal with dietary tetracycline, but, when reared without tetracycline or on host plants, only males will survive to adulthood. We used this strain in glasshouse-cages to study the effect of MS male *P. xylostella* releases on target pest population size and spread of *Bt* resistance in these populations.

Results: Introductions of MS-engineered *P. xylostella* males into wild-type populations led to rapid pest population decline, and then elimination. In separate experiments on broccoli plants, relatively low-level releases of MS males in combination with broccoli expressing Cry1Ac (*Bt* broccoli) suppressed population growth and delayed the spread of *Bt* resistance. Higher rates of MS male releases in the absence of *Bt* broccoli were also able to suppress *P. xylostella* populations, whereas either low-level MS male releases or *Bt* broccoli alone did not.

Conclusions: These results support theoretical modeling, indicating that MS-engineered insects can provide a powerful pest population suppressing effect, and could effectively augment current *Bt* resistance management strategies. We conclude that, subject to field confirmation, MS insects offer an effective and versatile control option against *P. xylostella* and potentially other pests, and may reduce reliance on and protect insecticide-based approaches, including *Bt* crops.

Keywords: *Bacillus thuringiensis*, Diamondback moth, Insect, Insecticide resistance management, Pest, *Plutella xylostella*, Sterile insect technique, Transgenic

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Background

Pest insects are a major threat to global food production, biodiversity conservation, and human and animal health [1–3]. Synthetic insecticides are widely used for control; however, potential off-target ecological damage, and the capacity of pest populations to develop resistance, has driven demand for alternative methods of pest control. Integrated pest management (IPM) approaches have been developed, utilizing multiple tools including biological insecticides (applied and expressed in transgenic crops), enhanced biological control, mating disruption, and the release of sterile insects (the sterile insect technique, SIT) to sustainably manage insect pest populations.

The SIT reduces target pest populations through sustained mass-releases of radiation-sterilized insects, thus reducing the frequency of mating between fertile insects [4]. SIT relies on the mate-seeking and mating behavior of released insects, and is therefore species-specific and can be effective against pests that are difficult to control by other methods. SIT has been successful in area-wide eradication and suppression programs against numerous crop pests [5]. Wider applicability of SIT is hindered by several challenges, including the negative effects on insect performance of sterilization by irradiation [6–10] and difficulty in conducting large-scale sex-sorting for male-only releases [11–13].

We have previously developed a male-selecting (MS) transgenic system to overcome these obstacles [14, 15]. In this system, pest colonies are engineered with tetracycline-repressible dominant female-specific lethal transgenes. Provision of tetracycline (or suitable analogues) to larval stages suppresses transgene lethality allowing mass rearing. Once released, mating between transgenic and wild insects results in mortality of female progeny (female-specific lethality) due to the absence of suitable quantities of tetracycline in the field, thereby reducing the reproductive potential of the target population [15–20]. Through targeting female progeny but allowing male transgene heterozygotes to survive to reproduce, the MS system is predicted to be significantly more efficient at suppressing populations than those which target both sexes (such as SIT) [19]. Additionally, this system avoids the negative impacts of irradiation on released insect competitiveness [21] and enables large-scale (off tetracycline) production of single-sex (male) release cohorts. Male-only releases can significantly improve per-male efficiency [22, 23] by concentrating the reproductive effort of released insects on wild females. For the Mediterranean fruit fly (medfly, *Ceratitis capitata*), SIT programs have, in tandem with sterilization by radiation, relied on translocation-based sex-sorting systems in which a dominant marker is translocated to the Y chromosome [24]. However, these traits are difficult to translate to new pest species, are unstable, and compromise insect productivity in mass rearing [25, 26].

The release of male insects carrying MS transgenes (“MS males”) has been shown to be effective in suppressing target pest populations in cage experiments against the mosquito *Aedes aegypti*, *C. capitata*, and the olive fly (*Bactrocera oleae*) [18, 20, 27]. However, the pest suppression potential of such a transgenic system has not yet been investigated in lepidopterans, which include many of the most destructive pests of forestry and agriculture worldwide [28]. Beyond this direct population-reducing effect, modeling suggests that releases of MS males into a target population may simultaneously provide an insecticide resistance management benefit. Mating between released males and wild females results in the survival of male transgene heterozygotes and the introgression of their background genetics into the wild pest population [29, 30]. With an insecticide-susceptible genetic background in released insects, this introgression will increase the frequency of susceptibility alleles within the target pest population.

This proposed mechanism of resistance management is analogous to that currently utilized in transgenic crops engineered to express insecticidal Cry toxin proteins from the bacterium *Bacillus thuringiensis*. A major advantage of these ‘*Bt* crops’ is their low environmental impact, with the effects of the toxin limited to target species both taxonomically (due to the high species-specificity of *Bt* toxins) and ecologically (as toxin expression is limited to crop tissue, which needs to be ingested to take effect). Cultivation of *Bt* crops, which primarily target lepidopteran and coleopteran pests, has increased rapidly over the past two decades, reaching 78.8 million hectares in 2014 [31]. Resistance in pest populations is an ongoing threat to transgenic *Bt* crop efficacy [32, 33]. The most widely applied resistance management strategy is known as high dose/refuge [34]. Here, the *Bt* toxin is expressed at sufficiently high levels for resistance to be functionally recessive and a proportion of the crop grown includes non-*Bt* varieties (the refuge). As with modeled MS-based resistance management, refugia therefore act as a source of susceptible alleles which introgress (via mating) into the pest population, reducing the frequency of *Bt*-resistant homozygotes. Although the high-dose/refuge strategy has been largely successful in delaying *Bt* resistance, the development of *Bt*-resistant populations has now been reported in the field, particularly in lepidopteran pests (reviewed in [32]). Additionally, recommendations on refuge size vary considerably depending on the cultivated species and the number of *Bt* transgenes expressed, but may be as high as 50 % of the crop, potentially exposing large areas to economic levels of damage [35]. Novel means of delaying *Bt* resistance, especially those which would function as effective pest control measures in their own right, would therefore be of economic and ecological benefit.

Models of population genetics and dynamics predict that release of MS males carrying insecticide susceptibility alleles could form effective components of insecticide resistance management (IRM) strategies for *Bt* crops [29, 30]. Such releases could substantially reduce refuge size requirements for equivalent levels of resistance management, or make refuges redundant altogether, as well as providing a potential remedial action to reverse the spread of resistance where present. Furthermore, the overall population suppression benefit of an integrated program combining *Bt* plants and MS insects is anticipated to be better than either alone. These effects are predicted at release rates significantly lower than those usually employed for population control in SIT programs.

Testing resistance management systems for *Bt* crops requires an insect that has evolved resistance to *Bt* proteins and plants that express such proteins. Diamond-back moth, *Plutella xylostella*, is a major pest of brassica crops costing an estimated US\$4–5 billion annually in losses and control costs worldwide [36]. *P. xylostella* was the first agricultural insect pest to have evolved resistance in the field to *Bt* proteins [37, 38]. Of the >500 insecticide-resistant arthropods documented, *P. xylostella* ranks second in the number of cases of resistance and is considered one of the most difficult insect pests to control [39]. Novel means of controlling this highly resistant pest are thus required [40]. Brassica plants have been developed that express *Bt* proteins [41–45] and the combination of *P. xylostella* and *Bt* brassica plants has served as an effective model system for studying resistance management of *Bt* proteins expressed in plants [46, 47]. We have developed a MS system in *P. xylostella* potentially suitable both as a novel control tool for growers, and as a model system for testing the predicted benefits of MS insect releases for managing resistance to *Bt* and other insecticides [15]. A strain of *P. xylostella* transformed with this system, called OX4319L, shows tightly controlled, highly penetrant female-specific lethality [15] and is sexually competitive against non-transgenic individuals [48]. Herein, we describe experiments that demonstrate the direct population-suppressing and *Bt* resistance management effects of releasing OX4319L males into wild-type populations.

Results

Population suppression

This experiment investigated the direct population suppression potential of the OX4319L MS transgene-carrying *P. xylostella* strain, independent of other control measures. Releases of transgene-homozygous OX4319L males into two experimental cages began 9 weeks after the initial wild-type introductions into the cages. At this point the population size, estimated by weekly consistency of egg production, in each cage was judged to have reached equilibrium (Fig. 1a). The first re-introductions of transgenic

progeny (as pupae, evidenced by positive screening of the DsRed2 fluorescent transgene marker) into the two treatment cages took place 2 weeks later (Fig. 1c), indicating successful mating by OX4319L males. The proportion of re-introduced pupae that were transgenic (fluorescence proportion) increased as releases continued into the treatment cages, eventually reaching 100 % 7 and 9 weeks after OX4319L releases began. Under the restrictive (non-tetracycline) conditions of this experiment, the transgenic (fluorescent) phenotype was restricted to a single genotype (male heterozygotes) as female transgene carriers are unable to survive to adulthood [15]. Transgenic males were therefore only able to mate with wild-type females and the fluorescence proportions recorded here are equal to twice the transgene allele frequency in the population [48]. The increasing introgression of MS transgenes into these treatment populations had a substantial effect on the population sex ratio and reproductive capacity. By week 15 (6 weeks after OX4319L releases began) the number of dead adult females collected in each of the treatment cages had decreased considerably relative to that of control cages (Fig. 1b), concurrent with a reduction in the reproductive output in these cages (Fig. 1a). As the generation time (egg to egg) of the insects in these experiments was approximately 3 weeks, these time periods (between initiation of MS male releases and suppression, approximately two generations) fit the hypothesis that introductions of the OX4319L transgene into the treatment populations were causing reductions in the number of females reaching adulthood, and thus the number of eggs being laid in the subsequent generation. Ten weeks after OX4319L releases began (week 19) the reproductive output of both treatment cages had dropped to 0, and no dead female moths were collected after this point. In this experimental protocol this equates to approximately three generations. Experiments were continued for another 2 weeks after egg-laying ceased, confirming that their populations were extinct.

Insecticide resistance management

This experiment investigated the predicted benefits to both resistance management and population control of combining *Bt* transgenic crops and MS males with *Bt*-susceptible genetic backgrounds. To achieve this, treatments were designed such that each pest control method on its own (*Bt* only and OX4319L release at low levels) would likely be insufficient for population control. The effect of the combination of treatments could then be compared with that of each one applied singly. In the *Bt* only treatment, the presence of resistance alleles in the founder population, and strong selection for these alleles in subsequent generations, were expected to result in a

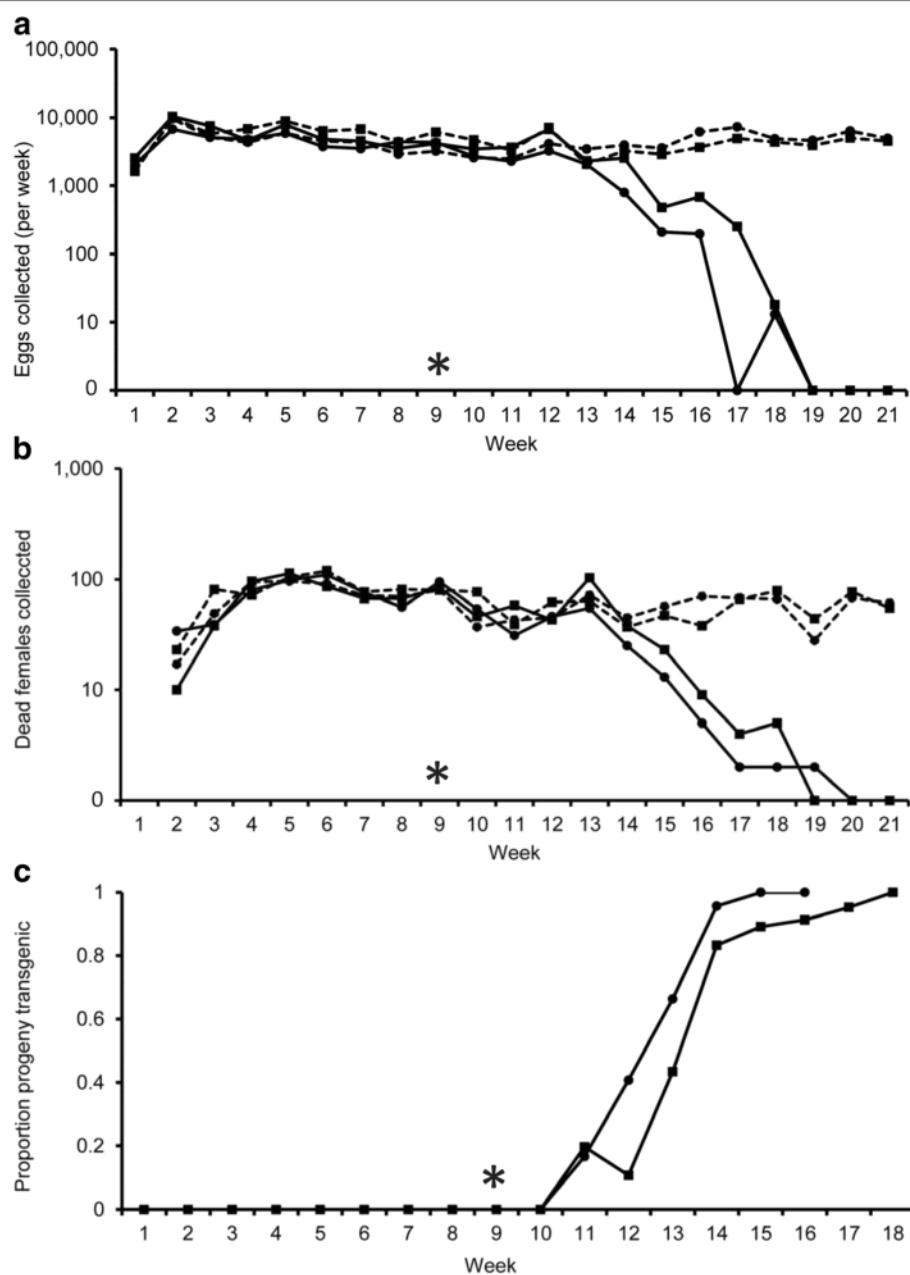


Fig. 1 Suppression of caged populations of *Plutella xylostella* by weekly introduction of OX4319L males. Graphs showing (a) number of eggs collected, (b) number of dead adult females collected weekly from cages, and (c) proportion of cage progeny that were transgenic re-entering the cages (fluorescence proportions), over the experimental period. With female moths present in the cages being only wild-type, transgenic progeny (including those re-introduced) were heterozygotes (restrictive conditions). Fluorescence proportions thus equate to twice the MS transgene allele frequency in the cage population at that time point. Solid lines represent OX4319L-treated populations (Cages 1 and 2, circular and square data-points, respectively). Dashed lines represent untreated control populations (Cages 3 and 4, circular and square data-points, respectively). In week 9, return of pupae into treatment and control cages was made proportional and release of OX4319L males into treatment cages began (marked with asterisk)

lack of effective control. In the low release rate OX4319L-only treatment, this was achieved by selecting a release rate predicted to be unable to prevent population growth under these conditions. Under this design, all treatment populations would persist for the duration of the

experimental period, allowing comparison of their population densities and resistance allele frequencies after multiple generations of treatment effects. In addition, a treatment where OX4319L males were released at a high rate (in the absence of *Bt*) was also conducted to act as an

investigation of this strain's suppression potential under more challenging conditions where the target pest population may be expanding rapidly.

In cages with *Bt* broccoli and no OX4319L releases, *P. xylostella* populations were well-controlled in Generation 1 (Fig. 2a), presumably due to the initially high frequency of genotypes susceptible to the *Bt* toxin. The

subsequent populations in these cages, now highly *Bt*-resistant due to the effects of strong selection in the initial generation, then increased rapidly until Generation 4. Similarly, as expected, low-level releases of OX4319L males into *P. xylostella* populations reared on non-*Bt* broccoli were not effective at preventing population growth, and this treatment was terminated at Generation

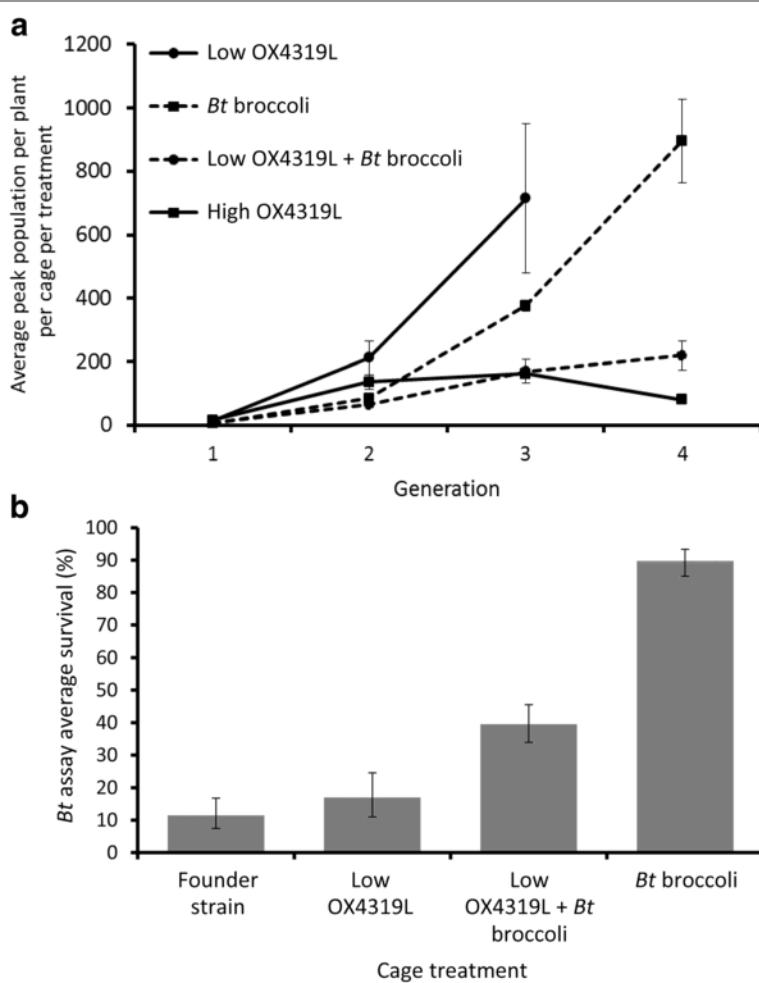


Fig. 2 Effects of *Bt* broccoli and OX4319L releases on caged *Plutella xylostella* populations over multiple generations. Caged populations were established with hybrid *Bt*-resistant/wild-type insects – the founder strain – with a low estimated frequency of homozygous-resistant individuals. **a** Graph shows the mean peak population size per plant, per generation, in four experimental treatments over the experimental period: Treatment 1, *Bt* broccoli, no OX4319L releases; Treatment 2, *Bt* broccoli, low-rate weekly OX4319L releases (release rate of 3:1 in Generation 1, increased to 5:1 in subsequent generations); Treatment 3, non-*Bt* broccoli, low-rate weekly OX4319L releases (identical release rates to Treatment 2); and Treatment 4, non-*Bt* broccoli, high-rate weekly OX4319L releases (release rate of 20:1 in Generation 1, increased to 40:1 in subsequent generations). Means were calculated from three experimental cage replicates, with the exception of Treatments 2 and 4, which were reduced to two and one cage replicates in Generation 3, respectively. Treatment 3 cages were terminated in Generation 3 as the insect populations had reached maximum capacity. Error bars represent standard error of the mean. **b** *Bt* survival assays. Mean survival of third-instar larvae from three experimental cage treatments and the founder strain used to begin these experimental treatments when exposed to a discriminating dose of *Bt* in artificial diet assays. *Bt* dose in this assay is high enough to ensure that only homozygous *Bt*-resistant individuals will survive (as in the high-dose/refuge strategy). This assay therefore indicates the proportion of each population remaining *Bt*-resistant (homozygous) and *Bt*-susceptible (heterozygous or homozygous-susceptible). For each cage, two *Bt* assays and one no-*Bt* control assay were performed. *Bt* assays in each cage were summed and means represent averages of each set of treatment cages corrected for control mortality. The assays took place using individuals from the final generation in which each treatment was run or, in the case of the founder strain, in the generation prior to the start of the experiment. Survival was corrected for control mortality prior to analysis and error bars represent Pearson's exact confidence intervals. Survival of insects from the low OX4319L release cages (Treatment 2) was not significantly different from the founder strain; other pairwise comparisons are significantly different (Table 1)

3. When *Bt* broccoli and low OX4319L releases were combined, however, the caged populations were well-controlled throughout, only increasing slowly at each generation. In Generation 3, the mean peak population counts recorded for the *Bt* broccoli-only and the low-rate OX4319L-only treatments did not differ significantly (Contrast 1). Concurrently, the mean peak population counts in the combined OX4319L + *Bt* broccoli treatment were significantly lower than those of the low-rate OX4319L-only treatment (Contrast 2), but not significantly different to that of the *Bt* broccoli-only treatment (Contrast 3) (Contrast 1: diff = 337, $P = 0.133$; Contrast 2: diff = -545, $P = 0.0272$; Contrast 3: diff = -207, $P = 0.315$). By Generation 4, however, populations in the *Bt* broccoli-only treatment were significantly larger than those where *Bt* broccoli was combined with low-rate OX4319L releases ($t = -4.84$, $P = 0.0084$). The high rate of OX4319L releases performed similarly to the combined OX4319L + *Bt*-broccoli treatment, but by Generation 4 had started to reduce the population in the last remaining cage. Due to the lack of replication in this treatment in Generation 4, it was excluded from statistical analysis.

Larval *Bt* survival assays conducted in the last generation of each treatment, and on the founder strain in Generation 0, showed a significantly reduced proportion of resistant individuals in treatments that combined *Bt* broccoli and low-rate OX4319L releases compared to those where *Bt* broccoli was used alone (Fig. 2b). Survival rates on *Bt* were low and not significantly different between the founder strain (11.5 %; CI, 7.4–16.8 %) and the low-rate OX4319L-only caged populations (17 %; CI, 11–24.5 %; Table 1). Both treatments in which larvae were exposed to *Bt* selection (Treatments 1 and 2) displayed significantly higher levels of survival on *Bt* compared to those where non-*Bt* broccoli plants were used, indicating high levels of selection against susceptible genotypes in these cages. However, significant differences between treatments with *Bt* plants were apparent. In the *Bt* broccoli-only treatment, *Bt* resistance rapidly increased in frequency in the population, as indicated by the high percentage survival rate (89.7 %; CI, 85–93.3 %). In the combined OX4319L and *Bt* broccoli treatment,

the survival rate was significantly lower (39.5 %; CI, 33.9–45.5 %), implying a significantly higher frequency of *Bt*-susceptible alleles (and thus individuals subject to *Bt* selection pressure) in the populations where low-level releases of OX4319L males had taken place.

Allele frequencies derived from these mean survival assay results indicated that the *Bt* resistance allele had increased in frequency over the experimental period in populations under *Bt* selection, from 0.36 in Generation 0 (founder strain) to an estimated 1.0 in the *Bt* broccoli treatment and 0.71 in the combined OX4319L and *Bt* broccoli treatment (both Generation 4). Fluorescence proportions determined for treatments in which OX4319L males were introduced estimated that 56.0 % (CI, 50.4–61.5 %) of the combined OX4319L and *Bt* broccoli treatment populations (Generation 4); 55.6 % (CI, 47.2–63.7 %) of the low OX4319L treatment populations (Generation 3); and 87.5 % of the high release rate OX4319L treatment population (Generation 3 – single replicate) carried the MS transgene. As in the population suppression experiment, these fluorescence proportions are equal to twice the MS transgene allele frequency in these populations [48].

Discussion

We found that sustained releases of male *P. xylostella* moths carrying a transgene which allows only males to survive to adulthood can have a direct suppressing effect on a target pest population, as previously shown for dipteran pests [18, 20, 27]. We also demonstrate that releases of such insects can significantly delay the spread of resistance to *Bt* in target pest populations by introgression of susceptibility alleles through male progeny, even when initial resistance levels are high. With the increasing number of cases of field resistance to *Bt* plants, transgenic systems such as the one examined here may play an important role in IRM programs aimed at delaying or overcoming the evolution of resistance to this valuable technology.

In stable target populations reared on artificial diet and an initial OX4319L over-flooding ratio of approximately 10:1, reduced numbers of females and suppression of the populations' reproductive capacity became evident approximately two generations after OX4319L

Table 1 Pairwise comparisons of survival assay data (from experiments described in Fig. 2b)

Comparison		Z value	P value
Founder strain	Low OX4319L	1.212	0.605
Founder strain	Low OX4319L + <i>Bt</i> broccoli	5.016	<0.001
Founder strain	<i>Bt</i> broccoli	8.557	<0.001
Low OX4319L	Low OX4319L + <i>Bt</i> broccoli	3.323	0.0048
Low OX4319L	<i>Bt</i> broccoli	-6.682	<0.001
Low OX4319L + <i>Bt</i> broccoli	<i>Bt</i> broccoli	-5.594	<0.001

Output was generated using an omnibus logit model for categorical data analysis, followed by post-hoc analysis using Generalized Linear Hypothesis Testing

male releases were initiated and these populations fell rapidly to extinction thereafter (after approximately three generations). This timeframe until extinction compares well with previous cage experiments exploring the pest suppression potential of female-lethal transgenes in *A. aegypti* (4.66 ± 0.88 mean generations until extinction; 8.5–10:1 over-flooding rate) [27], *B. oleae* (3 ± 0.0 ; 10:1) [18], and *C. capitata* (3 ± 0.0 ; 10:1) [20]. With such MS strains applied against isolated populations, as reported here, local eradication of the target pest is achievable as the over-flooding ratio increases as the target population falls. This effect would be lessened when applied in the field against non-isolated pest populations or when pests migrate between areas. However, even though *P. xylostella* is known to travel large distances in some circumstances [49–52], it does not typically travel far when host plants are available [53, 54]. Although complete isolation of *P. xylostella* and other pest species would be unlikely in most scenarios, efficient control of target populations is likely achievable with some degree of population isolation and/or with sufficient scale of application to reduce edge effects. Moreover, the SIT has previously demonstrated efficient control of other lepidopteran pests – codling moth (*Cydia pomonella*) and pink bollworm (*Pectinophora gossypiella*) – that are able to travel relatively large distances [55, 56].

In our experiments on broccoli, target populations were free to expand in protected environments devoid of limiting factors such as predators and adverse climatic effects. Here, OX4319L male release, with a high over-flooding ratio of up to 40:1, maintained a high level of control. As expected, low rates of OX4319L male releases alone – over-flooding ratio of up to 5:1 – did not prevent rapid population growth under these conditions. Whether a management strategy is able to prevent the expansion of a pest population is a function of that strategy's ability to counteract the reproductive rate (R_0) of the target species [57]. In the case of the lower rate OX4319L treatment, the proportion of matings won by these released insects was predicted to be low enough that the population's reproductive capacity would remain well above 1. Pests with a high R_0 , such as Lepidoptera, can represent a challenge to mating-based pest management approaches, and in some cases higher release rates may be required to counter this. However, in a controlled greenhouse setting, R_0 is artificially elevated, while in the field, mortality rates due to parasitism, predation, pathogen infection and environmental factors, especially in pre-adult stages, are likely to be high [40]; it is possible that these factors would reduce the release rate of OX4319L males required to control a target population, relative to release rates used in these experiments.

Populations maintained on *Bt* broccoli alone expanded rapidly after strong selection for *Bt* resistance. However,

low rates of OX4319L male release in combination with *Bt* broccoli significantly reduced population growth, controlling the target populations well when each treatment on its own failed to do so. This apparent synergistic effect supports model predictions that introgression of susceptibility alleles into a target population by released transgenic males could prevent or reverse the spread of resistance, thereby preserving susceptibility to *Bt* toxins [29, 30].

Consistent with the hypothesis underlying these models, results from our larval *Bt* survival assays showed significantly higher survival (frequencies of resistant homozygotes and, consequently, resistance alleles) in the *Bt* broccoli-only treatment compared with cages where low levels of OX4319L males were introduced in addition to *Bt* plants. Mean fluorescent protein marker frequencies in these combined OX4319L + *Bt* broccoli cages (56.0 %) displayed a high level of agreement with the proportions of these populations which were *Bt*-susceptible, once survival assay results were calibrated for homozygous-resistant mortality ($100 - 43.7 = 56.3$ %). These results are what would be expected if mating by released MS males were the source of *Bt*-susceptibility alleles in these populations, diluting the frequency of resistant (homozygous) genotypes and counteracting *Bt* resistance at a rate predicted by their mating success. Under this hypothesis, no such agreement would be expected if resistance alleles were not undergoing positive selection in the target population, as pre-existing susceptibility alleles would persist independent of those introgressed by MS males. Supporting this argument, populations treated with low-dose OX4319L releases, which received identical release rates of transgenic males to the low OX4319L + *Bt* broccoli treatment (resulting in a very similar population-level fluorescence ratio, 55.6 %) but were not exposed to *Bt* selection, showed a level of *Bt* susceptibility independent of their fluorescence proportions (mean proportion of individuals not surviving *Bt* assay = $100 - 18.4 = 81.6$ %). Taken together, these results provide an empirical demonstration of the mechanism by which MS transgenic insects carrying insecticide-susceptible genetic backgrounds can maintain insecticide efficacy, even in the face of continuous, strong selection for resistance.

These resistance management effects were explored using a well-established model system utilized for investigating IRM strategies for *Bt* crops and risks to non-target organisms [58–60]. Thus, although *Bt* crucifers are not yet commercially cultivated, the mechanism we demonstrate is broadly relevant to *Bt* crops. In addition, depending on the nature and inheritance of resistance, similar effects would be expected for *Bt* sprays or indeed any other insecticidal intervention against *P. xylostella* and other pests. The efficiency of a transgenic strain in providing these effects will likely depend on performance of its males in the field. Previous studies on

OX4319L indicate that this transgene insertion is associated with a small fitness cost in the laboratory [48], but its field performance has not yet been tested.

Theoretical population modeling studies have investigated the release of fertile non-transgenic insecticide-susceptible insects into *Bt* crop areas [29]. At present, the only feasible option for achieving sex separation in lepidopterans, at the scales required for release, is the MS system described herein, so in practice non-transgenic releases would comprise both males and females. In these models, introgression of susceptibility alleles from such releases also provided a degree of resistance management. However, released fertile females would lead to higher pest populations than MS releases, at least initially, and may necessitate increased insecticide usage in neighboring conventionally sprayed areas. For these reasons, an IRM program involving releases of fertile pest females would likely be unacceptable to growers and regulators.

The release of radiation-sterilized *P. gossypiella* has also been described as preventing development of resistance to *Bt*-expressing cotton [61]. However, these two systems (SIT and MS) provide fundamentally different ways of managing resistance. Whereas SIT may prevent increasing resistance by killing resistant insects before they have a chance to reproduce (any alternative population-suppression method would have the same effect), the MS phenotype additionally manages resistance by actively introgressing susceptibility alleles into the target population through the survival of male progeny. In areas of 100 % *Bt* crop cultivation these effects on resistance management may be similar, albeit with these benefits likely to be provided at lower release rates using MS males [22, 23]. However, if combined into existing *Bt*-IRM programs, MS releases may also be advantageous to the maintenance of susceptible populations within refuges as male MS heterozygotes within these areas will survive and continue to pass on susceptibility alleles in subsequent generations. Although some suppression of these refuge areas is expected under these conditions, our results demonstrate that, in the absence of other control measures such as *Bt*, low-level releases of MS males will have a minimal effect on population growth. Moreover, this mechanism may be of benefit to growers utilizing sprayed insecticides as introgressed susceptibility alleles will be spread through the natural mating behavior of the pest population between insecticide sprays. Future modeling studies may help to elucidate the relative benefits of these two potential alternatives to refuges in combating resistance to *Bt*.

Conclusions

The results described herein demonstrate that releases of MS transgene-carrying, insecticide-susceptible insects can provide two simultaneous effects on pest populations – direct population suppression through death of

female progeny and IRM by introgression of susceptibility alleles into target populations through surviving male progeny. Further laboratory experiments are required in order to disentangle the relative contributions of these two effects in suppressing pest populations on *Bt* plants. Nonetheless, our findings strengthen the argument that MS systems, such as OX4319L, could function as effective stand-alone tools against given pest species or as a highly compatible component of an integrated pest management program. If used as an alternative to broad-spectrum insecticides within such an IPM framework, the species-specific action of MS transgenes would reduce negative effects on non-target organisms including natural enemies which can further delay the evolution of resistance to *Bt* crops [60, 62, 63]. For successful IPM programs against *P. xylostella*, conservation of these natural enemies is important [40] and can provide more effective control than the use of broad-spectrum insecticidal sprays [64]. These conservation benefits are also likely to apply to other groups such as pollinators and birds which are currently threatened by the use of some agricultural insecticides [65].

Existing recommendations on minimum refuge size in some *Bt* crops, and compliance with these recommendations, may be inadequate for robust resistance management [32, 35]. MS insects carrying *Bt*-susceptible genetic backgrounds could help to reduce refuge requirements, or potentially replace or supplement refugia as a resistance management tool in *Bt* crops [29, 30]. Area-wide releases of transgenic MS insects in place of refugia could offer area-wide protection to growers and seed producers, for whom such compliance can be expensive and impractical. In addition, our results demonstrate that use of MS systems in combination with other pest management methods would reduce the required number of insects to be produced and released to achieve a given level of control.

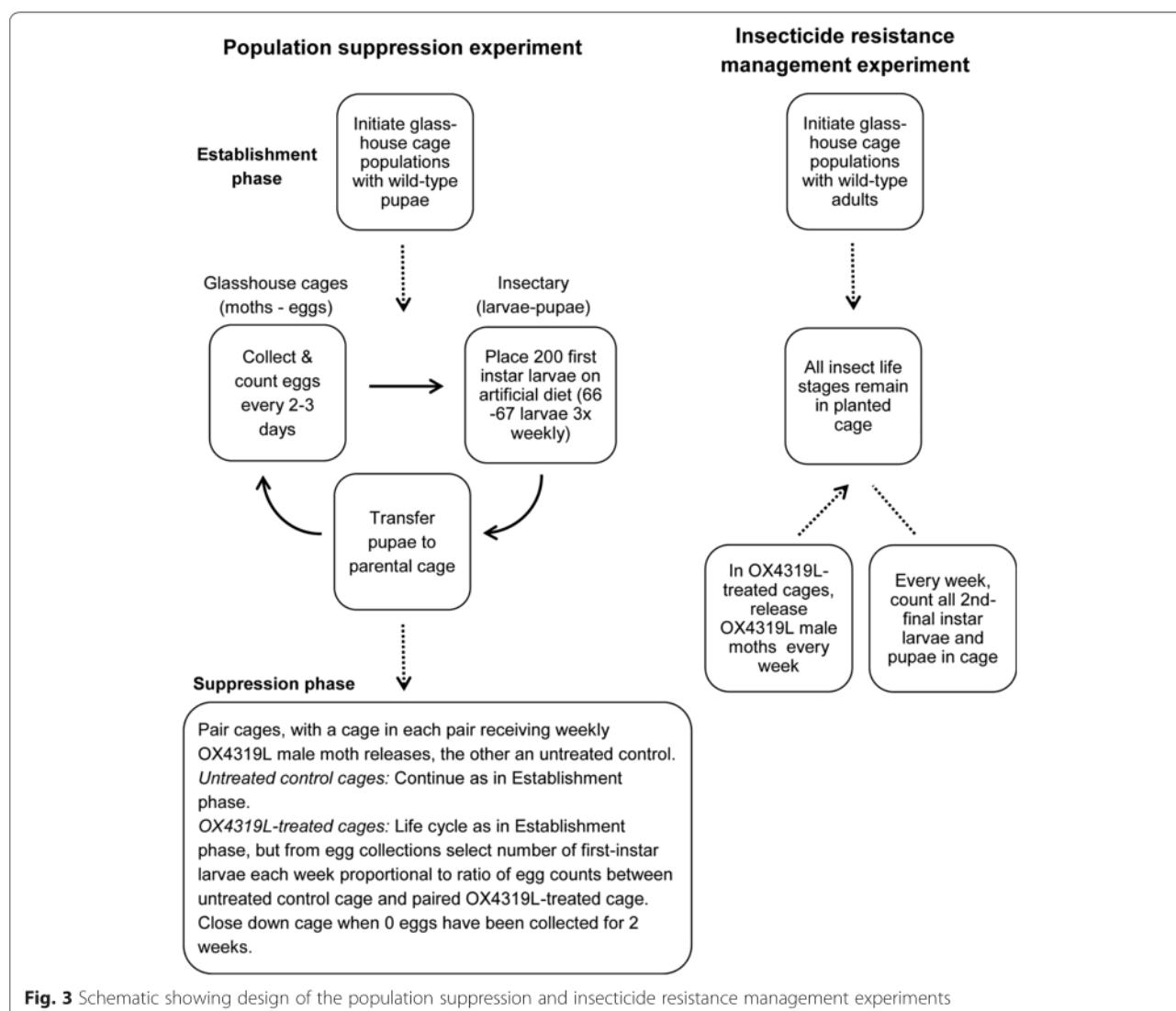
The utility of the MS system has been demonstrated here in a lepidopteran pest and elsewhere in dipteran pests [15, 16, 18, 20, 66], so further development in pests in these and other taxonomic groups appears feasible and should be evaluated. This technology represents a promising and sustainable pest management tool offering significant benefits to future IPM strategies.

Methods

A summary of the two experimental designs used is provided in Fig. 3. More detailed explanations of these designs and their aims are provided below.

Summary of experimental designs

The glasshouse cage population suppression experiment was designed to simulate a field scenario in which biotic and abiotic mortality factors maintain a stable pest



population, with adult moths mating and laying eggs in glasshouse cages, and larval rearing being conducted in a separate temperature-controlled room [18, 20, 27]. When weekly OX4319L male moth releases started, OX4319L-treated cages were paired with an untreated counterpart (control cage) and the number of pupae subsequently reintroduced into each treatment cage was made proportional to the ratio of eggs counted between the treatment cage and its paired control cage: this allowed the expected reduced number of females in OX4319L-treated cages to be reflected in reduced numbers of pupae entering the population in the next generation.

In contrast, the subsequent glasshouse cage IRM experiment was conducted on broccoli plants – some on *Bt* broccoli – as they offered a realistic model for many agricultural systems with transgenic crops. Artificially maintaining stable populations on plants, by accurately manipulating the number of progeny surviving, was

deemed impractical, and the populations were therefore allowed to expand freely in the near-absence of biotic and abiotic mortality factors, with all life stages of each experimental population residing in their respective cage. This second experiment provided a more rigorous test of the pest management potential of the OX4319L strain as the intrinsic growth rate of target populations was not artificially controlled and competitors from these target populations had the advantage of being reared on host plant material, as opposed to an artificial diet medium.

Experimental population structures

Generations in the population suppression experiment were continuous while those in the resistance management experiment were discrete, at least within the experimental period. In the population suppression experiment a continuous generational structure was achieved by

introducing pupae into cages three times per week. As the lifecycle of *P. xylostella* in this experiment was 3 weeks, this created a stable, mixed-age adult population with eggs being collected every 2–3 days over the experimental period. In the resistance management experiment conducted on broccoli, populations were founded by a single introduction of adult moths. Adults in this founding generation, and those in subsequent generations, were allowed to mate freely in their respective cages with females from the same generation ovipositing over a number of days. As such, within the experimental period generations remained discrete and were discernable by peaks in weekly population counts which, as expected, reached a maximum level once per generation (approximately every 3 weeks; see below). Generations did, however, begin to overlap over time.

The generation time of *P. xylostella* in the insecticide resistance experiment was approximately 20–23 days (incrementally: egg, 3–4 days; larvae, 13–14 days; pupae, 3–4 days; adult time to sexual maturity, 1 day). As experiments were conducted in a glasshouse, these time periods varied depending on outdoor weather conditions.

Population suppression experiment

Experiments were conducted in quarantine facilities at Rothamsted Research, Hertfordshire, UK in accordance with legislation concerning the contained use of GM organisms in the UK. Larval rearing took place in a temperature-controlled room and experimental cages were housed within a temperature-controlled glasshouse (both 25 °C and 16:8 light:dark photoperiod). As an efficient, easily replicable proxy for host crop infestation, larval rearing was conducted on artificial diet medium and followed the methods of Martins et al. [67]. All experimental populations were reared on non-tetracycline diet. Transgene-homozygous OX4319L males were produced on diet with chlortetracycline hydrochloride (100 µg/mL) and sexed manually prior to introduction. Experimental cages measured 120 × 100 × 120 cm (width × depth × height), with a zipped entrance at the front. Insects were added to the cages as pupae.

The experiment comprised two phases: establishment and suppression. During establishment, stable mixed-age populations of wild-type *P. xylostella* (wild-type background strain to OX4319L isolated in Vero Beach, Florida, USA) were established in each of the four cages. During suppression, weekly introductions of transgene-homozygous OX4319L males were made into two of the cages to investigate whether engineered female-specific lethality resulted in suppression of these populations. Throughout the experiment, cabbage-extract-baited Parafilm (Bemis Company Inc., Oshkosh WI, USA) pieces were hung from the roof of the cage to act as an artificial leaf oviposition substrate. These were replaced

three times per week (Monday, Wednesday, and Friday) and eggs collected on each sheet were counted. During each egg collection, dead adult moths and unclosed pupae were also collected from the cages, sexed, and counted. Enclosed adults were provided with sugar water-saturated cotton wool reservoirs, changed every 2–3 days.

Establishment phase

Cage populations were initiated by placing 200 unsexed wild-type pupae into each cage. Stable non-expanding populations were maintained in each cage to mimic the stabilizing effects of predation and other limiting factors in the wild. This was achieved by introducing a constant number of pupae back into the cages each week. A total of 200 first-instar larvae were selected each week to carry on the population, taken from the three weekly collections (67 larvae chosen from Monday collection, 67 from Wednesday, and 66 from Friday). These larvae were reared in plastic beakers on non-tetracycline diet, and after pupation were sexed and transferred back into the cage from which they had been collected as eggs, 2 weeks earlier. Tri-weekly egg collections (and subsequent tri-weekly pupal reintroductions) maintained stable, mixed-age populations as might be expected in the field, where adult moths would be continuously entering the population. The first two introductions of wild-type moths into each cage (weeks 1 and 2) originated from an independent laboratory colony. From this point onwards each cage population was self-sustaining.

Suppression phase

Once egg counts had stabilized (indicating stable populations) in week 9, two of the four cages were chosen as OX4319L treatment cages (Cages 2 and 4) and two as control cages (Cages 1 and 3). Cages were designated in a blocked design to minimize bias caused by uncontrolled abiotic factors. In non-treatment control cages, the protocol from the establishment phase was continued. Each OX4319L treatment cage was randomly paired with a control cage for the remainder of the experiment (Cages 1 and 2; Cages 3 and 4). The reintroduction of pupae into each OX4319L treatment cage followed the same protocol as for the establishment phase; however, the total number of early-instar larvae selected for rearing that week was made proportional to the ratio between the number of eggs collected for that treatment cage and its paired control cage for the week when these larvae were collected as eggs. This method ensured that the population suppression effect of female death of transgenic larvae, reflected later by reduced number of eggs collected, resulted in reduced numbers of pupae re-entering the OX4319L-treated cages relative to an untreated population.

After cages had been paired, weekly introductions of homozygous OX4319L males into the treatment cages began. The target over-flooding ratio (OX4319L to wild-type males entering the population) was set at 10:1. This release rate is in line with previous studies investigating the effect of female-lethal transgenes on caged populations [18, 20, 27] and much lower than the sterile:wild ratios that successful SIT programs have aimed to achieve against other Lepidoptera: *P. gossypiella* (60:1) [68]; *C. pomonella* (40:1) [69]; and painted apple moth, *Orgyia anartoides* (100:1) [70, 71]. This over-flooding ratio was calculated as 10× the mean male recruitment rate for the 3 weeks preceding OX4319L male introduction, and the numbers released were held constant for the remainder of the experiment (Cage 2: 990 OX4319L males; Cage 4: 980 OX4319L males). Males were released as pupae into the cages once per week (Wednesday).

Pupae reintroduced into treatment cages were screened for the DsRed2 fluorescent protein transformation marker and fluorescence proportions (proportion of the population which carried the transgene) recorded. Under the restrictive conditions of this experiment and the highly penetrant female-lethal phenotype of OX4319L [15], population-level transgene allele frequencies were equal to half the fluorescence proportion [48]. Each treatment cage's end-point – extinction of each population – was pre-defined as two consecutive weeks of zero eggs collected.

Insecticide resistance management (IRM) experiment

Experiments were conducted at Cornell University, New York State Agricultural Experiment Station, Geneva, NY, in accordance with legislation concerning the contained use of GM organisms in the USA. All *P. xylostella* experimental populations in this experiment were reared on broccoli plants and allowed to expand freely. Eleven cages, each 1.83 m × 0.91 m × 1.83 m (length × width × height), were placed in a temperature-controlled glasshouse with supplementary lights (23–27 °C, 16:8h light:dark photoperiod, and uncontrolled relative humidity). The 11 cages were assigned to four treatments: Treatment 1, *Bt* broccoli plants, no OX4319L release; Treatment 2, *Bt* broccoli plants, low-rate weekly OX4319L releases; Treatment 3, wild-type plants, low-rate weekly OX4319L releases; and Treatment 4, wild-type plants, high-rate weekly OX4319L releases. Treatments 1–3 were assigned three cages (replicates) while Treatment 4 was assigned two cages.

Experimental *Plutella xylostella* strains

Two strains were used: a hybrid *Bt*-resistant/wild-type strain used to generate starting populations in the cages (founder strain) and OX4319L. To generate the founder strain, 25 males from a homozygous Cry1Ac

Bt toxin-resistant stock colony (showing recessive, monogenic resistance to Cry1Ac toxins) [58] were crossed to 25 females from the homozygous-susceptible 'Geneva 88' colony; 59 of the F1 male progeny from this cross were then mated to 100 females from the Geneva 88 strain. A total of 250 males and 250 females from this cross were then mated to produce the founder strain. These crosses provide an expected resistance allele frequency of 0.25 and an expected homozygous-resistant frequency of 0.0625. At all stages of founder strain production and maintenance, progeny were reared on wild-type broccoli plants in large numbers (>500 pupae collected per generation) to minimize the effects of inbreeding and genetic drift. However, *Bt* survival assay results (Fig. 2b) suggest that this frequency had increased to 0.36 ($r = \sqrt{0.1265}$) by the start of the experiment.

Experimental broccoli cultivars

Two strains of broccoli (*Brassica oleracea* L.) plants were used: a wild-type cultivar (Packman) and a transgenic strain engineered to express high levels of the *Bt* toxin Cry1-Ac [41]. Together, this transgenic plant cultivar and the Cry1-Ac-resistant *P. xylostella* strain comprise a well-established model system used to study the dynamics of transgenic crops and their resistance management [58, 59]. Cry1-Ac toxin production was verified by screening the plants (4–5 weeks old) with susceptible Geneva 88 strain neonates. Leaf assays of transgenic plants also killed 100 % of OX4319L/Cry1-Ac hybrids, indicating high levels of *Bt* toxin expression.

Restocking of plant material

All cages started with 20 broccoli plants of their respective cultivar. Plants were replaced after 4 weeks, or when defoliated due to larval feeding, by cutting them at their base and placing them on the replacement plants to allow larvae to transfer. If moth populations grew beyond the capacity of the maximum food supply (estimated by exceeding their cage's plant material within one generation), the cage was terminated.

Establishment of *P. xylostella* populations in cages

In all treatments, replicates were initiated by the release of *P. xylostella* adults from the founder strain into the cages. In treatments involving *Bt* plants, 200 randomly selected adults were released. In treatments involving non-*Bt* plants, seven males and females (total 14 adults) were released into the cages. Due to *Bt* selection, this gave approximately equal starting population densities in Generation 1 in all cages.

Male-selecting transgenic insect treatments

OX4319L-homozygous males for release into the cages were produced by rearing egg collections from a stock

colony in the absence of tetracycline in larval feed. Releases in this experiment were proportional: daily estimates of adult male recruitment for each cage were used to calculate a daily male release number for that cage, dependent on the release rate pre-determined for that treatment. A proportional release rate, rather than a constant number, was applied to reduce the likelihood of population extinction and thereby allow exploration of the effect of MS transgene releases on population dynamics and resistance allele frequency.

Release rates were selected in advance based on the outcomes of predictive deterministic models, investigating the effects of each experimental treatment on population size.

In Treatments 2 and 3 (low-OX4319L release treatments), the intended over-flooding ratio was 5:1 (transgenic: wild-type males). However, due to insect rearing limitations this was limited to 3:1 in Generation 1, and increased to 5:1 thereafter. These low release rates, in combination with *Bt* plants, were predicted to maintain a relatively constant population size, but insufficient to cause population suppression when used alone. Similarly, the over-flooding rates in cages with high-OX4319L releases, initially 20:1 and increased to 40:1 in Generation 2 as production capacity increased, were predicted to be sufficient for population suppression when used alone.

Where small populations are expanding in the absence of limiting biotic or abiotic factors, stochastic effects make it difficult to accurately predict the rate of population increase. In response to higher-than-predicted population growth and limited plants available, the cages with *Bt* broccoli plants and low-rate weekly OX4319L releases were reduced to two replicates in Generation 3 (terminated cage selected at random). In Generation 4, the number of plants in each treatment with *Bt* broccoli only (no OX4319L releases) cage was reduced to five (while maintaining per-plant population density levels) in response to limited plant availability. This was achieved by randomly harvesting 25 % of the leaves on each plant within the cage, removing all insect and plant material from the cage, and then restocking the cage with five new plants and returning harvested leaves. As generations were still discrete, this manipulation was timed during a period when the vast majority of insects in the population were present as larvae or pupae on plants, allowing accurate culling of the population. In Generation 3, requirements for OX4319L male moths exceeded production capacity, so the treatment with high-rate weekly OX4319L releases was reduced to one replicate only.

Data collection

Population size estimation

The numbers of second to fourth instar larvae and pupae on each plant in each cage were counted once per week.

Bt resistance assays

With the exception of cages with high-rate OX4319L releases, *Bt* resistance data were collected from all cages in their final generation. As each cage reached maximum egg-laying potential (judged by female recruitment data in each population, collected from eclosion cages), eggs were collected from 8–10 leaves selected at random from each cage. These eggs were transferred to filter paper by paintbrush and resulting larvae were reared on chlortetracycline hydrochloride-augmented artificial diet (100 µg/mL). For each cage, two replicated *Bt* survival assays and one control assay were performed. For *Bt* assays, chlortetracycline hydrochloride-augmented diet was poured into 30 mL plastic pots and 500 µL of 10 ppm *Bt* (Dipel®, Valent BioSciences Corp., Libertyville, IL) – shown previously to discriminate between homozygous-resistant and other genotypes [46] – pipetted onto the dried surface. Non-*Bt* controls were prepared in the same way, with no added *Bt* solution. At third instar, larvae were transferred onto the air-dried diet surface. Exact numbers of larvae per replicate differed between cages according to availability of eggs. A minimum of 33 larvae per pot were used for the first *Bt* replicate; 11 per pot for the second *Bt* replicate; and all control replicates contained 20 larvae. Mortality was assessed 72 h later with surviving larvae taken to be homozygous for the resistance allele. For comparison, the founder strain was subjected to the same assay, in the generation prior to experimental initiation.

Bt resistance allele frequency estimation

Bt assay survival data from the founder strain, *Bt* broccoli alone, and low OX4319L + *Bt* broccoli treatments were used to calculate frequency of the *Bt* resistance allele, as conducted in previous studies utilizing this model system [46, 47, 58]. Under strong *Bt* selection (preliminary assays showed 100 % *Bt* susceptibility in homozygous susceptible and OX4319L/resistant heterozygotes) in the absence of resistance management efforts (as in the *Bt* broccoli-only treatment) it was expected that the *Bt* resistance allele would reach fixation from Generation 1 onwards (all individuals homozygous resistant). Deviation from 100 % *Bt* assay survival in this treatment thus represents mortality to a small proportion of homozygous-resistant individuals under these conditions. This reduction in survival was used to calibrate the results of the assay for other treatments prior to calculation of allele frequencies. Once calibrated, the square of the proportion surviving gave an estimate of the *Bt* allele frequency in the founder strain (under Hardy-Weinberg equilibrium). In the low OX4319L + *Bt* broccoli treatment, the calibrated survival rate represents the proportion of homozygous resistant individuals in the population, with the remainder of (non-surviving) individuals assumed to be the (heterozygous) offspring

of resistant females and OX4319L males (as the only source of susceptibility alleles in these populations). The validity of this assumption was explored by comparing the percentage of non-surviving individuals with DsRed2 fluorescence proportions. If the mechanism of resistance management proposed in this experiment were functioning as predicted (released MS males providing susceptibility alleles through mating, maintaining *Bt* efficacy), we would expect to see high levels of agreement between population-level *Bt* susceptibility and fluorescence proportions in the low OX4319L + *Bt* broccoli treatment (where OX4319L males were hypothesized to be the sole providers of susceptibility alleles) but no such agreement in the low OX4319L only treatment populations, which received identical doses of MS transgenic males (and thus would be expected to show similar fluorescence proportions to the combined OX4319L + *Bt* treatment) but where *Bt* resistance alleles were not under selection. The *Bt* resistance allele frequency in the low OX4319L + *Bt* broccoli treatment was calculated as $[X + 0.5(1-X)]$, where X is the calibrated survival rate.

Fluorescence proportion assays

The population-level fluorescence proportion (proportion of the population carrying the transgene) was estimated for all cages into which OX4319L males were released, in the final generations they were conducted. The exception to this was the high OX4319L release treatment where this assay was conducted in the penultimate (third) generation due to the small population size of this treatment cage in Generation 4. Collection of eggs followed procedures described for *Bt* resistance assays with larvae being reared to pupation on chlortetracycline hydrochloride-augmented artificial diet (100 µg/mL) in 500 mL Styrofoam pots. At pupation, individuals were screened for presence/absence of the DsRed2 fluorescent protein marker. Each cage acted as a replicate with a minimum of 75 larvae per assay pot.

Male recruitment rate estimation

For those cages requiring OX4319L male introductions, daily male recruitment rate was assessed by the removal of four randomly selected plants/cage/week. These were placed into smaller cages in an adjacent glasshouse maintained in the same environmental conditions. The number and sex ratio of the adults eclosing from these plants were recorded daily, and eclosed adults were then returned to their respective experimental cages. From this data an estimate of the eclosion rates in the main cage was made, from which the number of OX4319L males required to achieve the respective over-flooding ratio for each cage was calculated.

Statistical analyses

For comparisons between treatment peak population densities in Generation 3, ANOVA was used, followed by Tukey's HSD for pair-wise comparisons. For Generation 4, a *t*-test was used as only two treatments were compared. For *Bt* assay results, results from the two *Bt* assays within each cage were summed to avoid pseudo-replication and then corrected for control mortality using a Henderson-Tilton correction. For comparisons between treatments, a Logit model for Categorical Data Analysis was used followed by pair-wise comparisons using Generalized Linear Hypothesis Testing with each cage acting as a replicate. Error estimates for mean proportions were calculated using Pearson's exact confidence intervals. Data were analyzed using R (Version 2.15.0).

Abbreviations

Bt: *Bacillus thuringiensis*; IPM: Integrated pest management; IRM: Insecticide resistance management; MS: Male-selecting; SIT: Sterile insect technique.

Competing interests

TH-S, NM, AW, TM, KG, SW, and LA are, or were, employees or sponsored students of Oxitec Ltd, which therefore provided salary or stipend and other support for the research program. Also, all these employees have shares or share options in Oxitec Ltd. NA has an indirect interest in shares in the company Oxitec Ltd. Both Oxitec Ltd and the University of Oxford have one or more patents or patent applications related to the subject of this manuscript.

Authors' contributions

All authors were involved in experimental design. TH-S, NM AW, TM, JY, and HC performed experiments. TH-S analyzed results. TH-S, NM, NA, SW, TS, and LA wrote the paper. All authors read and approved the final manuscript.

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Description of genetic elements in *Plutella xylostella* strains OX4319L-Pxy, OX4319N-Pxy and OX4767A-Pxy

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Components listed as in plasmid maps

Construct component name	Construct component type	Component function	Donor	Detailed description
<i>piggyBac 5'</i>	Vector sequence	Germline transformation	<i>piggyBac</i> from <i>Trichoplusia ni</i> (moth)	5' end of <i>piggyBac</i> . <i>piggyBac</i> is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding <i>piggyBac</i> transposase under the control of the <i>Drosophila melanogaster</i> hsp70 promoter. One of the inverted terminal repeats that flank the wild-type <i>piggyBac</i> transposase in <i>piggyBac</i> has been removed in the helper plasmid so that the helper plasmid cannot, itself integrate even though it encodes for active <i>piggyBac</i> transposase.
<i>piggybac 3'</i>	Vector sequence	Germline transformation	<i>piggyBac</i> from <i>T. ni</i> (moth)	As above (Handler & Beeman, 2003)
polyA	Regulatory sequence 3'UTR	Stabilize mRNA	Virus	Regulatory sequence that helps stabilize mRNA
nls	Nuclear localization sequence	Localises DsRed2 protein into the nuclei of cells	synthetic	NLS causes DsRed2 protein to accumulate within the nuclei of cells. Allows for spatial patterning of protein expression, which is useful to distinguish fluorescence patterns
DsRed2	Protein coding sequence	Express DsRed2	<i>Discosoma Sp</i> (coral)	This allows the expression of a fluorescent protein. The transgenic DBM with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenicDBM can be envisioned.
Target site A	Synthetic sequence	Target site	synthetic	Target recognition site in construct for integrase or recombinase, which is not used in this particular strain and project.
Intron	Regulatory	Requirement	<i>Drosophila</i>	Stabilizes mRNA and required for translation of

	sequence for 5'UTR	for translation	<i>melanogaster</i>	mRNA
<i>ie1/Hr5</i>	Enhancer /Promoter sequences	Control expression of DsRed2	<i>Autographa californica</i> nuclear polyhedrosis virus (AcMNPV)	Promoter from <i>immediate-early-1</i> gene and <i>hr5</i> enhancer region
<i>tetOx7</i>	Synthetic regulatory sequence	Control of gene expression in a tet-repressible manner	Synthetic	Enhancer region to control gene expression
PBW <i>dsx</i> genomic region	splicing	Gives female specificity	<i>Pectinophora gossypiella</i>	Contains an alternative splicing intron allowing sex-specific expression of sequence inserted into the female-specific exon (in this case tTAV).
<i>hsp70</i>	promoter sequence	Minimal promoter	<i>Drosophila melanogaster</i>	Minimal promoter to enable transcription of gene
PBW <i>dsx</i> genomic region	splicing	Gives female specificity	<i>Pectinophora gossypiella</i>	Contains an alternative splicing intron allowing sex-specific expression of sequence inserted into the female-specific exon (in this case tTAV).
tetR	Protein coding region (Gene)	Component of tTAV protein	Tn10-specified tetracycline-resistance operon of <i>E. coli</i> (Gossen & Bujard, 1992)	In the presence of the antibiotic tetracycline tetR does not bind to its operators located within the promoter region of the operon and allows transcription (Gossen & Bujard, 1992)
tTAV (comprising of tet R and VP16 domains)	Protein coding region (Gene)	Expression tTAV protein	<i>Escherichia coli</i> (bacterium) and Herpes simplex (virus)	tTAV is a tet-responsive transcriptional factor. It is a fusion of the tetR from <i>E.coli</i> and the VP16 transcriptional activator from HSV. By combining tetR with the C-terminal domain of VP16 from HSV, known to be essential for the transcription of the immediate early viral genes a hybrid transactivator was generated that stimulates minimal promoters fused to tetracycline operator (tetO) sequences. These promoters are silent in the presence of low concentrations of tetracycline, which prevents the tetracycline-controlled transactivator (tTA) from binding to tetO sequences.
Dro K10	Regulatory sequence 3'UTR	Stabilize mRNA	<i>Drosophila melanogaster</i>	Regulatory sequence that helps stabilize mRNA
VP16	Protein coding region	Component of tTAV protein	Herpes simplex (virus)	Component of synthetic transcription factor tTA

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Threatened or endangered species present at the release site

A search was carried out on the IUCN red list of threatened species (<http://www.iucnredlist.org/search>; accessed 12th August 2013) according to the following search criteria:

Show taxa:
Species
Search by taxonomy:
ANIMALIA
Search by location:
New York
(Native)
Search by systems:
Terrestrial
Match any habitat:
1. Forest
2. Savanna
3. Shrubland
4. Grassland
5. Wetlands (inland)
6. Rocky areas (eg. inland cliffs, mountain peaks)
7. Caves and Subterranean Habitats (non-aquatic)
8. Desert
14. Artificial/Terrestrial
16. Introduced vegetation
17. Other
18. Unknown
Match any threat:
1. Residential & commercial development
2. Agriculture & aquaculture
3. Energy production & mining
4. Transportation & service corridors
5. Biological resource use
6. Human intrusions & disturbance
7. Natural system modifications
8. Invasive & other problematic species & genes
9. Pollution
10. Geological events
11. Climate change & severe weather
12. Other options
Search by assessment:
Categories: CR, EN, VU, DD

This search found only seven species from which only one species, the New Cottontail Rabbit (*Sylvilagus transitionalis*), whilst this is not an aquatic species and has the potential for habitat overlap with the diamondback moth this species this species is a herbivore which is unlikely to directly interact with the released moth.

Further searches on the New York States Department for Environment (<http://www.dec.ny.gov/animals/7494.html>; accessed 12th August 2013) indicate that there are a number of endangered and threatened animals in the state which are not listed on the IUCN red list. Evaluation of these species for animals which might have a habitat which overlaps with the agricultural pest, diamondback moth, has been carried out and is presented in Table 1.

Overall there are a number of birds which could be present around abandoned agricultural land or nearby open grasslands however occurrence of any special concern bird species in a large highly managed farmland is unlikely. Insects form the diet of many small mammals, reptiles and birds however there is no one species which is reliant on the diamond back moth as a diet source. None of the species listed on the IUCN red list and New York States Department of Environment were reliant on any one species as a food source therefore the impact that this release of diamondback moths would have on the endangered, threatened or special concern animal populations is negligible.

Table 1. Species which could interact with the released Diamondback moths and are present in New York State and are Endangered, threatened or are of special concern.

Common name (latin name)	Distribution	Threat	New York Status	Habitat overlap with diamondback moth
Loggerhead Shrike (<i>Lanius ludovicianus</i>)	Most of Northern America from South Canada to South Mexico.	Threats to this species are unclear however it has been suggested that abandonment of farms and orchards have removed breeding sites. Roadkills and pesticide contamination could also be factors.	Endangered	Feed on beetles, grasshopper sand small rodents therefore it is unlikely that this species will have a direct interaction with the diamondback moth however this species is found in agricultural land.
Vesper Sparrow (<i>Pooecetes gramineus</i>)	Open grassy areas in North America	This species requires bare ground as breeding territory, abandonment of farms and regrown of forest areas threaten this species.	Special concern	This species has a diet consisting of insects and seeds. In New York this species is commonly found in the Erie-Ontario Plain and the central Appalachians and is not anticipated to be present in currently managed farmland.
Grasshopper Sparrow (<i>Ammodramus savannarum</i>)	Common throughout much of the United States and Southern Canada.	Threats include mowing of grasslands, use of pesticides and loss of grassland by plant succession.	Special concern	This species breeding in meadows, pastures, hayfields and croplands. There could be a habitat overlap between the diamondback moths and this species however interactions are likely to be limited as this is a widespread species and the proposed trial is small.
Golden-Winged Warbler (<i>Vermivora chrysoptera</i>)	Breeds throughout north central and north-eastern United States	Maintenance of early successional fields is required to preserve this species.	Special concern	This species breeds in early successional habitats therefore it could be present on any abandoned farmlands near to the release site. This is limited potential for habitat overlap and interaction with this species.

Oxitec GE OX4319L-Pxy

Additional CBI justification:

The identity of these research strains is highly proprietary and their disclosure could be expected to under-mine the developer Oxitec's competitive position as the market leader.

Oxitec has developed innovative new solutions to controlling harmful insect pests. If competitors knew what methodologies Oxitec has developed or what materials it uses in those methods, often after significant investment of time and resources, those competitors could adopt these proprietary materials and methods for their own benefit without themselves having to expend the same level of resources that Oxitec devoted to develop these materials and methods. Oxitec therefore maintains this type of information in secrecy and considers information about its research efforts to constitute confidential business information.

The reports OX4319L construct sequencing and OX4319L molecular characterisation characterise the strain and would give competitors insight into re-creation of the strain and its unique molecular construction. This represents considerable investment of time and effort for the developer Oxitec and would provide competitors with detailed and direct information regarding the construction of the strain, which would be highly advantageous to competitors if it were in the public domain.

The report OX4319L tTAV expression levels characterise the strain and would give competitors insight into some of the unique attributes of the strain and how the genetic engineering construct is expressed. This represents considerable investment of time and effort for Oxitec and would provide competitors with detailed and direct information regarding the strain, which would be highly advantageous to competitors if it were in the public domain.

The collection of this information is authorized by the Plant Protection Act of 2000. The information will be used to determine eligibility to receive all types of permits. No permit will be issued until this application has been approved.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE

APPLICATIONS FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

1. NAME, ADDRESS, TELEPHONE, AND EMAIL OF APPLICANT

Name: (b)(6)
 Position: Professor
 Organization: Cornell University/NYSAES
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 FAX: (b)(6)
 Alternate: (b)(6)
 Email 1: (b)(6) cornell.edu
 Email 2:

2. INTRODUCTION TYPE

- Importation
- Interstate Movement
- Interstate Movement and Release
- Release

3. PERMIT TYPE

- Standard
- Permit
- Courtesy
- Permit

4. PURPOSE OF PERMIT

- Industrial Product
- Pharmaceutical Product
- Phytoremediation
- Traditional

5. CONFIDENTIAL BUSINESS INFORMATION VERIFICATION (CBI)

Does this application contain CBI? Yes No

CBI Justification:

N/A

6. REQUEST TYPE

New Amendment Renewal

Amendment/Renewal Description:

Previous Permit Number(s):

7. MEANS OF MOVEMENT

Import by air; releases manually from the ground/vehicles.

8. VARIANCE VERIFICATION

Have you previously applied for variance(s) that you wish to apply to this permit? Yes No

Variance Number(s):

If so, describe in a brief summary how the variance will be applied:

N/A

9. REGULATED ARTICLE

Scientific Name: Plutella xylostella

Common Name: Diamondback moth

Cultivar and/or Breeding Line:

OX4319L-Pxy

Any biological material (e.g., culture medium, or host material) accompanying the regulated Article during movement:

Artificial insect diet.

Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed, and produced:

All final engineering of the transforming constructs was performed at Oxitec Ltd, in the United Kingdom.

The genes used from the donor organisms and the piggyBac-derived portions of the vectors used to build the transforming construct were cloned off-site. The recipient organism is the moth, *Plutella xylostella*, which is endemic in temperate regions around the world, including the USA. The recipient *Plutella xylostella* strain for the transformation was a wild-type strain obtained from Syngenta (public limited company), UK, which has been reared in Oxitec insectaries since 2008.

Processes, Procedures, and Safeguards Description:

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

This is a permit request for seasonal releases (April to October) of a female-lethal, genetically marked diamondback moth (maximum 30,000 moths/week), in brassica fields at the Cornell University research station, Geneva NY.

Males of the transgenic moths will be released in cultivated brassica plots and biological parameters of these moths, such as dispersal and persistence, measured using traps, for example baited with synthetic sex pheromone. The moths of the OX4319L-Pxy strain carry a stable, heritable marker the DsRed2 fluorescent protein, viewed by fluorescence microscope or detected by PCR and their female progeny die in the absence of a dietary repressor (tetracycline or suitable analogues supplied in their artificial diet). The male-selecting (female-lethal) penetrance of the strain is >99% (Jin et al. 2013). The marker provides a means of distinguishing released moths from wild moths, and female-lethality is a self-limiting trait in the wild.

All genetically modified moths will be reared in insectaries at Cornell University, Geneva NY. The facilities and their general operation have been inspected and approved through a previous importation permit (12-227-102m). Larval rearing will be conducted in quarantine using the same approved procedures as in this previous permit. Only moths homozygous for the conditional lethal transgene, reared off tetracycline, will be released. Adult moths will be transported in sealed containers, with at least two layers of containment, labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle; for each batch the number of containers and identity of member of staff supervising the release will be recorded.

The transgenic diamondback moths encode no toxic or allergen proteins. The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA-CFSAN in the USA for human safety, and they raised no objections to its use in corn plants. This involved an assessment of the amino acid sequence using bioinformatics analyses in accordance with the Guidance provided by Codex (2003), the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, as well as the toxicity of the protein using bioinformatics analysis. The amino acid sequence in OX4319L-Pxy is the same as that evaluated in the NPC. It has been further evaluated in an Environmental Assessment (EA) by the USDA (http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf), which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk.

Additional EAs on another GE moth, GE pink bollworm, expressing fluorescent genes similar to DsRed2 have also been conducted (<http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm>) and concluded that it was unlikely to present any hazard to the environment.

The other protein coding region, tTAV, is regulated by sequences from the sex-determination gene, doublesex, from pink bollworm (*Pectinophora gossypiella*), that produce different splice variants in males and females: the female transcript comprises coding sequence for the tetracycline-repressible transcription factor, tTAV, which interacts with the upstream tetracycline response element, tetO (or tRE), to form a positive-feedback loop that results in insect lethality prior to adulthood. Under the control of the doublesex sex-alternate splicing, lethality is induced only in females. The tTAV amino acid sequence in OX4319L-Pxy has also been evaluated independently using the bioinformatics analyses provided by Codex (2003) for both potential allergenicity and toxicity. No homologies with known allergens or toxins were determined. This study is available on request.

Tetracycline can be provided to the insect in larval artificial diet to suppress female death and permit colony rearing in the laboratory. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

This female-specific lethal trait was previously discussed in a USDA Environmental Impact Statement published in October 2008, entitled Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs, which concluded that the use of genetically engineered fruit flies and pink bollworm in APHIS plant pest control programs were the environmentally preferred alternative (Record of Decision (Federal Register Vol 74 (87) 21314 2009).

Reference:

Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology, 2:160-166.

10. ARTICLE SUPPLIER AND/OR DEVELOPER

<u>Name</u>	<u>Location</u>	<u>Contact Information</u>
(b)(6)	Oxitec Ltd 71 Milton Park Abingdon OX144RQ County: Oxford	Day Telephone: 0044-1235-832393 FAX: Email: (b)(6) oxitec.com

11. PHENOTYPES/GENOTYPE

1) Phenotypic Designation Name: visual marker; repressible lethality

WARNING: Any use of ePermits to make materially false, fictitious, or fraudulent statements or representations is subject to civil penalties of up to \$250,000 (7 U.S.C. § 7734(b)) or punishable by a fine of not more than \$10,000, or imprisonment of not more than 5 years, or both (18 U.S.C. §1001).

Identifying Line(s):	OX4319L-Pxy
Construct(s):	OX4319
Mode of Transformation:	Direct injection
Phenotype Description: A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.	The introduced genetic material in the diamondback moth comprises three protein coding regions, one for marking the insects and two for inducing death before the insect reaches adulthood (in this instance, females only). The former allows the expression of a DsRed2 fluorescent protein originally derived from a coral (<i>Discosoma</i> sp.). The transgenic diamondback moth with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenic diamondback moth can be envisioned. The non-modified diamondback moth has no fluorescent protein gene; therefore, it does not fluorescence when illuminated under the same light frequency. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

Phenotype(s)

MG - Visual marker; DsRed2 Fluorescent Protein Expression

Genotype(s)

Screenable Marker

Gene: DsRed2 **from** *Discosoma* sp. - Screenable marker gene DsRed2 from *Discosoma* spp - Allows the expression of a fluorescent protein from *Discosoma* spp. Fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5iel promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by intense illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. Expression of a fluorescent protein will therefore permit released modified moths to be distinguished from unmodified.

Vector Sequence: piggyBac (non-autonomous) **from** piggyBac from *Trichoplusia ni* (moth) - Transformation Vector from *Trichoplusia ni* (moth) - Effects germline transformation of diamondback moth from piggyBac from *Trichoplusia ni* (moth) - 3' end of piggyBac. piggyBac is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element. In the construct used for transformation of the pink bollworm, the transposase gene of the piggyBac element was irreversibly destroyed by insertion of the transgene. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding piggyBac transposase under the control of the *Drosophila melanogaster* hsp70 promoter. One of the inverted terminal repeats that flank the wild-type piggyBac transposase in piggyBac has been removed in the helper plasmid so that the helper plasmid cannot itself integrate even though it encodes for active piggyBac transposase.

Repressible lethality

Gene: tTAV **from** *Escherichia coli* (bacterium) and *Herpes simplex* (virus) - Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from *Escherichia coli* with VP16 from a type 1 herpes simplex virus. The tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (tetO) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline, it will induce expression

from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and Drosophila melanogaster with no known adverse effects. Unmodified *Plutella xylostella* do not have a tTAV activity.

Regulatory sequence: doublesex genomic region **from** Pink bollworm, *Pectinophora gossypiella* - Female-specificity is conferred using truncated sex-alternate splicing sequences from the doublesex gene of *Pectinophora gossypiella*. Sequence encoding tTAV is inserted into this splicing sequence, allowing for the expression of tTAV in a sex-specific manner, resulting in a conditional female-lethal system (Jin et al. 2013).

A full list of construct components is provided in the attached Table of genetic elements.

References:

- Berger SL, et al. 1990 Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* 61, 1199-1208.
- Damke H, et al. 1995 Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Meth Enzymol* 257, 209-220.
- Gillespie JP, et al. 1997 Biological mediators of insect immunity. *Annu Rev Entomol* 42, 611-643.
- Gong P, et al. 2005 A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat Biotechnol* 23, 453-456.
- Gossen M, et al. 1994 Inducible gene expression systems for higher eukaryotic cells. *Curr Opin Biotechnol* 5, 516-520.
- Gossen M, and Bujard H 1992 Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc Natl Acad Sci USA* 89, 5547-5551.
- Salghetti S, et al. 2001 Regulation of transcriptional activation domain function by ubiquitin. *Science* 293, 1651-1653.
- Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2:160-166.

12. INTRODUCTION

Release Site

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Research Farm North	<p>NY County: Ontario Proposed Release Start Date: 7/1/2016 Proposed Release End Date: 12/31/2017 No. of Releases: Quantity:</p> <p>up to 72/year, up to 30,000 moths/week up to 10 acres</p>	
Location Unique ID:	RFN1097	
Location GPS Coordinates:	(b)(4)	
Release Site History:	Managed agricultural, cropping, research. Managed agricultural land around release site.	
Critical Habitat Involved?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	

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13. DESIGN PROTOCOLS

Production Design

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

The diamondback moth (*Plutella xylostella*) strain OX4319L-Pxy shows a tetracycline-repressible female-lethal phenotype, which could serve as an insecticide-free means of controlling pest populations of *Plutella xylostella* in the field in a species-specific manner. Successful pest control will rely upon strong performance of released males, in terms of female-seeking behavior and mating competitiveness. We will seek to measure relevant performance traits in one or more mark-release-recapture field experiments. We will also conduct caged trials to support information about relevant performance traits, including release rates.

Mark-release-recapture studies. In the mark-release-recapture experiments, we will release up to 10,000 male OX4319L-Pxy moths per release (up to 30,000 males per week) from a single point in an experimental field of up to 10 acres planted with brassicas (e.g. cabbage or broccoli). The anticipated release point is (b)(4) and brassicas will be planted up to a (b)(4) (b)(4) from the release point. Exact coordinates will be provided upon planting. Movement patterns of moths within the field will be assessed using sticky traps and mating stations.

Mating stations will consist of confined, wild-type females that attract males. Traps will be collected at least once per week and the recaptured moths screened for the fluorescence marker. Additional PCR screening will be conducted to validate this visual screening. Females from the mating stations will be screened for whether they mated to OX4319L-Pxy males.

Sticky traps baited with synthetic sex pheromone will be placed around the field, up to 1 km from the release point, to capture male moths and detect the presence of any OX4319L-Pxy *Plutella xylostella*. Traps will be collected at least once per week and moths screened for the fluorescence marker indicating they are OX4319L-Pxy *Plutella xylostella*. Additional PCR screening will be conducted to validate this visual screening.

To permit an overlapping series of releases in the experimental field, OX4319L-Pxy male moths will sometimes be additionally marked, for example using different-colored fluorescent powders, which are commonly used in such field experiments with insects (reviewed by Hagler & Jackson 2001 Ann Rev Entomol 46:511-543). Powder-marked USA-derived wild-type moths may also be used in mark-release-recapture experiments to provide a comparison with OX4319L-Pxy male *Plutella xylostella*. Each experimental field will be surrounded by an approximately 10-m-wide border free of potential host plants. Upon completion of the experiment, an insecticide (e.g. Coragen-chlorantraniliprole) will be sprayed on the plants and surrounding area to kill remaining *Plutella xylostella* larvae and adults. Pheromone-baited traps will be deployed at the trial site that will continue until no OX4319L-Pxy male moths are recaptured for 2 consecutive weeks.

Data from these field experiments will provide information on dispersal and field longevity of OX4319L-Pxy male moths.

Field cage studies. During the cage experiments, we will: conduct mating competition experiments between male moths of OX4319L-Pxy and recently-colonized wild-type male moths, for wild-type female mates; assess longevity of OX4319L-Pxy male moths and wild-type male moths in field cages; assess the reproductive rate of *Plutella xylostella* in field cages; and test suppression of field cage populations of wild-type *Plutella xylostella* through releases of OX4319L-Pxy male moths. Cages will consist of screened cages approximately 24ft x 12ft x 6ft (L x W x H) covering a metal frame that does not permit ingress or egress of *Plutella xylostella* moths.

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

All OX4319L-Pxy moths used in the trials will be reared as larvae on non-tetracycline artificial diet. Releases will be conducted from the ground or vehicle on Cornell University's New York State Agricultural Research Station. Releases will be conducted up to five times per week, depending on experimental requirements.

The area around the release sites (up to 1000 m radius from release site) will be monitored with traps (e.g. sticky traps baited with synthetic sex pheromone). Traps will be collected at least weekly to count the number of OX4319L-Pxy moths and wild moths captured on each trap. Samples in the laboratory will be screened for presence of the DsRed2 fluorescent marker, using fluorescence microscopy, and this will be validated by PCR detection of the DNA construct in selected samples. Some non-viable insect samples will be sent to Oxitecs labs in the UK for the PCR analysis. Prior to each field release, samples from each cohort of male OX4319L-Pxy moths will be screened for the fluorescent marker and sexed. Only male moths - which do not directly damage crops - will be released so the effect on the crop will therefore be negligible: male activity is restricted to finding and mating with females, imbibing liquids from plants, and taking shelter during the day.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the

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regulated article at each of the intended destinations:

Adult genetically modified moths will be transported in sealed containers labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle by authorized personnel.

The conditional lethality expressed by the construct carried by OX4319L-Pxy means that female progeny from matings with Oxitec male insects die in the absence of tetracycline, and the trait is therefore unlikely to persist in the environment. Other mitigation measures include the lack of known sexually compatible relatives of *Plutella xylostella* in the USA; the piggyBac transposable element used for the transformation has no endogenous functioning transposase, rendering it non-autonomous (it cannot mobilize itself); the release area will be monitored extensively with traps to attract and collect *Plutella xylostella* moths; the release field is no larger than 10 acres; the *Plutella xylostella* can be sprayed with insecticide at any time in the case of observed adverse events; the OX4319L-Pxy *Plutella xylostella* will be securely managed and contained in production and transport; and all viable insects reared for this trial that are not required for release or additional analysis will be devitalized by freezing.

Final Disposition Method: Destruction/Devitalization Other Storage in Contained Facility

Final Disposition Description: All unused genetically modified eggs, larvae, pupae and moths not released, or not needed in the mass-rearing, will be frozen at a minimum of $-15^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 48 h to destroy all life stages.

14. ATTACHMENTS**Attachments**

BRS Permit 13-297-102r-a1 (3/16/2016 @ 08:43 AM)
BRS importation permit 12-227-102m (3/16/2016 @ 08:42 AM)
CBI Justification statement- Amended (5/31/2016 @ 03:51 AM)
Cornell University field site with GPS (3/16/2016 @ 08:43 AM)
Jin et al 2013-Scientific publication- description of the technology (3/16/2016 @ 08:38 AM)
OX4319L chlortetracycline sensitivity-CBI deleted (3/16/2016 @ 08:46 AM)
OX4319L chlortetracycline sensitivity-contains CBI (3/16/2016 @ 08:45 AM)
OX4319L construct sequencing- Contains CBI (3/16/2016 @ 08:47 AM)
OX4319L construct sequencing-CBI Deleted (3/16/2016 @ 08:47 AM)
OX4319L molecular characterisation-CBI deleted (3/16/2016 @ 08:49 AM)
OX4319L molecular characterisation-Contains CBI (3/16/2016 @ 08:48 AM)
OX4319L tTAV expression levels-CBI Deleted (3/16/2016 @ 08:50 AM)
OX4319L tTAV expression levels-Contains CBI (3/16/2016 @ 08:50 AM)
Plutella OX4319L Phenotype-Genotype text for submission 16March2016 (3/16/2016 @ 09:04 AM)
Scientific publication- Harvey-Samuel et al 2014 (3/16/2016 @ 08:44 AM)
Scientific publication- Harvey-Samuel et al 2015 (3/16/2016 @ 08:45 AM)
Table of genetic elements-Contains CBI (3/16/2016 @ 08:51 AM)
Threatened or endangered species (3/16/2016 @ 08:51 AM)

15. ADDITIONAL INFORMATION

i) Similar introductions were previously authorised under permit number: 13-297-102r-a1 and importation under permit number 12-227-102m

ii) Data from some of the research reports submitted under permit number: 13-297-102r-a1 have now been described in the peer reviewed publications noted below:

Harvey-Samuel, T., Ant, T., Gong, H., Morrison, N.I., and Alphey, L. (2014). Population-level effects of fitness costs associated with repressible female-lethal transgene insertions in two pest insects. *Evol Appl* 7, 597-606.

Harvey-Samuel, T., Morrison, N.I., Walker, A.S., Marubbi, T., Yao, J., Collins, H.L., Gorman, K., Davies, T.G., Alphey, N., Warner, S., et al. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biol* 13, 49.

16. COURTESY JUSTIFICATION

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(b)(6) hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON

(b)(6)

18. DATE

March 16, 2016

The collection of this information is authorized by the Plant Protection Act of 2000. The information will be used to determine eligibility to receive all types of permits. No permit will be issued until this application has been approved.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
BIOTECHNOLOGY REGULATORY SERVICE

APPLICATIONS FOR PERMIT OR COURTESY PERMIT UNDER 7 CFR 340

(Genetically Engineered Organisms or Products)

1. NAME, ADDRESS, TELEPHONE, AND EMAIL OF APPLICANT

Name: (b)(6)
 Position:
 Organization: Cornell University/NYSAES
 Organization Unique ID:
 Address: 630 W. North St.
 (b)(6)
 Geneva, NY 14456
 County/Province:
 Township/Island:
 Day Telephone: (b)(6)
 FAX:
 Alternate:
 Email 1: (b)(6)@cornell.edu
 Email 2:

2. INTRODUCTION TYPE

- Importation
- Interstate Movement
- Interstate Movement and Release
- Release

3. PERMIT TYPE

- Standard
- Permit
- Courtesy
- Permit

4. PURPOSE OF PERMIT

- Industrial Product
- Pharmaceutical Product
- Phytoremediation
- Traditional

5. CONFIDENTIAL BUSINESS INFORMATION VERIFICATION (CBI)

Does this application contain CBI? Yes No

CBI Justification:

N/A

6. REQUEST TYPE

New Amendment Renewal

Amendment/Renewal Description:

Previous Permit Number(s):

7. MEANS OF MOVEMENT

Import by air; releases manually from the ground/vehicles.

8. VARIANCE VERIFICATION

Have you previously applied for variance(s) that you wish to apply to this permit? Yes No

Variance Number(s):

If so, describe in a brief summary how the variance will be applied:

N/A

9. REGULATED ARTICLE

Scientific Name: Plutella xylostella

Common Name: Diamondback moth

Cultivar and/or Breeding Line:

OX4319L-Pxy

Any biological material (e.g., culture medium, or host material) accompanying the regulated Article during movement:

Artificial insect diet.

Country and locality where the donor organism, recipient organism, and vector or vector agent were collected, developed, and produced:

All final engineering of the transforming constructs was performed at Oxitec Ltd, in the United Kingdom.

The genes used from the donor organisms and the piggyBac-derived portions of the vectors used to build the transforming construct were cloned off-site. The recipient organism is the moth, *Plutella xylostella*, which is endemic in temperate regions around the world, including the USA. The recipient *Plutella xylostella* strain for the transformation was a wild-type strain obtained from Syngenta (public limited company), UK, which has been reared in Oxitec insectaries since 2008.

Processes, Procedures, and Safeguards Description:

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This is a permit request for seasonal releases (April to October) of a female-lethal, genetically marked diamondback moth (maximum 30,000 moths/week), in brassica fields at the Cornell University research station, Geneva NY.

Males of the transgenic moths will be released in cultivated brassica plots and biological parameters of these moths, such as dispersal and persistence, will be measured. The moths of the OX4319L-Pxy strain carry a stable, heritable marker the DsRed2 fluorescent protein, viewed by fluorescence microscope or detected by PCR and their female progeny die in the absence of a dietary repressor (tetracycline or suitable analogues supplied in their artificial diet). The male-selecting (female-lethal) penetrance of the strain is >99% (Jin et al. 2013). The marker provides a means of distinguishing released moths from wild moths, and female-lethality is a self-limiting trait in the wild.

All genetically modified moths will be reared in insectaries at Cornell University, Geneva NY. The facilities and their general operation have been inspected and approved through a previous importation permit (12-227-102m). Larval rearing will be conducted in quarantine using the same approved procedures as in this previous permit. Only moths homozygous for the conditional lethal transgene, reared off tetracycline, will be released. Adult moths will be transported in sealed containers, with at least two layers of containment, labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle; for each batch the number of containers and identity of member of staff supervising the release will be recorded.

The transgenic diamondback moths encode no toxic or allergen proteins. The DsRed2 marker protein has been evaluated in a New Protein Consultation by the FDA-CFSAN in the USA for human safety, and they raised no objections to its use in corn plants. This involved an assessment of the amino acid sequence using bioinformatics analyses in accordance with the Guidance provided by Codex (2003), the lability of the protein in simulated gastric fluid (SGF) and an examination of the gene source and history of exposure, as well as the toxicity of the protein using bioinformatics analysis. The amino acid sequence in OX4319L-Pxy is the same as that evaluated in the NPC. It has been further evaluated in an Environmental Assessment (EA) by the USDA (http://www.aphis.usda.gov/brs/aphisdocs/08_33801p_dpra.pdf), which concluded that the corn transformation event that contained the DsRed2 gene was unlikely to become a plant pest risk.

Additional EAs on another GE moth, GE pink bollworm, expressing fluorescent genes similar to DsRed2 have also been conducted (<http://www.gpo.gov/fdsys/pkg/FR-2006-04-19/html/E6-5878.htm>) and concluded that it was unlikely to present any hazard to the environment.

The other protein coding region, tTAV, is regulated by sequences from the sex-determination gene, doublesex, from pink bollworm (*Pectinophora gossypiella*), that produce different splice variants in males and females: the female transcript comprises coding sequence for the tetracycline-repressible transcription factor, tTAV, which interacts with the upstream tetracycline response element, tetO (or tRE), to form a positive-feedback loop that results in insect lethality prior to adulthood. Under the control of the doublesex sex-alternate splicing, lethality is induced only in females. The tTAV amino acid sequence in OX4319L-Pxy has also been evaluated independently using the bioinformatics analyses provided by Codex (2003) for both potential allergenicity and toxicity. No homologies with known allergens or toxins were determined. This study is available on request.

Tetracycline can be provided to the insect in larval artificial diet to suppress female death and permit colony rearing in the laboratory. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

This female-specific lethal trait was previously discussed in a USDA Environmental Impact Statement published in October 2008, entitled Use of Genetically Engineered Fruit Fly and Pink Bollworm in APHIS Plant Pest Control Programs, which concluded that the use of genetically engineered fruit flies and pink bollworm in APHIS plant pest control programs were the environmentally preferred alternative (Record of Decision (Federal Register Vol 74 (87) 21314 2009)).

Reference:

Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. ACS Synthetic Biology, 2:160-166.

10. ARTICLE SUPPLIER AND/OR DEVELOPER

<u>Name</u>	<u>Location</u>	<u>Contact Information</u>
(b)(6)	Oxitec Ltd 71 Milton Park Abingdon OX144RQ County: Oxford	Day Telephone: (b)(6) FAX: (b)(6) Email: (b)(6)

11. PHENOTYPES/GENOTYPE

- 1) Phenotypic Designation Name:** visual marker; repressible lethality

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Identifying Line(s):	OX4319L-Pxy
Construct(s):	OX4319
Mode of Transformation:	Direct injection
Phenotype Description: A description of the anticipated or actual expression of the altered genetic material in the regulated article and how that expression differs from the expression in the non-modified parental organism.	The introduced genetic material in the diamondback moth comprises three protein coding regions, one for marking the insects and two for inducing death before the insect reaches adulthood (in this instance, females only). The former allows the expression of a DsRed2 fluorescent protein originally derived from a coral (<i>Discosoma</i> sp.). The transgenic diamondback moth with the marker gene fluoresces when excited by illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no ecological or other consequences resulting from incorporation of these markers into the transgenic diamondback moth can be envisioned. The non-modified diamondback moth has no fluorescent protein gene; therefore, it does not fluorescence when illuminated under the same light frequency. Neither piggyBac transposase activity nor any antibiotic resistance is conferred to the transgenic diamondback moth by the introduced genetic material.

Phenotype(s)

MG - Visual marker; DsRed2 Fluorescent Protein Expression

Genotype(s)

Screenable Marker

Gene: DsRed2 **from** *Discosoma* sp. - Screenable marker gene DsRed2 from *Discosoma* spp - Allows the expression of a fluorescent protein from *Discosoma* spp. Fluorescent protein of the GFP superfamily (DsRed2) under the control of a hr5iel promoter/enhancer sequence, which is from *Autographa californica* nuclear polyhedrosis virus (AcMNPV). A transgenic diamondback moth with the marker gene will fluoresce when excited by intense illumination of the appropriate wavelength. These fluorescent proteins, which have been used as markers in a wide range of vertebrate and invertebrate species, confer no known competitive advantage or disadvantage to the recipient, and no adverse ecological effect or other consequences resulting from incorporation of these markers into the transgenic diamondback moth are envisioned. Expression of a fluorescent protein will therefore permit released modified moths to be distinguished from unmodified.

Vector Sequence: piggyBac (non-autonomous) **from** piggyBac from *Trichoplusia ni* (moth) - Transformation Vector from *Trichoplusia ni* (moth) - Effects germline transformation of diamondback moth from piggyBac from *Trichoplusia ni* (moth) - 3' end of piggyBac. piggyBac is a DNA (deoxyribonucleic acid) transposable element that, only when its ITR (inverted terminal repeats) are intact, is capable of integrating DNA flanking by element-specific DNA into other DNA through mediation of a transposase encoded by an ORF (open reading frame) within the element. In the construct used for transformation of the pink bollworm, the transposase gene of the piggyBac element was irreversibly destroyed by insertion of the transgene. Transformation was effected by introducing, with the transforming construct, a helper plasmid that supplied transposase activity but was itself unable to transpose into other DNA. This transposition-defective helper plasmid has an ORF encoding piggyBac transposase under the control of the *Drosophila melanogaster* hsp70 promoter. One of the inverted terminal repeats that flank the wild-type piggyBac transposase in piggyBac has been removed in the helper plasmid so that the helper plasmid cannot itself integrate even though it encodes for active piggyBac transposase.

Repressible lethality

Gene: tTAV **from** *Escherichia coli* (bacterium) and *Herpes simplex* (virus) - Tetracycline-repressible transcriptional activator from tTAV is a synthetic fusion of the tetR protein from *Escherichia coli* with VP16 from a type 1 herpes simplex virus. The tTA protein binds to and activates expression from the tetracycline response element (tRE), which includes multiple copies of the specific DNA sequence to which tTA binds (tetO) (Gossen et al., 1994; Gossen & Bujard, 1992). tTAV also binds tetracyclines with high affinity; the tetracycline-bound form of tTAV does not bind DNA. tTAV therefore acts as a tetracycline-regulated switch. In the absence of tetracycline, it will induce expression

from tRE, whereas in the presence of tetracycline it will not. High-level expression of tTAV is thought to be deleterious to cells as it can repress their normal transcription; low-level expression has no known effect other than activation of tRE (Berger, et al., 1990; Damke et al., 1995; Gillespie et al., 1997; Gong et al., 2005; Gossen and Bujard, 1992; Salghetti et al., 2001). tTAV has been used in fungi, plants, mice and Drosophila melanogaster with no known adverse effects. Unmodified *Plutella xylostella* do not have a tTAV activity.

Regulatory sequence: doublesex genomic region **from** Pink bollworm, *Pectinophora gossypiella* - Female-specificity is conferred using truncated sex-alternate splicing sequences from the doublesex gene of *Pectinophora gossypiella*. Sequence encoding tTAV is inserted into this splicing sequence, allowing for the expression of tTAV in a sex-specific manner, resulting in a conditional female-lethal system (Jin et al. 2013).

A full list of construct components is provided in the attached Table of genetic elements.

References:

- Berger SL, et al. 1990 Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors. *Cell* 61, 1199-1208.
- Damke H, et al. 1995 Tightly regulated and inducible expression of dominant interfering dynamin mutant in stably transformed HeLa cells. *Meth Enzymol* 257, 209-220.
- Gillespie JP, et al. 1997 Biological mediators of insect immunity. *Annu Rev Entomol* 42, 611-643.
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- Gossen M, and Bujard H 1992 Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc Natl Acad Sci USA* 89, 5547-5551.
- Salghetti S, et al. 2001 Regulation of transcriptional activation domain function by ubiquitin. *Science* 293, 1651-1653.
- Jin L, et al. 2013 Engineered female-specific lethality for control of pest Lepidoptera. *ACS Synthetic Biology*, 2:160-166.

12. INTRODUCTION

Release Site

<u>Location Name & Description</u>	<u>Location Address</u>	<u>Contact(s)</u>
1) Research Farm North	<p>NY County: Ontario Proposed Release Start Date: 7/1/2016 Proposed Release End Date: 12/31/2017 No. of Releases: Quantity:</p> <p>up to 72/year, up to 30,000 moths/week up to 10 acres</p>	
Location Unique ID:	RFN1097	
Location GPS Coordinates:	(b)(4)	
Release Site History:	Managed agricultural, cropping, research. Managed agricultural land around release site.	
Critical Habitat Involved?	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No	

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13. DESIGN PROTOCOLS

Production Design

A detailed description of the purpose for the introduction of the regulated article including detailed description of the proposed experimental and/or production design:

The diamondback moth (*Plutella xylostella*) strain OX4319L-Pxy shows a tetracycline-repressible female-lethal phenotype, which could serve as an insecticide-free means of controlling pest populations of *Plutella xylostella* in the field in a species-specific manner. Successful pest control will rely upon strong performance of released males, in terms of female-seeking behavior and mating competitiveness. We will seek to measure relevant performance traits in one or more mark-release-recapture field experiments. We will also conduct caged trials to support information about relevant performance traits, including release rates.

Mark-release-recapture studies. In the mark-release-recapture experiments, we will release up to 10,000 male OX4319L-Pxy moths per release (up to 30,000 males per week) from a single point in an experimental field of up to 10 acres planted with brassicas (e.g. cabbage or broccoli). The anticipated release point is (b)(4) and brassicas will be planted (b)(4) (b)(4) from the release point. Exact coordinates will be provided upon planting. Movement patterns of moths within the field will be assessed using sticky traps and mating stations.

Mating stations will consist of confined, wild-type females that attract males. Traps will be collected at least once per week and the recaptured moths screened for the fluorescence marker. Additional PCR screening will be conducted to validate this visual screening. Females from the mating stations will be screened for whether they mated to OX4319L-Pxy males.

Sticky traps baited with synthetic sex pheromone will be placed outside the field, up to 1 km from the release point, to capture male moths and detect the presence of any OX4319L-Pxy *Plutella xylostella*. Traps and mating stations will be collected at least once per week and moths screened for the fluorescence marker indicating they are OX4319L-Pxy *Plutella xylostella*. Additional PCR screening will be conducted to validate this visual screening.

To permit an overlapping series of releases in the experimental field, OX4319L-Pxy male moths will sometimes be additionally marked, for example using different-colored fluorescent powders, which are commonly used in such field experiments with insects (reviewed by Hagler & Jackson 2001 Ann Rev Entomol 46:511-543). Powder-marked USA-derived wild-type moths may also be used in mark-release-recapture experiments to provide a comparison with OX4319L-Pxy male *Plutella xylostella*. Each experimental field will be surrounded by an approximately 10-m-wide border free of potential host plants. Upon completion of the experiment, an insecticide (e.g. Coragen-chlorantraniliprole) will be sprayed on the plants and surrounding area to kill remaining *Plutella xylostella* larvae and adults. Pheromone-baited traps will be deployed at the trial site that will continue until no OX4319L-Pxy male moths are recaptured for 2 consecutive weeks.

Data from these field experiments will provide information on dispersal and field longevity of OX4319L-Pxy male moths.

Field cage studies. During the cage experiments, we will: conduct mating competition experiments between male moths of OX4319L-Pxy and recently-colonized wild-type male moths, for wild-type female mates; assess longevity of OX4319L-Pxy male moths and wild-type male moths in field cages; assess the reproductive rate of *Plutella xylostella* in field cages; and test suppression of field cage populations of wild-type *Plutella xylostella* through releases of OX4319L-Pxy male moths. Cages will consist of screened cages approximately 24ft x 12ft x 6ft (L x W x H) covering a metal frame that does not permit ingress or egress of *Plutella xylostella* moths.

Destination or Release Description

A detailed description of the intended destination (including final and all intermediate destinations), uses, and/or distribution of the regulated article (e.g., greenhouses, laboratory, or growth chamber location; field trial location, pilot project location; production, propagation, and manufacture location; proposed sale and distribution location):

All OX4319L-Pxy moths used in the trials will be reared as larvae on non-tetracycline artificial diet. Releases will be conducted from the ground or vehicle on Cornell University's New York State Agricultural Research Station. Releases will be conducted up to five times per week, depending on experimental requirements.

The area around the release sites (up to 1000 m radius from release site) will be monitored with traps (e.g. sticky traps baited with synthetic sex pheromone). Traps will be collected at least weekly to count the number of OX4319L-Pxy moths and wild moths captured on each trap. Samples in the laboratory will be screened for presence of the DsRed2 fluorescent marker, using fluorescence microscopy, and this will be validated by PCR detection of the DNA construct in selected samples. Some non-viable insect samples will be sent to Oxitecs labs in the UK for the PCR analysis. Prior to each field release, samples from each cohort of male OX4319L-Pxy moths will be screened for the fluorescent marker and sexed. Only male moths - which do not directly damage crops - will be released so the effect on the crop will therefore be negligible: male activity is restricted to finding and mating with females, imbibing liquids from plants, and taking shelter during the day.

Confinement Protocols

A detailed description of the proposed procedures, processes, and safeguards which will be used to prevent escape and dissemination of the

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regulated article at each of the intended destinations:

Adult genetically modified moths will be transported in sealed containers labeled as follows "CORNELL UNIVERSITY GENETICALLY MODIFIED MOTHS FOR RELEASE AT CORNELL UNIVERSITY'S NEW YORK STATE AGRICULTURAL EXPERIMENT STATION - AUTHORIZED PERSONNEL ONLY". Insects will be transported by hand or in a vehicle by authorized personnel.

The conditional lethality expressed by the construct carried by OX4319L-Pxy means that female progeny from matings with Oxitec male insects die in the absence of tetracycline, and the trait is therefore unlikely to persist in the environment. Other mitigation measures include the lack of known sexually compatible relatives of *Plutella xylostella* in the USA; the piggyBac transposable element used for the transformation has no endogenous functioning transposase, rendering it non-autonomous (it cannot mobilize itself); the release area will be monitored extensively with traps to attract and collect *Plutella xylostella* moths; the release field is no larger than 10 acres; the *Plutella xylostella* can be sprayed with insecticide at any time in the case of observed adverse events; the OX4319L-Pxy *Plutella xylostella* will be securely managed and contained in production and transport; and all viable insects reared for this trial that are not required for release or additional analysis will be devitalized by freezing.

Final Disposition Method: Destruction/Devitalization Other Storage in Contained Facility

Final Disposition Description: All unused genetically modified eggs, larvae, pupae and moths not released, or not needed in the mass-rearing, will be frozen at a minimum of $-15^{\circ}\text{C} \pm 5^{\circ}\text{C}$ for 48 h to destroy all life stages.

14. ATTACHMENTS**Attachments**

BRS Permit 13-297-102r-a1 (3/16/2016 @ 08:43 AM)
BRS importation permit 12-227-102m (3/16/2016 @ 08:42 AM)
CBI Justification statement- Amended (5/31/2016 @ 03:51 AM)
Cornell University field site with GPS (3/16/2016 @ 08:43 AM)
Jin et al 2013-Scientific publication- description of the technology (3/16/2016 @ 08:38 AM)
OX4319L chlortetracycline sensitivity-CBI deleted (3/16/2016 @ 08:46 AM)
OX4319L chlortetracycline sensitivity-contains CBI (3/16/2016 @ 08:45 AM)
OX4319L construct sequencing- Contains CBI (3/16/2016 @ 08:47 AM)
OX4319L construct sequencing-CBI Deleted (3/16/2016 @ 08:47 AM)
OX4319L molecular characterisation-CBI deleted (3/16/2016 @ 08:49 AM)
OX4319L molecular characterisation-Contains CBI (3/16/2016 @ 08:48 AM)
OX4319L tTAV expression levels-CBI Deleted (3/16/2016 @ 08:50 AM)
OX4319L tTAV expression levels-Contains CBI (3/16/2016 @ 08:50 AM)
Plutella OX4319L Phenotype-Genotype text for submission 16March2016 (3/16/2016 @ 09:04 AM)
Scientific publication- Harvey-Samuel et al 2014 (3/16/2016 @ 08:44 AM)
Scientific publication- Harvey-Samuel et al 2015 (3/16/2016 @ 08:45 AM)
Table of genetic elements-Contains CBI (3/16/2016 @ 08:51 AM)
Threatened or endangered species (3/16/2016 @ 08:51 AM)

15. ADDITIONAL INFORMATION

i) Similar introductions were previously authorised under permit number: 13-297-102r-a1 and importation under permit number 12-227-102m

ii) Data from some of the research reports submitted under permit number: 13-297-102r-a1 have now been described in the peer reviewed publications noted below:

Harvey-Samuel, T., Ant, T., Gong, H., Morrison, N.I., and Alphey, L. (2014). Population-level effects of fitness costs associated with repressible female-lethal transgene insertions in two pest insects. *Evol Appl* 7, 597-606.

Harvey-Samuel, T., Morrison, N.I., Walker, A.S., Marubbi, T., Yao, J., Collins, H.L., Gorman, K., Davies, T.G., Alphey, N., Warner, S., et al. (2015). Pest control and resistance management through release of insects carrying a male-selecting transgene. *BMC Biol* 13, 49.

16. COURTESY JUSTIFICATION

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(b)(6)

hereby certify that the information in this application and all attachments is complete and accurate to the best of my knowledge and belief.

I acknowledge this is not an application to move or import select agents, the genes expressing select agents, or the toxins made by the select agents, as described in 9 CFR 121.

I will not introduce the regulated articles described in this application until APHIS has deemed the application complete and has granted the permit. By signing this permit, I agree to comply with any and all state, local, and tribal laws and regulations that may apply to the introduction of the articles described in this applications.

If there are any changes to the information disclosed in this application, I will contact APHIS.

17. SIGNATURE OF RESPONSIBLE PERSON**18. DATE**

(b)(6)

March 16, 2016