Corrections

APPLIED BIOLOGICAL SCIENCES

Correction for "Female-specific flightless phenotype for mosquito control," by Guoliang Fu, Rosemary S. Lees, Derric Nimmo, Diane Aw, Li Jin, Pam Gray, Thomas U. Berendonk, Helen White-Cooper, Sarah Scaife, Hoang Kim Phuc, Osvaldo Marinotti, Nijole Jasinskiene, Anthony A. James, and Luke Alphey, which appeared in issue 10, March 9, 2010, of *Proc Natl Acad Sci USA* (107:4550–4554; first published February 22, 2010; 10.1073/pnas.1000251107).

The authors note that their conflict of interest statement was omitted during publication. The authors declare that those authors affiliated with Oxitec Limited (as noted in the author list) are or were employees or collaborative students of this company, which therefore provided salary and other support for the research program. Also, such employees may have shares or share options in Oxitec Limited. Both Oxitec Limited and Oxford University have one or more patents or patent applications related to the subject of this paper.

MICROBIOLOGY

Correction for "Lsr2 is a nucleoid-associated protein that targets AT-rich sequences and virulence genes in *Mycobacterium tuberculosis*," by Blair R. G. Gordon, Yifei Li, Linru Wang, Anna Sintsova, Harm van Bakel, Songhai Tian, William Wiley Navarre, Bin Xia, and Jun Liu, which appeared in issue 11, March 16, 2010, of *Proc Natl Acad Sci USA* (107:5154–5159; first published January 20, 2010; 10.1073/pnas.0913551107).

The authors note that author Bin Xia should have been listed as an additional corresponding author. The corrected author line and correspondence footnote appear below. The online version has been corrected.

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Female-specific flightless phenotype for mosquito control

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Dengue and dengue hemorrhagic fever are increasing public health problems with an estimated 50–100 million new infections each year. *Aedes aegypti* is the major vector of dengue viruses in its range and control of this mosquito would reduce significantly human morbidity and mortality. Present mosquito control methods are not sufficiently effective and new approaches are needed urgently. A "sterile-male-release" strategy based on the release of mosquitoes carrying a conditional dominant lethal gene is an attractive new control methodology. Transgenic strains of *Aedes aegypti* were engineered to have a repressible female-specific flightless phenotype using either two separate transgenes or a single transgene, based on the use of a female-specific indirect flight muscle promoter from the *Aedes aegypti Actin-4* gene. These strains eliminate the need for sterilization by irradiation, permit male-only release ("genetic sexing"), and enable the release of eggs instead of adults. Furthermore, these strains are expected to facilitate area-wide control or elimination of dengue if adopted as part of an integrated pest management strategy.

Aedes aegypti | dengue virus | sterile insect technique | genetic control | population suppression

Dengue fever, including dengue hemorrhagic fever and dengue shock syndrome, is an increasing public health problem, with an estimated 50–100 million new infections per year (1–3). It is a mosquito-borne viral disease and *Aedes aegypti* is the principal vector. *Aedes aegypti* is an invasive species spread inadvertently around the world by human trade and travel and is now distributed widely in tropical and subtropical regions, most notably in urban environments where it has adapted to breed in artificial containers and refuse. This increase in the geographic distribution of the vector is accompanied by the emergence of the viruses and disease in new areas. There is currently no vaccine or specific therapeutic drug available for dengue; therefore, control focuses on the mosquito. Bed nets are largely ineffective against this day-biting mosquito, making source reduction and space spraying the mainstays of control. However, the mosquito breeds in a wide variety of containers, and finding and treating sufficient numbers of them is extremely challenging or impossible for even the most well-funded and organized programs. New approaches and vector control tools are needed urgently.

The sterile insect technique (SIT) is an environmentally friendly, species-specific population control method used for area-wide control of several major agricultural pests (4–6). *Aedes aegypti* is a particularly suitable target for suppression or local elimination through SIT, because it is readily reared and manipulated in the laboratory, suitable for mass rearing (7), and is found at relatively low densities in disease-endemic locales (1, 3). However, despite encouraging trials in the 1970s (8–9), there are currently no large-scale SIT programs against this or any other mosquito species.

The use of SIT against mosquitoes is problematic, due mainly to the fitness costs and operational difficulty of irradiation, and the density-dependent nature of the target mosquito populations (10–12). In addition, male-only release is preferred for mosquito SIT due to the biting nuisance and potential disease transmission caused by adult females. Distribution of mosquitoes also may be more problematic than for current SIT target species, for example, *Ceratitis capitata* (Medfly) and *Cochliomyia hominivorax* (New World Screwworm), because adult mosquitoes are less robust and more likely to suffer damage during transit and release. These issues could be mitigated by the use of insects engineered to carry specific novel traits, including conditional genetic sterilization or lethality, that removes the need for irradiation (10, 12–14). Insects engineered to carry a female-specific lethal (or otherwise incapacitating) gene could be used to remove females prior to release (8, 11, 14–16). A system based on a lethal gene [release of insects carrying a dominant lethal (RIDL); (14)] that acts late in development would prevent mosquitoes from becoming adults, the only harmful life stage, yet enable them to survive and compete at the larval stage, when density-dependent competition occurs (17). Modeling this system predicts that fewer male mosquitoes of a late-lethal strain need to be released as compared to those carrying an early-lethal gene or irradiated strain to achieve an equivalent level of control of a target population (12, 18). We reported previously the construction of repressible lethal strains of insects using the "tet-off" gene expression system based on a synthetic tetracycline-repressible transactivator (tTA) (12, 14, 16, 19, 20). Here we describe the construction of transgenic *Ae. aegypti* that combine all of the genetic features necessary to produce highly penetrant, dominant, late-acting, female-specific lethality. The promoter derived from the *Aedes aegypti Actin-4 (AeAct-4)* gene leads to the expression of tTA in a stage-, tissue-, and sex-specific manner resulting in female-specific RIDL strains for population suppression of the most significant vector of dengue viruses.

Results

AeAct-4 is expressed in female pupae, predominantly in the indirect flight muscles (IFMs) (21). Further analysis of this gene revealed a low-abundance transcript in males, which through alternative splicing, contains an additional 244 nucleotides in the 5′ UTR relative to the female transcript (Fig. 1*A* and Fig. S1). The biological basis for this differential splicing is unknown, but the
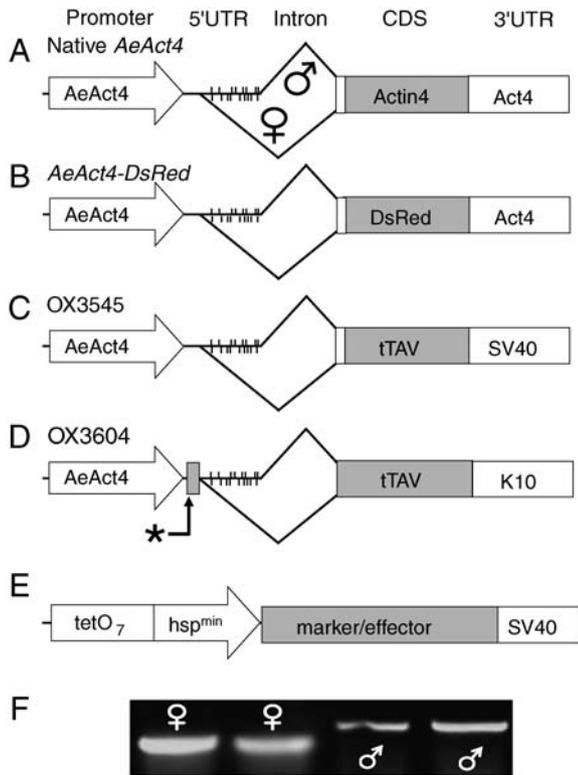
Fig. 1. Schematic representation of *Actin4* gene and the constructs used: (*A*) native *Actin4* gene; (*B*) *AeAct-4*-DsRed; (*C*) OX3545; (*D*) OX3604; (*E*) marker construct OX3576 (tRE-DsRed2) and the effector constructs OX3547 (tRE-Nipp1Dm) and OX3582 (tRE-michelob_x); (*F*) alternative splicing of *AeAct-4* in OX3604C; RT-PCR products from females (583 bp) and males (826 bp). (*A–E*) Origins of coding region (CDS) and 3′ UTR sequences are indicated [K10 indicates the *Drosophila* gene *fs(1)K10*]. Short vertical lines above and below the thick horizontal line represent potential translation start (ATG) and stop codons, respectively. The first ATG of the female splice variant of *AeAct-4* is that of the *AeAct-4* coding region; the male splice variant has multiple 5′-end ATGs. (*D*) OX3604 has an additional engineered 5′-end ATG, indicated with an asterisk; this leads to the alternatively spliced intron being within the tTAV open reading frame (ORF), consequently this ORF is interrupted by stop codons in the male splice variant. (*E*) tTA response element (tRE) comprises multiple copies of the tTA binding sequence, tetO, and a minimal promoter (hsp^min), here from *Drosophila* hsp70.

sequence of the male-specific product contains several start and stop codons that may lead to premature initiation or termination of translation and thereby reduce the amount of functional AeAct-4 protein made from these transcripts.

A putative promoter region from *AeAct-4* was used to drive expression of the reporter gene product, DsRed, in transgenic *Ae. aegypti* (Fig. 1*B*). Two independent lines analyzed both exhibited strong red fluorescence in the IFMs of female pupae and adults (see Fig. 2). These results support the use of the *AeAct-4* regulatory sequences to drive expression of a repressible deleterious or lethal system in *Ae. aegypti*. Because IFMs are not a vital tissue in the vinegar fly, *Drosophila melanogaster* (22, 23), we predicted that their disruption in mosquitoes would result in a flightless phenotype, rather than lethality. However, the inability to fly is likely to be a lethal condition in the field, with adult females suffering a reduced ability to escape water after emerging, and an increased vulnerability to predation in and around breeding sites, as well as an inability to fly to locate a blood meal and mate. Indeed, the *AeAct-4* regulatory DNA provides one of the last opportunities in development to kill females before blood feeding, and is therefore ideal for a late-acting RIDL trait.

Seven independent lines of an *AeAct4*-tTA construct (OX3545, Fig. 1*C*) were made and crossed to a transgenic line carrying a DsRed reporter gene under control of a tetracycline responsive element (tRE), comprising multiple copies of the tetO sequence, to which tTA binds, together with a minimal promoter (Fig. 1*E*). *AeAct4*-mediated expression of tTA in these dual transgene hybrid mosquitoes produces the tTA transactivator that then binds the operator and triggers expression of the reporter gene product. All *AeAct4*-tTA, tRE-DsRed progeny displayed strong red fluorescence in the developing IFMs of late fourth-instar female larvae, as well as in pupae and adults (Fig. S2). DsRed expression appeared to be fully repressible by tetracycline (Tc); equivalent insects reared in medium containing 30 µg/mL Tc ("reared on Tc") showed no such fluorescence. Three of seven *AeAct4*-tTA lines also showed DsRed expression in other female tissues, one of which also showed DsRed expression in males, although never as strongly as in the female IFMs. Weak expression of DsRed in other lines or tissues might not have been observed due to the confounding presence of the transformation marker. We interpret these observations to indicate that, although the *AeAct-4* promoter fragment used directs expression reliably in female IFMs, it is also sensitive to its chromosomal context ("position effect"). Furthermore, and contrary to our original expectation, the sex-specific alternative splicing does not always prevent expression of tTA in males in this construct, as exhibited by expression of DsRed in the flight muscles of males when certain lines of *AeAct4*-tTA were crossed with tRE-DsRed.

OX3545 strains carrying the *AeAct4*-tTA construct were crossed to transgenic lines OX3547 and OX3582, containing lethal effector genes *Nipp1Dm* (24, 25) and *michelob_x* (26), respectively, under tRE control. A high proportion of females (69.8–98.3%; Table 1) carrying both tTA and effector genes were flightless when reared in the absence of Tc, showing that expression of a suitable effector in the IFMs can cause a flightless phenotype.

In the course of these experiments, we observed some flightless females in the OX3545 lines (Table 1), even though no effector gene was present. Although expression of tTA at moderate levels appears to be innocuous, expression at high levels can be deleterious, disrupting transcription and the ubiquitin-dependent protein degradation system (20, 27–30). Indeed, tTA can be used as the effector molecule in a repressible lethal system (12, 16, 19). We infer that the *AeAct-4* fragment used represents a strong promoter that expresses tTA at a level close to the threshold for IFM damage, and that expression is modulated by position effects so that different lines show the flightless phenotype to a greater or lesser extent. Furthermore, we reasoned that the observed flightless phenotype of insects expressing tTA was due to VP16, the domain of tTA that can cause cellular toxicity (31). Additional expression of VP16 should therefore increase the penetrance of this phenotype. Furthermore, this would be combined with the potential advantage of VP16 having low toxicity in basal or off-target expression, and consequent minimal effect on male fitness, or indeed on females reared in the presence of Tc.

The specificity of the system was refined further by exploiting the sex-specific alternative splicing observed in the native *AeAct-4* RNA. Along with promoter specificity, alternative splicing plays a key role in regulating gene expression in many developmental processes, including sex determination, but has not been used widely in genetic engineering (16). A start codon (ATG) was introduced adjacent to the 5′ end of the male-specific exon (Fig. 1*D*). In the female-specific splice variant, this ATG is in frame with tTA with no intervening stop codons, but it interposes a frame shift and multiple stop codons in male-specific transcripts. This is predicted to prevent the expression of functional tTA protein in males, even if the promoter is active. A construct, OX3604, was made that combines both components of the expression system. The driver component consists of the regulatory region and sex-specific intron of *AeAct-4* and tTA with an engineered start codon at the 5′ end of the sex-specific intron,

Fig. 2. Phenotype of OX3604C crossed with OX3576 (tRE-DsRed2) and reared in the absence of tetracycline. (*A*) Functional elements of OX3604. 3′ UTR sequences and *piggyBac* ends have been omitted for clarity. Protein coding regions are as follows, from left to right: (*i*) tTAV under the control of a promoter fragment from *AeAct4*. OX3604C has an engineered translation initiation codon (ATG) 5′ to the alternatively spliced intron of *AeAct4* (AeAct4; Fig. 1), so this intron is within the tTA open reading frame; (*ii*) VP16 under control of tRE, and hence responsive to tTAV protein; and (*iii*) fluorescent marker (DsRed2) under control of the Hr5-IE1 promoter. (*B*) late fourth-instar female larvae. White (*C*) and fluorescent (*D*) light micrographs of male and female pupae from OX3604C alone (*Top*) and OX3604C + tRE-DsRed (*Bottom*). Male and female adults (OX3604C + tRE-DsRed) under white (*E*) and fluorescent light (*F*). Strong red fluorescence is visible in the IFMs of females, but not of males. Some background red fluorescence is visible from the transformation markers, especially the punctate overall fluorescence in *B*, the developing IFMs are the bright pair of spots in each thorax (for one larva these are indicated by white arrows).

and an effector component has the VP16 effector under the control of Tc operator (Fig. 1*D* and *E*).

A line of OX3604 (OX3604C) was recovered that had a single insertion of the transgene. OX3604C was crossed with a line, OX3576, with the tet response element linked to DsRed (tRE-DsRed2; Fig. 1*E*) and reared in the absence of Tc to analyze the expression of tTA. Strong DsRed2 expression was seen exclusively in the developing IFM tissue of female fourth-instar larvae, pupae, and adults (Fig. 2). Unlike some OX3545 lines, OX3604C did not drive expression of tRE-DsRed2 in males, perhaps

because the integration of the alternative splicing into the protein coding region provides an additional level of regulation. Amplification of transgene products showed that, although males produce some RNA, transcripts encoding functional tTA were present only in females (Fig. 1F).

OX3604C mosquitoes reared in the absence of Tc showed a highly penetrant (99–100%), dominant, female-specific flightless phenotype (Table 1, Movie S1, and Movie S2). When this strain was reared in the presence of 30 μg/mL of Tc, only 0.3% of females showed the flightless phenotype, not significantly different from wild type (Table 1; $\chi^2 = 0.089$, df = 1, $p = 0.765$). As expected, most males could fly irrespective of Tc (3.2% flightless without Tc; 2.1% with Tc, compared with 0–2.4% for other genotypes; Table S1).

Discussion

We show that it is possible to engineer late-acting, repressible, tissue-specific, and female-specific transgene expression to cause a flightless phenotype in female *Ae. aegypti*. Further studies are needed to assess the mating competitiveness of males of specific strains, and their operational effectiveness in suppressing wild mosquito populations. Owing to the obvious incapacitation of females in the field, the flightless phenotype can be considered equivalent to lethality for a RIDL strain. Flightless females also are effectively sterile, being unable to attract and mate males as courtship and mating depend on the wing oscillation "song" (32). The development of this technology has all the advantages of the previous RIDL system (12), including late-acting lethality, but also allows any life stage of the insect to be released. Releasing eggs into natural or artificial breeding sites would widen considerably the range of distribution options, possibly with significant reduction in costs. *Aedes aegypti* eggs can be stored for several months, are lightweight (~10 ng each), compact, and easy to store and distribute. Distribution of eggs to communities, public bodies, and/or private citizens would allow much greater community involvement than is typical for current SIT programs. Community engagement and involvement is now seen as crucial to long-term sustainability of vector control programs (33–35). Furthermore, the tolerance of *Ae. aegypti* eggs to desiccation and long-term storage makes it possible to stockpile eggs prior to the start of a control program (18), allowing the initial releases to be larger than the maximum sustainable output of the rearing facility. The substantial technological achievements described in this paper have the potential to increase the efficacy and efficiency of the RIDL technique in the control of *Aedes aegypti* and potentially other mosquito species.

Materials and Methods

Plasmid Construction. OX3545 was constructed by amplifying a 3363 bp DNA fragment containing the *AeAct-4* putative promoter and first intron (21) from *Ae. aegypti* genomic DNA using the oligonucleotide primers 5′-ACTGG-GAAGTTGACGTTGATATAGAGC-3′ and 5′-GGTGGTTTACTAGTGGCGCCGCTT-CAAGGTCCGTTAG-3′. The amplification product was cloned at the 5′ end of tTAV linked to a SV40 3′ UTR, which was previously constructed (16, 19). tTAV is a variant of tTA optimized for expression in *Drosophila* by eliminating potential cryptic splice sites and rare codons in the *tet*R region. This gene cassette was inserted into a *piggyBac* vector containing Hr5-IE1 promoter driving DsRed (16, 19). OX3604 consists of a translation initiation codon and Kozak sequence, CCACCATG, engineered into the *AeAct-4* 5′ UTR 42 bp before the 5′ donor site of the intron. The promoter and the 5′ UTR–intron were amplified using primer pairs, 5′-GAATTGAACTTCACCGCTGCTTG-3′, 5′-GGTTTCACCGGTCCGTATCGAACTGTACCAGAGCG-3′; and 5′-GGTACAGT-CGGACCGGCCACCATGGAAACCGAGGATAACGACGAAG-3′, 5′-GCATCGAGC-TAGCCGTCACACGTTTTGGCGCCGCTTC-3′, respectively. The two amplification products were ligated at the *Rsr*II restriction endonuclease recognition site and the ligated product cloned at the 5′ end of the fusion gene, ubiquitin-tTAV-k10 3′ UTR. The engineered start codon was in frame with the tTAV coding sequence. This gene cassette was inserted into a *piggyBac* vector containing Hr5-IE1 promoter driving DsRed (16, 19). *AeAct-4*-DsRed was constructed by amplifying the putative promoter of *AeAct-4* using the primers 5′-CAGAGCTCGGGATCTCGATCACACTG-3′ and 5′-TGCCGCGGTGGCGCCGC-TTCAAGG-3′. The 3′ UTR of *AeAct-4* also was amplified using the primers 5′-TAGCGGCCGCCGATTGTACTGATTAC-3′ and 5′-GATCTAGACGTACGCAT-GAGCCTACTCC-3′. Amplification products were cloned into pSLfa1180fa (36), which already contained DsRed. This entire insert then was excised using *Asc*I flanking sites and inserted into the donor plasmid pMos[3xP3-eGFPaf] (36). tRE-DsRed2, tRE-Mx, and tRE-Nipp1 gene cassettes also were made by gene amplification. The effector genes (*michelob_x* and *Nipp1Dm*) were amplified from genomic DNA, using primers: Michx: 5′-CCACCATGTGTA-GAAACATGGCAATCGCA-3′ and 5′-TCAATACACCTTTCGCAGCAAGTAGT-3′; Nipp1: 5′ CCACCATGCAGTTCTACGCTAACAG-3′ and 5′- AACGTTAA AAGTC-TAATAAACAA-3′. DsRed2 was amplified using 5′-CCACCATGGCCTCCTCC-GA-3′ and 5′-TAAGATACATTGATGAGTTTGG-3′. The tRE (teto7-hsp70 minimal promoter) and SV40 3′ UTR were excised from OX3545 using *Pac*I and *Eag*I sites and inserted into the *attB* vector containing 3xP3-DsRed-SV40 transformation marker (37). The effector genes were cloned between tetO and SV40 using the *Asis*I and *Spe*I sites.

Mosquito Rearing, Transformation, and Analysis. Mosquitoes were reared using standard protocols (38). Preblastoderm *Ae. aegypti* [Asian strain or site-specific (37) Bangkok strain] G0 embryos were collected and prepared for microinjection as described (39). Helper (*piggyBac* transposase or, for tRE-marker/effector constructs, φC31 integrase (37) was provided as mRNA. Embryo microinjection was performed as described (40). Embryos were coinjected at a final concentration of 0.3 mg/mL donor and 0.7 mg/mL helper plasmid in injection buffer (37), placed at 27 °C, 80% relative humidity, and aestivated for 5 days before hatching. Surviving adult males and females were pooled (20–25 females per pool and 3 males per pool) and mated to the host strain. G1 larvae were screened for positive marker gene expression using a Leica MZFLIII or an Olympus SZX12 fluorescence microscope. Positive G1 progeny then were used to establish transgenic families. The proportion of flightless mosquitoes was assayed by collecting pupae daily, allowing them to emerge into individual shell vials plugged with cotton wool, and observing adults ~24 h after emergence for the ability to fly. Adults were deemed able

Table 1. Flight ability of transgenic females

Cross lines	Tet	Wild type		*Nipp1Dm* (OX3547)		*michelob_x* (OX3582)	
		n	Flightless %	*n*	Flightless %	*n*	Flightless %
OX3545D	+	90	4.4	9	0.0	16	18.8
	−	83	6.0	43	69.8	9.0	77.8
OX3545F	+	178	13.5	147	0.7	147	13.6
	−	219	5.0	174	98.3	88	95.5
OX3545H	+	99	26.3	27	55.6	88	30.7
	−	234	22.6	133	97	86	89.5
Wild-type	+	910	0.9	160	0.0	178	0.6
	−	1,705	1.2	165	1.8	228	7.0
OX3604C	+	354	0.3	—	—	—	—
	−	996	100	—	—	—	—

OX3604C and OX3545 (D, F, H) lines were crossed either with wild-type, with OX3547 (tRE-Nipp1Dm), or with OX3582 (tRE-michelob_x) mosquitoes. Progeny from these crosses were reared in the presence (+) or absence (−) of tetracycline (Tet) and flight assays performed on the progeny. Data from female progeny are shown (for males, see Table S1). *n*, total number of female adults analyzed; %, percentage of females that could not fly.

to fly if they could take off from the water surface when the vial was agitated.

Molecular Analysis. Transcripts were analyzed by reverse-transcriptase gene amplification (RT-PCR), using SuperScript™ III One-Step RT-PCR System (Invitrogen), according to the manufacturer's instructions, and the following oligonucleotide primers: Agexon1F, 5′-GGAAACCGAGGATAACGACGAAGG-3′; TETRR1, 5′-GCGGAACGACTTGGCGTTATTGCG-3′. Primer Agexon1F anneals to the *AeAct-4* 5′ UTR 43 bp before the 5′-end donor site of the intron; TETRR1 anneals to the tTAV sequence 540 bp after 3′-end acceptor site of the intron. The female spliced form of the amplification product is expected to have a size of 583 bp, whereas the male spliced form of the amplification product is 826 bp long. All major products were sequenced, and the electropherogram and sequence data were analyzed using Vector NTi (Invitrogen).

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