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Engineered Female-Specific Lethality for Control of Pest Lepidoptera

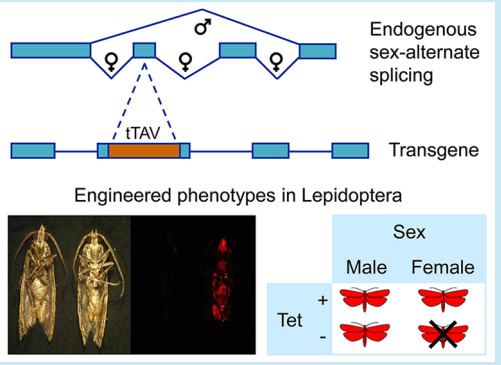
Li Jin,[†,‡,§] Adam S. Walker,[†,§] Guoliang Fu,[†,‡,§] Timothy Harvey-Samuel,[†,‡,§] Tarig Dafa'alla,[†] Andrea Miles,[†] Thea Marubbi,[†] Deborah Granville,[†] Nerys Humphrey-Jones,[†] Sinead O'Connell,[†] Neil I. Morrison,[†] and Luke Alphey*[,†,‡]

[†]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 strains[8] 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-CTC test crosses (Figure 2b). In the absence of CTC, all strains showed high female mortality (89−100%) and low male

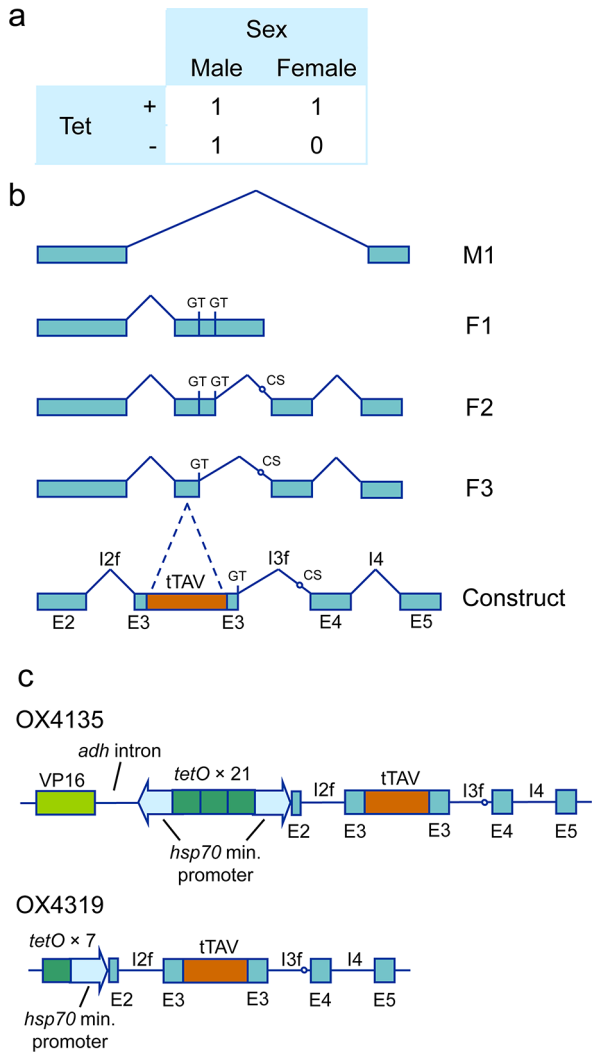

Figure 1. Splicing of pink bollworm *doublesex* (*Pgdsx*) and its incorporation in constructs for transformation of pink bollworm and diamondback moth. The transgenic construct provides a genetic circuit that responds to inputs of sex and presence of tetracycline in the larval diet and gives an output 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 (*Hr5ie1-DsRed2*) and *piggyBac* sequences for genomic insertion.

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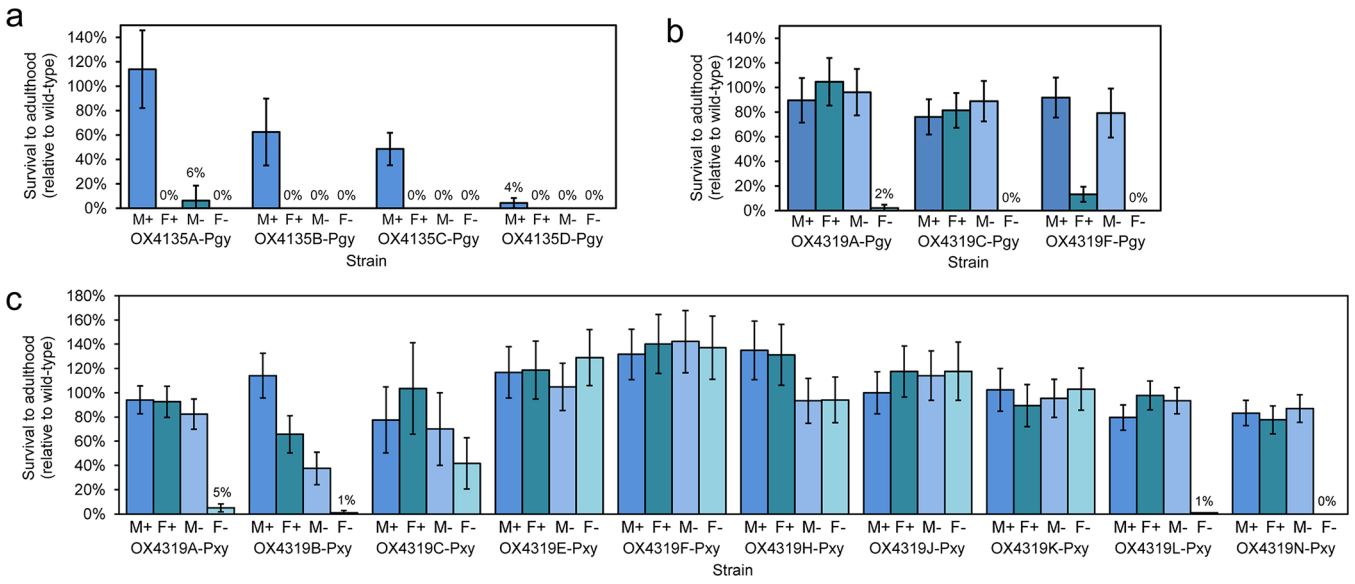

Figure 2. Tests for CTC-repressible, female-specific lethality in transformed pink bollworm and diamondback moth strains. (a) Survival to adulthood of transgenic strains of pink bollworm heterozygous for OX4135 insertions, reared on and off chlortetracycline (CTC). (b) Survival to adulthood of transgenic 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.

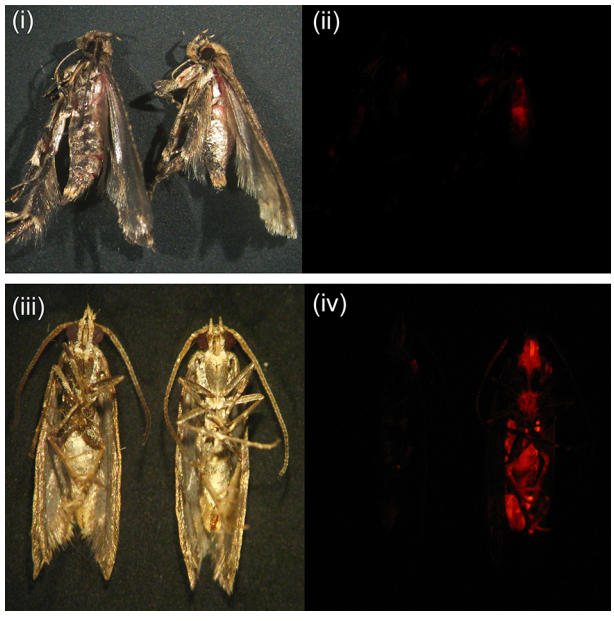

Figure 3. DsRed2 marker in pink bollworm (i, ii) and diamondback moth (iii, iv) strains transformed with OX4319. Images are in white light (i and iii) or under red fluorescent protein excitation filters.

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

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

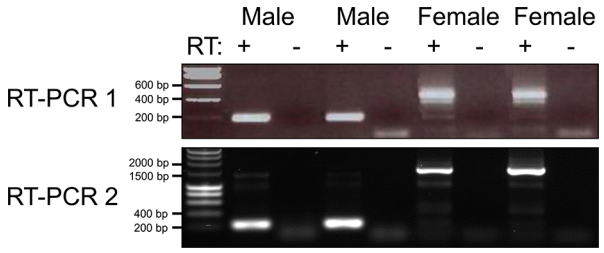

Figure 4. Gel images showing reverse transcription PCR analysis of sex-alternate splicing of endogenous *Pgdsx* and a *Pgdsx* minigene (samples from two males and two females in each). RT-PCR 1: amplified cDNA from wild-type pink bollworm using the primer pair targeting the common exons for male and 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-

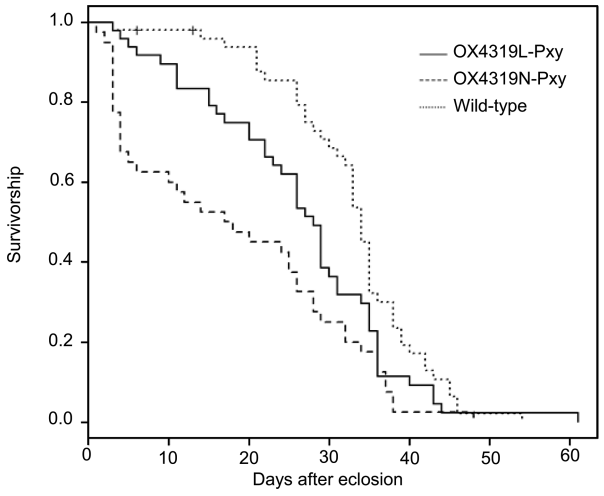

Figure 5. Kaplan–Meier object showing longevity of wild-type, OX4319L-Pxy, and OX4319N-Pxy male adult diamondback moths. OX4319 strains were transgene-homozygous.

type males showed the highest longevity (31.7 days ± 1.43 se), although not significantly different to that of OX4319L-Pxy (25.9 days ± 1.73). OX4319N-Pxy male longevity, however, was significantly lower (18.3 days ± 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 (χ^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

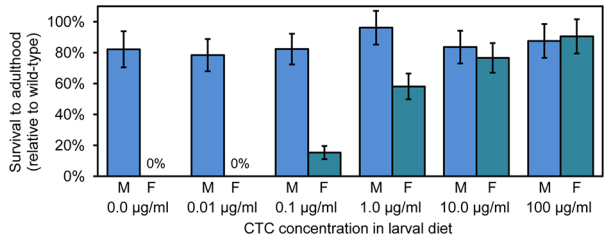

Figure 6. Survival to adulthood of OX4319L-Pxy-heterozygous diamondback moth reared on different concentrations of CTC in larval diet. Survival is expressed relative to that of wild-type counterparts. "M", male survival; "F", female survival. Error bars indicate 95% confidence intervals.

females survived to adulthood at CTC concentrations up to 0.01 µg/mL, 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.[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

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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 Dsx5r.

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): Pbwdsx1, Pbwdsx2, Pbwdsx3, and Pbwdsx4.

3′ RACE was performed using 3′ kit (Clontech, Mountain View, California) according to the manufacture'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 strainwere 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 DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Espoo, Finland). Primers were 3816RTEX1, RTCOTETR1, pbwdsx3, and RTPBWexon2R. Amplification conditions were as follows (Supplementary Table S4): 50 °C, 2 min; 95 °C, 15

min; 40 × (94 °C, 10 s; 58 °C, 30 s; 72 °C, 30 s; 80 °C for 2 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: CI = SR ± (1.96 × SE); where SE = (SR/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.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: luke.alphey@oxitec.com.

Author Contributions

[§]These authors contributed equally to this work.

Notes

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■ ABBREVIATIONS

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

■ REFERENCES

(1) Bloem, K. A., Bloem, S., and Carpenter, J. E. (2005) Impact of moth suppression/eradication programmes using the sterile insect technique or inherited sterility, in *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management* (Dyck, V. A., Hendrichs, J., and Robinson, A. S., Eds.), pp 677−700, Springer, The Netherlands.

(2) Knipling, E. (1955) Possibilities of insect control or eradication through the use of sexually sterile males. *J. Econ. Entomol. 48*, 459−469.

(3) Dyck, V. A., Hendrichs, J., and Robinson, A. S. (Eds.) (2005) *Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management*, Springer, The Netherlands.

(4) Bloem, S., and Carpenter, J. (2001) Evaluation of population suppression by irradiated Lepidoptera and their progeny. *Florida Entomol. 84*, 165−171.

(5) Blomefield, T., Bloem, S., and Carpenter, J. (2010) Effect of radiation on fecundity and fertility of codling moth *Cydia pomonella* (Linnaeus) (Lepidoptera: Tortricidae) from South Africa. *J. Appl. Entomol. 134*, 216−220.

(6) Rendón, P., McInnis, D., Lance, D., and Stewart, J. (2000) Comparison of medfly male-only and bisexual releases in large scale field trials, in *Area-Wide Control of Fruit Flies and Other Insect Pests* (Tan, K., Ed.), pp 517−525, Penerbit Universit Sains Malaysia, Pulau Pinang.

(7) Rendón, P., McInnis, D., Lance, D., and Stewart, J. (2004) Medfly (Diptera:Tephritidae) genetic sexing: large-scale field comparison of males-only and bisexual sterile fly releases in Guatemala. *J. Econ. Entomol. 97*, 1547−1553.

(8) Franz, G. (2005) Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique, in *Sterile Insect Technique. Principles and Practice in Area-Wide Integrated Pest Management* (Dyck, V. A., Hendrichs, J., and Robinson, A. S., Eds.), pp 427−451, Springer, The Netherlands.

(9) Strunnikov, V. (1975) Sex control in silkworms. *Nature 255*, 111−113.

(10) Marec, F., Neven, L., Robinson, A., Vreysen, M., Goldsmith, M., Nagaraju, J., and Franz, G. (2005) Development of genetic sexing strains in Lepidoptera: from traditional to transgenic approaches. *J. Econ. Entomol. 98*, 248−259.

(11) Morrison, N. I., Franz, G., Koukidou, M., Miller, T., Saccone, G., Alphey, L., Beech, C., Nagaraju, J., Simmons, G., and Polito, L. (2011) Genetic improvements to the sterile insect technique for agricultural pests. *AsPac J. Mol. Biol. Biotechnol. 18*, 275−295.

(12) Henneberry, T. J., and Naranjo, S. E. (1998) Integrated management approaches for pink bollworm in the southwestern United States. *Int. Pest Manage. Rev. 3*, 31−52.

(13) Suckling, D., Barrington, A., Chhagan, A., Stephens, A., Burnip, G., Charles, J., and Wee, S. (2007) Eradication of the Australian painted apple moth *Teia anartoides* in New Zealand: Trapping, inherited sterility, and male competitiveness, in *Area-Wide Control of Insect Pests* (Vreysen, M., Robinson, A., and Hendrichs, J., Eds.), pp 603−615, Springer, The Netherlands.

(14) Gong, P., Epton, M., Fu, G., Scaife, S., Hiscox, A., Condon, K., Condon, G., Morrison, N., Kelly, D., Dafa'alla, T., Coleman, P., and Alphey, L. (2005) A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. *Nat. Biotechnol. 23*, 453−456.

(15) Fu, G., Condon, K. C., Epton, M. J., Gong, P., Jin, L., Condon, G. C., Morrison, N. I., Dafa'alla, T. H., and Alphey, L. (2007) Female-specific insect lethality engineered using alternative splicing. *Nat. Biotechnol. 25*, 353−357.

(16) Morrison, N. I., Simmons, G., Fu, G., Walker, A., O'Connell, S., Dafa'alla, T., Walters, M., Claus, J., Tang, G., Jin, L., Marubbi, T., Epton, M., Staten, R., Miller, E., Miller, T., and Alphey, L. (2012) Engineered repressible lethality for control of the pink bollworm, a lepidopteran pest of cotton. *PLoS ONE 7*, No. e50922.

(17) Fu, G., Lees, R. S., Nimmo, D., Aw, D., Jin, L., Gray, P., Berendonk, T. U., White-Cooper, H., Scaife, S., Kim Phuc, H., Marinotti, O., Jasinskiene, N., James, A. A., and Alphey, L. (2010) Female-specific flightless phenotype for mosquito control. *Proc. Natl. Acad. Sci. U.S.A. 107*, 4550−4554.

(18) Phuc, H. K., Andreasen, M. H., Burton, R. S., Vass, C., Epton, M. J., Pape, G., Fu, G., Condon, K. C., Scaife, S., Donnelly, C. A., Coleman, P. G., White-Cooper, H., and Alphey, L. (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Bio.l 5*, 11.

(19) Labbé, G. M., Scaife, S., Morgan, S. A., Curtis, Z. H., and Alphey, L. (2012) Female-specific flightless (fsRIDL) phenotype for control of *Aedes albopictus*. *PLoS Neglected Trop. Dis. 6*, e1724.

(20) Gossen, M., and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc. Natl. Acad. Sci. U.S.A. 89*, 5547−5551.

(21) Thomas, D. D., Donnelly, C. A., Wood, R. J., and Alphey, L. S. (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science 287*, 2474−2476.

(22) Simmons, G. S., McKemey, A. R., Morrison, N. I., O'Connell, S., Tabashnik, B. E., Claus, J., Fu, G., Tang, G., Sledge, M., Walker, A. S., Phillips, C. E., Miller, E. D., Rose, R. I., Staten, R. T., Donnelly, C. A., and Alphey, L. (2011) Field performance of a genetically engineered strain of pink bollworm. *PLoS One 6*, e24110.
(23) Suzuki, M. G., Ohbayashi, F., Mita, K., and Shimada, T. (2001) The mechanism of sex-specific splicing at the doublesex gene is different between *Drosophila melanogaster* and *Bombyx mori*. *Insect Biochem. Mol. Biol. 31*, 1201−1211.
(24) Ohbayashi, F., Suzuki, M. G., Mita, K., Okano, K., and Shimada, T. (2001) A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 128*, 145−158.
(25) McInnis, D. O., Lance, D. R., and Jackson, C. G. (1996) Behavioural resistance to the sterile insect release technique by the Mediterranean fruit fly (Diptera: Tephritidae) in Hawaii. *Ann. Entomol. Soc. Am. 89*, 739−744.
(26) FAO/IAEA/USDA (2003) *Manual for Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies v5.0*, IAEA, Vienna, Austria.
(27) Wang, X.-P., Fang, Y.-L., and Zhang, Z.-N. (2005) Effect of male and female multiple mating on the fecundity, fertility, and longevity of diamondback moth, *Plutella xylostella* (L.). *J. Appl. Entomol. 129*, 39−42.
(28) 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. *J. Environ. Qual. 34*, 2082−2085.
(29) Alphey, N., Bonsall, M. B., and Alphey, L. (2011) Modeling resistance to genetic control of insects. *J. Theor. Biol. 270*, 42−55.
(30) Koyama, J., Kakinohana, H., and Miyatake, T. (2004) Eradication of the Melon Fly *Bactrocera cucurbitae* in Japan: Importance of behaviour, ecology, genetics and evolution. *Ann. Rev. Entomol. 49*, 331−349.
(31) Alphey, N., Coleman, P. G., Donnelly, C. A., and Alphey, L. (2007) Managing insecticide resistance by mass release of engineered insects. *J. Econ. Entomol. 100*, 1642−1649.
(32) Alphey, N., Bonsall, M., and Alphey, L. (2009) Combining pest control and resistance management: synergy of engineered insects with *Bt* crops. *J. Econ. Entomol. 102*, 717−732.
(33) Martins, S., Naish, N., Walker, A., Morrison, N., Scaife, S., Fu, G., Dafa'alla, T., and Alphey, L. (2012) Germline transformation of the diamondback moth, *Plutella xylostella* L., using the *piggyBac* transposable element. *Insect Mol. Biol. 21*, 414−421.
(34) Alan Dever, G. E. (1984) *Epidemiology in Health Services Management*, Aspen Systems Corporation, Rockville, MD.