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Female-specific insect lethality engineered using alternative splicing

Guoliang Fu[1,3], Kirsty C Condon[1–3], Matthew J Epton[2], Peng Gong[2], Li Jin[1], George C Condon[1], Neil I Morrison[1,2], Tarig H Dafa'alla[1] & Luke Alphey[1,2]

The Sterile Insect Technique is a species-specific and environmentally friendly method of pest control involving mass release of sterilized insects that reduce the wild population through infertile matings[1–5]. Insects carrying a female-specific autocidal genetic system offer an attractive alternative to conventional sterilization methods[6,7] while also eliminating females from the release population[7–10]. We exploited sex-specific alternative splicing in insects to engineer female-specific autocidal genetic systems in the Mediterranean fruit fly, *Ceratitis capitata*. These rely on the insertion of cassette exons from the *C. capitata transformer* gene into a heterologous tetracycline-repressible transactivator such that the transactivator transcript is disrupted in male splice variants but not in the female-specific one. As the key components of these systems function across a broad phylogenetic range, this strategy addresses the paucity of sex-specific expression systems (e.g., early-acting, female-specific promoters) in insects other than *Drosophila melanogaster*. The approach may have wide applicability for regulating gene expression in other organisms, particularly for combinatorial control with appropriate promoters.

The Mediterranean fruit fly, or medfly, is one of the most economically important agricultural pest insects in the world. It is a true fruit fly (family Tephritidae); this group also contains many other important pest species. The Sterile Insect Technique (SIT) has been used extensively for the control of medflies, and also on a smaller scale against other tephritids. Although early SIT programs released both males and females, it is now considered highly desirable to release only males[9,10]. For some species, sterile females are potentially damaging, either to agriculture (e.g., medflies) or public health (e.g., mosquitoes). Co-release of sterile females may also cause the sterile males to court these co-released females instead of seeking out wild females. In some species, sufficient sexual dimorphism exists for efficient sex separation by physical methods. Alternatively, a suitable sexual dimorphism can be induced by genetics; such strains are known as genetic-sexing strains. Sexing strains established using classical genetics have a recessive, selectable marker on an autosome and a covering translocation to the Y chromosome[11]. Various selectable markers have been used, including insecticide resistance and pupal color, but perhaps the most sophisticated is the medfly temperature-sensitive-lethal (tsl) system[10,11]. The mutations and chromosome rearrangements required to construct these strains inevitably impose various fitness penalties; for the tsl strains these are clearly outweighed by the benefits, but are still undesirable. Although these systems are triumphs of classical genetics, their development is lengthy and uncertain. Furthermore, none of the special chromosomes created can be transferred to another species.

Recombinant DNA methods have the potential to produce alternative genetic sexing systems, with proof of principle provided in the model insect *D. melanogaster*[7,8]. One attractive feature of such systems is the prospect that they might be translated from one species to another much more readily than translocation-based systems. Furthermore, a specific strategy termed 'release of insects carrying a dominant lethal' (RIDL) could provide both genetic sexing and 'sterilization' from the same construct[6,7,12], which strains developed by classical genetics cannot readily accomplish. Such a scheme would involve mass-rearing a strain homozygous for a repressible female-specific dominant lethal under permissive conditions. The release generation would be reared under restrictive conditions (without the repressor), killing the females and providing a male-only population for release. These released males would mate with wild females, and their female progeny, lacking the repressor chemical, would die. This would reduce the reproductive capacity of the population, thereby potentially controlling it[6,7,13,14].

A severe limitation in the development of this approach in insects besides *D. melanogaster* has been the general lack of characterized gene expression systems capable of conferring female-specific expression at early developmental stages. We developed a system for the control of gene expression to overcome this problem, and used it to construct pest strains with the necessary properties for this approach.

The proof-of-principle experiments in *D. melanogaster* used the tetracycline-repressible transcription factor (tTA)[15] system to control the expression of an effector molecule by a tetracycline response element (tRE), and yolk protein promoters to confer female specificity[7,8]. These promoters are not ideal, as they may not conserve their female specificity between species[16] and also act later in development than is desirable. Furthermore, these promoters contain strong

[1]Oxitec Limited, 71 Milton Park, Oxford OX14 4RX, UK. [2]Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK. [3]These authors contributed equally to this work. Correspondence should be addressed to L.A. (luke.alphey@zoo.ox.ac.uk).

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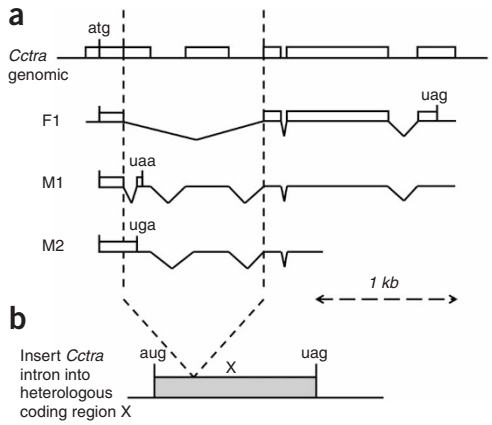

Figure 1 Sex-specific expression by alternative splicing of *Cctra*. (**a**) Sex-specific alternative splicing of medfly *transformer* (*Cctra*). *Cctra* is spliced in females to produce three transcripts: F1, M1 and M2. F1 encodes functional Tra protein, but M1 and M2 do not because they include additional exons with in-frame stop codons (redrawn from ref. 19). Males produce only transcripts M1 and M2 and therefore do not produce functional Tra protein. (**b**) Use of *Cctra* intron to regulate gene expression. If this alternatively spliced intron were to retain its function in a heterologous coding region, females, but not males, would produce functional protein X.

transcriptional enhancers, making it difficult to combine the two components of the expression system (promoter-tTA and tRE-effector) in a single construct. We therefore attempted a different approach, using alternative splicing. Along with promoter specificity, alternative splicing plays a key role in regulating gene expression in many developmental processes, including sex determination[17–20], but has not been widely used in genetic engineering.

Two key sex-determination genes of insects, *transformer* (*tra*) and *doublesex* (*dsx*), are both regulated by sex-specific alternative splicing[17,20]. The *tra* coding region is interrupted by additional exons in males; only females produce a splice variant encoding functional Tra protein. We have exploited this female-specific excision of a cassette exon to prevent expression of an engineered protein in males, thereby making its expression female-specific (**Fig. 1**).

As the sequence elements necessary for the sex-specific alternative splicing of medfly *transformer* (*Cctra*) are unclear[19], we analyzed transgenic medflies containing constructs with varying amounts of *Cctra*-derived exonic sequence flanking the *Cctra* intron. These experiments suggested that it might be possible to use the intron with no *Cctra*-derived exonic sequence. This would give greater flexibility in the use of the strategy illustrated in **Figure 1**, which could otherwise only produce fusion proteins including all or part of *Cctra*. Further preliminary transgenic experiments suggested that the dinucleotides TG…GT immediately adjacent to the intron might be important for correct splicing. We inserted the *Cctra* female-specific intron into the tTAV-coding region (between TG and GT nucleotides) in a positive-feedback configuration previously shown to give repressible dominant lethality in medflies[21] to make constructs LA3077 and LA3097 (**Fig. 2**). tTAV is a variant of tTA optimized for expression in *D. melanogaster*. Transgenic medflies carrying LA3077 or LA3097 processed the *Cctra* intron correctly. The F1 variant appeared only in females and sequence analysis showed that all splice variants and junctions corresponded to the predicted forms (**Figs. 1** and **2**).

Constructs equivalent to LA3077 and LA3097, but without the *Cctra* intron, give tetracycline-repressible, dominant lethality in transgenic medflies[21]. We found that LA3077 and LA3097 correspondingly gave repressible female-specific lethality, predominantly as pupae (**Fig. 3** and **Supplementary Tables 1** and **2** online). Genetic sexing systems made by classical genetics using visible markers such as pupal color also allow sex separation as pupae, though requiring individual examination and sorting. Embryonic female-specific lethality, as seen in the medfly tsl strains, would be preferable as this eliminates diet consumption by unwanted females. Engineered early-acting, tetracycline-repressible lethality may be achievable by use of suitable embryonic promoters[22].

The LA3097 lines show highly penetrant, dominant female-specific lethality (LA3097A, 98%; LA3097B and LA3097C, both 100%, **Fig. 3**) and in this regard are suitable for genetic sexing and/or a female-specific RIDL strategy as outlined above. On a tetracycline-containing diet, two lines (LA3077A and LA3097B) showed a small but significant ($P < 0.002$ and $P < 0.0001$, respectively) loss of females. This may indicate incomplete suppression by this concentration of tetracycline (100 μg/ml), or a tetracycline-independent effect. Similar effects, varying from one insertion line to another, were observed previously[21]. Survival of transgenic female progeny did not seem to be affected by maternal tetracycline (mothers raised on 0 or 100 μg/ml tetracycline). We have also shown previously that constructs of similar

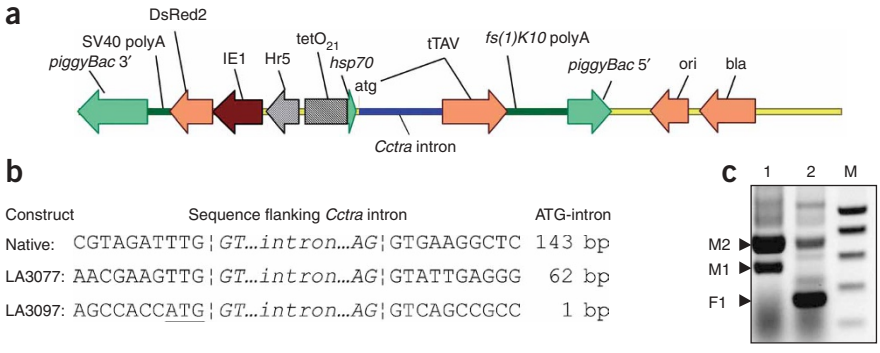

Figure 2 Sex-specific lethal constructs. (**a**) Diagrammatic representation of plasmid pLA3097, linearized at the 3′ end of the *piggyBac* transposon. Within the ends of the transposon are two functional segments: the marker (hr5-IE1-DsRed2-SV40 3′)[21] to allow detection of transgenic individuals by fluorescence and the tTAV expression cassette ($tetO_{21}$-*Dmhsp70* minimal promoter – Cctra:tTAV – *fs(1)K10* 3′). The tTAV expression cassette acts by a positive feedback mechanism to induce lethality[21], but here its expression and therefore lethality is limited to females by a *Cctra*-derived alternative splicing system. The principal difference between pLA3077 and pLA3097 is the location of the *Cctra* intron. (**b**) Intron-exon boundary sequences of native *Cctra* and *Cctra*-based constructs. The sequence immediately flanking the first intron of *Cctra* F1 is shown in its native context and as inserted in tTAV coding sequence in constructs LA3077 and LA3097. Actual splice junctions, as determined by RT-PCR and sequencing, are marked (¦). The start codon of tTAV is underlined. The distances between the start codon (ATG) and the *Cctra* intron are also shown. (**c**) *Cctra* intron splices correctly in a heterologous context. Gel photo of RT-PCR from male (lane 1) and female (lane 2) LA3097C transgenic Medflies. Lane M, DNA size standards: 200–1,000 bp in 200-bp increments (Eurogentec SmartLadder). Band corresponding to splicing in the F1 pattern (reconstituting the tTAV open reading frame) is seen only in females.

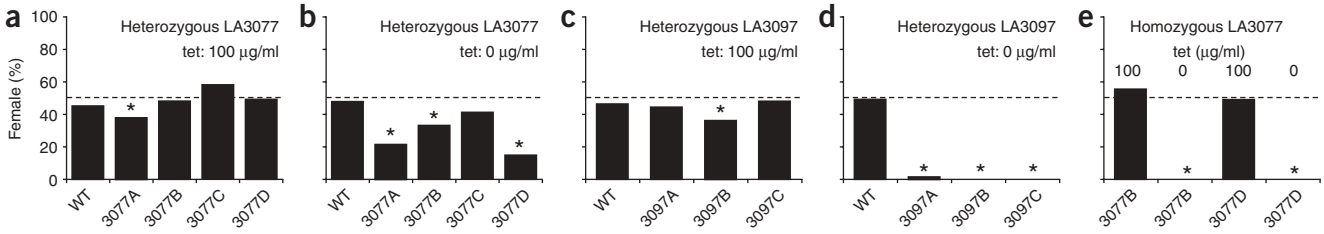

Figure 3 Repressible female-specific lethality in transgenic Medflies. (**a**–**d**) Seven independent, autosomal insertion lines of LA3077 and LA3097 were generated in Medflies (LA3077A-D and LA3097A-C). All lines were determined to be single insertions based on segregation analysis and sequencing each insertion site. Males heterozygous for LA3077 (**a**,**b**) or for LA3097 (**c**,**d**) were crossed to wild-type (WT) females. The progeny were raised on a diet supplemented with tetracycline to 100 μg/ml (**a**,**c**) or on a normal diet (**b**,**d**). Adult progeny were scored for fluorescence, that is, presence of the construct, and for sex. Graphs show the proportion of females of each class; the dotted line at 50% across each graph represents the expected sex ratio in the absence of female-specific effects. The sex ratio of the nonfluorescent (WT) sibling controls did not differ significantly between lines ($\chi^2 = 16.221$, $P > 0.05$, d.f. = 13, see **Supplementary Table 1** online); these are shown aggregated in the first column of **a**–**d**. Other columns are labeled with the construct name and a letter to represent the insertion line (e.g., LA3077A, LA3077B). (**e**) Effect of increased copy number of LA3077 on the penetrance of the female-specific lethality. Homozygous lines of two insertions of LA3077 were constructed (LA3077B and LA3077D). Homozygous males and females were held and mated on a tetracycline-supplemented or normal diet and their progeny were reared on the same diet as the parents; the numbers of males and females surviving to adulthood were scored (see also **Supplementary Table 2** online). Crosses where significant female-specific lethality was observed (chi-squared test, $P < 0.01$) are marked with an asterisk. Data are the sum of at least three independent experiments.

design, but lethal to both males and females rather than females only, need not have significant deleterious effects on adult male longevity or mating competitiveness[21]. LA3077 is less effective than LA3097 (**Fig. 3**). There are several slight differences between these constructs that might cause this; one possibility is that the M1 and M2 splice variants of LA3077 might express small fragments of tTAV that interfere with the normal dimerization and function of tTAV. Although the LA3077 lines gave female-specific dominant lethality in the absence of tetracycline (20–80% fewer heterozygous LA3077 females than wild-type females or heterozygous LA3077 males), this is not sufficiently penetrant for 'genetic sterilization'[6,7]. However, for genetic sexing, the female-specific lethality would operate on the mass-reared insects themselves, rather than on their progeny after mating to wild type, and therefore on homozygotes rather than heterozygotes. We therefore examined LA3077 homozygotes. LA3077B and LA3077D homozygotes both gave 100% female lethality (**Fig. 3e**). Therefore, efficient genetic sexing is readily provided by this construct design.

Alternative splicing is potentially an extremely powerful tool for genetic engineering, but may have been avoided until now because there are several potential difficulties. Selection between different potential splice variants is generally thought to depend on exonic splice enhancers and silencers[17,23]; performance of an intronic fragment may therefore depend on the context in which it is placed. Earlier attempts using alternative splice acceptors from *D. melanogaster tra* (*Dmtra*) had variable success—an *Act5C-Dmtra*-β-galactosidase fusion showed sex-specific splicing, whereas a similar *Dmtra-InaZ* fusion did not, even though both had substantial amounts of 3′ exonic material from *Dmtra*[24]. Another potential difficulty concerns saturation of the response capacity. The factors regulating alternative splicing are thought to be in relatively short supply, so that the alternative splicing pathway may be saturated if too much pre-mRNA is provided[25–27]. In order for the female-specific, positive-feedback system to be lethal, large amounts of tTAV must be produced[21], and therefore high levels of F1-type splicing are required in females. The demonstration that this occurs implies that the Tra/Tra2-dependent sex-specific splicing system is not easily saturated. For the *Cctra* system, a third problem is inefficiency: a substantial proportion of the pre-mRNA (primary transcript) in females is processed in the M1 and M2 forms (**Fig. 1**); these do not produce functional protein and therefore tend to attenuate the lethality of LA3077 and LA3097 relative to previous non-sex-specific constructs[21]. However, although this effect occurs, the lethality of both constructs shows the system functions as desired.

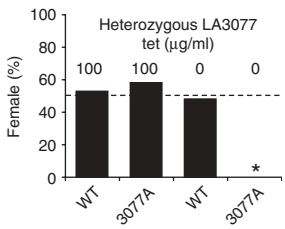

Figure 4 Conservation of function of a *Cctra*-based construct. *D. melanogaster* was transformed with LA3077. Heterozygous males were crossed to wild-type (WT) females and adult progeny analyzed for fluorescence, that is, presence of the LA3077 construct, and for sex. The sex ratio of both nonfluorescent (WT) progeny and transgenic (3077A) progeny was normal on tetracycline-supplemented diet (see **Supplementary Table 3** online); however, no transgenic female progeny were recovered from normal diet (leftmost two columns: tetracycline-supplemented diet, rightmost two columns: normal diet).

A major potential benefit of genetic engineering is the possibility of rapidly translating success from one species to another, which is essentially impossible for classical genetics. Genetic sexing strains would be highly desirable for many species of tephritid fruit fly. To determine whether our *Cctra*-based system would splice correctly in other species, we used the vinegar fly *D. melanogaster*, which diverged from the Tephritidae ~120–150 million years ago[28]. Two independent lines of transgenic *D. melanogaster*, each carrying LA3077, gave dominant, tetracycline-repressible, female-specific lethality (**Fig. 4**, data not shown and **Supplementary Table 3** online). As these were 100% penetrant, we did not transform *D. melanogaster* with LA3097, which was created later. On close examination, we found subtle differences in the splicing of the *Cctra* intron between medfly and *D. melanogaster* (**Supplementary Fig. 1** online); nonetheless, the crucial F1 splice variant is identical and completely female-specific in both species. In *D. melanogaster*, *tra* is regulated by *Sex-lethal* (*Sxl*) rather than being autoregulated. However, our data indicate that the system regulating *Cctra* is functionally conserved in *D. melanogaster*.

In other words, DmTra/DmTra-2 can recognize and process *Cctra*-derived transcripts correctly, possibly through the putative Tra/Tra-2 binding sites previously observed in the *Cctra* intron[19]. CcTra protein can promote the female-specific splicing of known *tra* targets in *D. melanogaster*[29]. Together, these observations suggest that the Tra/Tra-2 splicing system is functionally conserved between these species despite the low overall sequence identity between CcTra and DmTra. This further emphasizes the likely wide applicability of the alternative splicing system described here. We therefore expect that constructs of this type will function in most or all higher Diptera, including economically important Tephritidae such as Natal fruit fly (*Ceratitis rosa*), Melon fruit fly (*Bactrocera cucurbitae*), Queensland fruit fly (*Bactrocera tryoni*) and South American fruit fly (*Anastrepha fraterculus*).

Our results demonstrate the potential of controlling gene expression through alternative splicing. We used alternative splicing to confer female specificity on a conditional promoter, but many other combinations are possible. For example, expression specifically in female embryos only would be extremely difficult by conventional methods—promoters with this specificity are unknown, even in *D. melanogaster*. However, one could combine an embryo-specific promoter, for example from *serendipity α* (*sryα*)[22], with a suitable splicing system. Such combinatorial control can provide patterns and precision that would be difficult to obtain by any other method. Furthermore, as alternative splicing systems of varying tissue and sex specificity are known from a wide range of eukaryotes, such combinatorial control for precise regulation of gene expression may be of wide applicability.

METHODS

Plasmid construction. In essence, the orientation of the marker in pLA928 (ref. 21) was reversed and the copy number of tetO increased to 21 to give pLA1124. The *Cctra* intron[19] was then inserted into tTAV to generate pLA3077 and pLA3097. While cloning fragments of *Cctra*, we found an additional small intron 3′ to the alternatively spliced intron; this is included in **Figure 1**. The key features of, and differences between, pLA3077 and pLA3097 are shown in **Figure 2**. Note that the *hsp70* promoter used is the proximal promoter fragment only, including the transcription start and a short leader sequence; this is not predicted to respond to heat shock. tTAV is a variant of tTA optimized for expression in *D. melanogaster* by eliminating potential cryptic splice sites and rare codons in the *tet*R region[21]. The *fs(1)K10* sequence provides a 3′ UTR and polyadenylation signal; it has no sex-specific function[21]. The complete predicted sequences of plasmids pLA3077 and pLA3097 are provided in **Supplementary Figures 2** and **3** online.

Fruit fly rearing and genetics. Medfly transformation and rearing was performed as previously described[21]. Toliman wild-type strain was used both for breeding experiments and as the background strain for transformation. The recipient strain for *D. melanogaster* transformation was w^{1118}; SamI236 (ref. 30).

Molecular analysis. Transcripts were analyzed by reverse-transcriptase PCR (RT-PCR), using SuperScript III One-Step RT-PCR System (Invitrogen), according to the manufacturer's instructions, and the following oligonucleotides:
HSP: 5′-CAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTA-3′
TETRR1: 5′-GCGGAACGACTTGGCGTTATTGCG-3′
TETRR2: 5′-CCAGTGTCTCGTATTGCTTTTCGGTC-3′
HSPAC: 5′-GCTAAACAATCTGCAGGTACCCTGGCG-3′
VP16: 5′-GCCCTCGATGGTAGACCCGTAATTG-3′

All major RT-PCR products were sequenced, using the above oligonucleotides and BigDye v3.1 sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Reaction products were run on Applied Biosystems automated sequencers in the central sequencing facility, Department of Zoology, Oxford University. Electropherogram and sequence data were analyzed using Vector NTi (Invitrogen).

Requests for materials. luke.alphey@zoo.ox.ac.uk.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS
G.F. and K.C.C. contributed equally to this work. G.F. and L.J. made the DNA constructs and assisted with the molecular analysis of transgenics. K.C.C. and G.C.C. created the transgenic medfly lines described and conducted most of the phenotypic and molecular analysis. M.J.E. and P.G. performed preliminary experiments in medflies. N.I.M. performed the *D. melanogaster* experiments. T.H.D. provided advice. L.A. conceived and supervised the project and wrote the paper. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS STATEMENT
The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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