

# Genetic elimination of dengue vector mosquitoes

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**An approach based on mosquitoes carrying a conditional dominant lethal gene (release of insects carrying a dominant lethal, RIDL) is being developed to control the transmission of dengue viruses by vector population suppression. A transgenic strain, designated OX3604C, of the major dengue vector, *Aedes aegypti*, was engineered to have a repressible female-specific flightless phenotype. This strain circumvents the need for radiation-induced sterilization, allows genetic sexing resulting in male-only releases, and permits the release of eggs instead of adult mosquitoes. OX3604C males introduced weekly into large laboratory cages containing stable target mosquito populations at initial ratios of 8.5–10:1 OX3604C:target eliminated the populations within 10–20 weeks. These data support the further testing of this strain in contained or confined field trials to evaluate mating competitiveness and environmental and other effects. Successful completion of the field trials should facilitate incorporation of this approach into area-wide dengue control or elimination efforts as a component of an integrated vector management strategy.**

genetic control | sterile insect technique | cage trial

Dengue fever is a rapidly emerging arthropod-borne viral disease threatening one-third of the world's population (1). In the absence of effective drugs and vaccines, mitigation efforts focus on controlling the primary mosquito vector, *Aedes aegypti*. However, current control methods are inadequate and new methods are needed urgently (2, 3). A key challenge in the control of *Ae. aegypti* is finding and treating each of the many breeding sites of this mosquito, which oviposits in diverse natural and artificial containers (4–6). Chemical control is increasingly restricted due to potential human toxicity, mortality in nontarget organisms, insecticide resistance, and other environmental impacts (7–9). Release of insects carrying a dominant lethal (RIDL) is a genetic control strategy derived from classical sterile insect technique (SIT) that provides a new solution to the challenges facing current control efforts (10–14).

SIT is a species-specific, environmentally friendly control method that involves the rearing, sterilization, and release of large numbers of disabled insects (15, 16). These sterile insects mate with wild insects in the target population, thereby reducing the reproductive potential of the target and, if sufficient numbers of sexually competitive insects can be released, achieving local control or even elimination. Large-scale SIT programs have suppressed or eliminated a number of major agricultural pests (15). SIT approaches for mosquito control have been tried (17–19) and continue to be proposed (20, 21). Factors that may have limited the success of these initial mosquito SIT programs and that are of continued concern in proposed SIT applications include reduced mating competitiveness and residual fertility of irradiated males (22, 23), the need to release exclusively males (male mosquitoes do not take blood meals) (20, 24), and reduction of density-dependent larval mortality due to early acting lethality (22, 25, 26). These limitations may be overcome using recombinant DNA technology to engineer repressible dominant-lethal transgenes for an RIDL strategy (4–7).

The *Ae. aegypti* OX3604C strain has the genetic features necessary to produce highly penetrant, dominant, late-acting, female-specific lethality (14). These transgenic mosquitoes carry genes that impose a tetracycline-repressible flightless-female phenotype. This phenotype is effectively lethal because flightless females cannot mate, seek hosts, or avoid predators. Importantly, they cannot serve as vectors for dengue viruses. Here we describe the results of testing this strain for its efficacy in suppressing a target wild-type population of mosquitoes in laboratory-based, large-cage trials.

## Results

OX3604C (14) was outcrossed to a genetically diverse laboratory strain (GDLS) of *Ae. aegypti* derived from a mixture of equal numbers of 10 geographically distinct collections made in 2006 from Chiapas, Mexico (27). The outcrossed OX3604C males released in this experiment are expected to be homozygous for the transgene in a genetic background that is approximately 96.9% GDLS. The rationale for the outcrossing procedure was to create an OX3604C strain containing a similar genetic background to that of the target population and to maintain the competitive fitness of OX3604C males (28). The objective of this study is to assess the efficacy of the RIDL strategy using this outcrossed OX3604C strain to eradicate target populations of GDLS *Ae. aegypti* in large (232–504 ft<sup>3</sup>) laboratory cages.

Target populations of GDLS were established in six cages (Fig. S1) over the course of 12 weeks prior to the release of OX3604C (Figs. 1 and 2). Stable egg and adult densities in all six cages were achieved by Week 6. Three of the six cages were assigned randomly at Week 13 as treatment cages and approximately 900 OX3604C males, representing release ratios of 8.5–10 OX3604C:1 GDLS, were released into them weekly thereafter (Fig. S2). The weekly numbers of larvae returned to the treatment cages were adjusted relative to the weekly return rate in the control cages (held constant at 200 second-instar larvae/week) to reflect any impact of OX3604C release on egg production.

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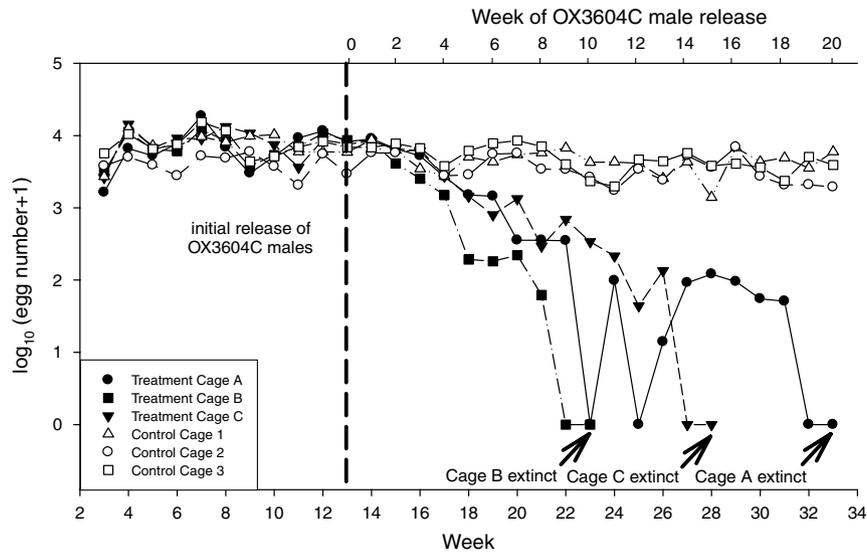
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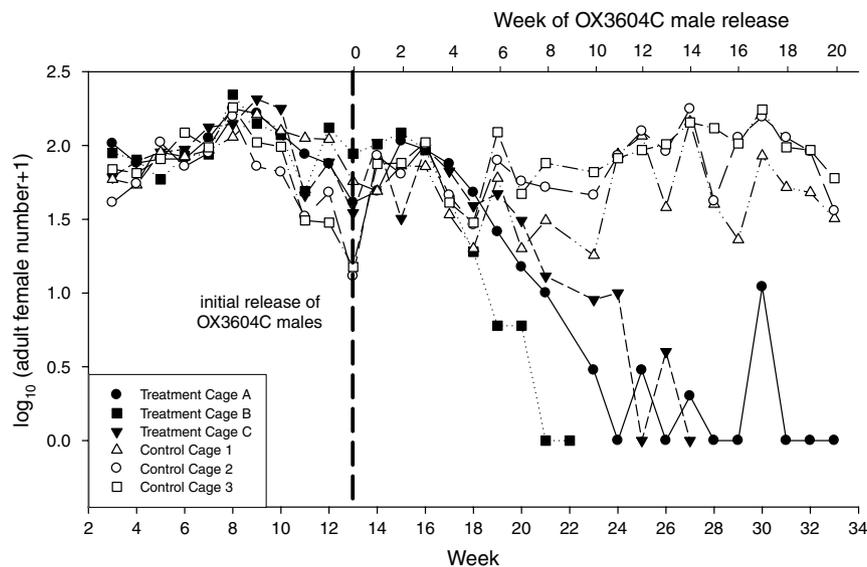
**Fig. 1.** Egg production in treatment and control cages. Weekly egg production is shown for each control and treatment cage. Production numbers were stable in all cages by Week 4 after population establishment. After OX3604C male release was initiated (vertical dashed line) in the treatment cages (Week 13; Week 0 PR, top time axis), egg production in the control cages continued to be stable but gradually declined in the treatment cages, with a clear reduction relative to controls by Week 5 PR. The populations in the treatment cages became extinct (defined as two consecutive weeks with no egg production) by weeks 10, 15, and 20 PR (arrows).

Reductions in egg and adult numbers in treatment cages relative to control cages became evident by Weeks 5–6 and 6–8 postrelease (PR), respectively, of OX3604C (Figs. 1 and 2). Transgene introgression into the caged populations was detected first during Week 3 PR by the presence of the DsRed marker gene (4, 5) in random samples of larvae hatched from eggs collected from treatment cages (Fig. 3) (29). The majority of larvae in all treated cages carried the transgene by Week 8 PR. All larvae expressed DsRed in two of the treatment cages; however, the percentage varied over time in the third cage and never reached 100% (Fig. 3). Subsequently, cage populations reached extinction (defined as two weeks without eggs) by 10, 15, and 20 weeks PR (Fig. 1).

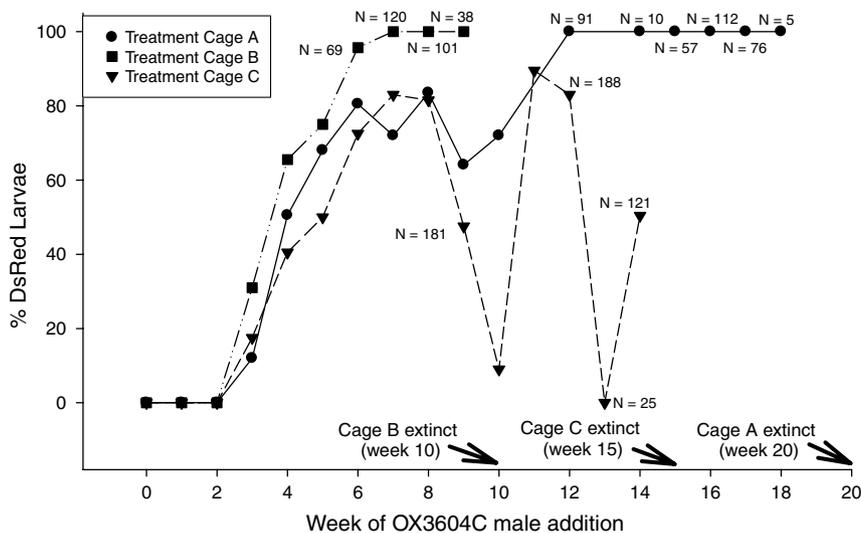
### Discussion

We show that the OX3604C strain can cause elimination of a target population within an epidemiologically relevant time frame. In addition, release ratios increased as the experiments proceeded, favoring the elimination of the target population despite the fact that actual numbers of released males remained fixed. The utilization of the genetically engineered male mosquitoes to find target females mitigates human involvement in detecting the breeding sites and relieves the need to introduce toxic chemicals into the environment.

The variation in the time to eradication (10–20 weeks PR) could represent the differences in the cage sizes, cage conditions, or stochastic variants among replicates. Variation also could result from differential longevity among inseminated target



**Fig. 2.** Adult female populations in treatment and control cages. Adult female populations were sampled weekly with BGS-Traps for one hour (no lure). The numbers of trapped females are shown. Populations had declined in all treatment cages by eight weeks PR while the populations in the control cages continued at the prerelease levels. In all treatment cages, numbers of trapped females reached zero prior to extinction (defined as two consecutive weeks without eggs; Fig. 1 and text). The mosquitoes in the control cages were collected and counted at the end of the experiment. Six hundred eighteen adult females were recovered (222, 185, and 211 from cages 1, 2, and 3, respectively). Compared with the Week 33 BGS-Trap catch of 31, 35, and 59 females, this indicates a trapping efficiency in the weekly monitoring of approximately 20%.



**Fig. 3.** Progeny genotypes in the treatment cages. Random samples of eggs from each weekly collection were hatched and the larvae screened for DsRed. Two hundred larvae were screened unless otherwise marked. The percentage of DsRed larvae reached 100% in two of the three treatment cages prior to extinction. The percentage of larvae positive for the OX3604C construct in the third cage varied over time and never reached 100%. The missing data from Cage A, Week 19, was due to the eggs collected not hatching. As expected, the frequency of the transgene in the larvae from collected eggs increased before adult female numbers or egg production decreased.

females, and this also is expected to be a key factor in the time scale for suppression. Females typically mate only once and then deposit eggs fertilized by sperm stored in the spermathecae (29, 30). Therefore, the genotype of these eggs reflects the mating choice of the female shortly after her emergence as an adult. Furthermore, with our definition of extinction, a single long-lived female can delay extinction by several weeks, even though she has mated with an OX3604C male and will produce no flying female progeny. This appears to have happened in Cage A where all eggs produced from Week 12 PR were transgenic, but extinction did not occur until Week 20 PR. Alternately, a rare nonhomozygous male in the OX3604 release population could have led to the more protracted extinction time.

We conclude that the release of outcrossed OX3604C males can successfully eliminate target populations of *Ae. aegypti* in a laboratory caged system. We also conclude that OX3604C males competed effectively with target males for target females, as revealed by the production of offspring expressing DsRed. Future field trials need to account for the rate of immigration of wild-type males and females into target populations. This includes movement of mosquitoes from outside the treatment area into it and the emergence in the treatment areas of estivated embryos in seasonally flooded breeding sites. These circumstances represent key differences from a fully contained cage trial. Our data support the transition to field trials with the expectation that release of outcrossed OX3604C or similar mosquitoes will result in a similar significant local suppression or elimination of the target mosquito populations.

## Materials and Methods

**Outcrossed Homozygous OX3604C.** The outcrossed OX3604C homozygous strain of genetically engineered males released in this experiment was established by first outcrossing the OX3604C strain (14) to a GDLS established in 2006 (27). Virgin male OX3604C were caged with virgin female GDLS, and a cage containing the reciprocal cross was established. The resulting  $F_1$  heterozygous adults were allowed to intercross, eggs were hatched, and 1,000 larvae expressing DsRed were collected, reared to adults, and mated individually to virgin GDLS adults. This was repeated for four additional generations so that as much as 96.9% ( $1 - (1/32)$ ) of the genome could consist of GDLS. The frequency of the RIDL construct was increased through selection of DsRed larvae for four generations to generate a homozygous line of the outcrossed OX3604C strain. A total of 250 single-pair families then were established using only DsRed mosquitoes. Eggs were collected and hatched from each family and those families with wild-type larvae discarded. DsRed-

only mosquitoes were reared to adults, sib-mated, and the resulting larvae screened again for the DsRed marker. Any families with even a single wild-type larva were discarded and the remaining families were expected to be homozygous. These families were then intercrossed to provide the outcrossed OX3604C strain.

**Target *Ae. aegypti* Population.** Target populations were established in each of the six large laboratory cages (Fig. S1) over the course of 12 weeks using a GDLS derived from 10 geographically distinct populations in Chiapas, Mexico, in 2008 (28). Each cage was accessed through a series of three sleeves, two of which opened to shelves, and the third opening to the cage floor, which allowed introduction and removal of a BG-Sentinel Mosquito Trap™ (BGS-Trap Biogents AG; Fig. S1). The shelves in each cage held a total of six oviposition containers (OPs, three on each shelf; Fig. S1), which were glass Pyrex containers. All OPs were filled with 600 ml tap water, which was changed weekly, and were lined with white paper towels (Scott® brand, Kimberly-Clark) for oviposition. The caged populations of mosquitoes were reared and maintained under a 12:12 LD photoperiod at  $27^\circ\text{C} \pm 2^\circ\text{C}$  and  $82\% \pm 3\%$  humidity. Larvae were fed ground brewer's yeast tablets ad libitum. Adult mosquitoes were provided with raisins and allowed to feed weekly on restrained mice.

The method by which the target populations were established was conducted in two parts, initiation and maintenance. Initiation (Weeks 0–3) consisted of adding 300 second-instar GDLS larvae (50/OPs) for an initial population of 1,200 mosquitoes. Females began to oviposit at Week 3, after which the population was maintained (Weeks 4–12). Eggs laid by existing females were returned to their respective cages as second-instar larvae at a return rate of 200/week (approximately 33/OPs). Egg and adult densities were monitored weekly during target-population establishment. Egg densities were determined by counting the total number of eggs laid weekly in each cage. Adult sample collection commenced in Week 3. Adults were sampled placing a BGS-Trap into each cage for 1 h weekly. Adults were counted and sexed on cold plates and then returned to their respective cages. The adults experienced 5–25% mortality associated with trapping and handling.

**Release of OX3604C Males.** Weekly releases consisted of hatching 2,100 OX3604C eggs without tetracycline (to produce the flightless-female phenotype) per treatment cage and then rearing these larvae to pupae. Pupae were collected over the course of 3 d and placed in a covered pupal emergence container (PEC; Fig. S1) within each treatment cage. The PEC remained covered to prevent oviposition by females but was uncovered once daily to allow males to escape. Sexing the pupae was unnecessary because emerging females either drowned in the PEC or fell to the floor of the cage, where they died. We estimate that 2,100 OX3604C eggs per week led to the weekly addition of approximately 1,800 total pupae (approximately 900 male pupae) to each treatment cage. This release number was kept constant, and at the

first OX3604C male release (Week 13) it was 8.5–10 times the weekly return rate of 200 second-instar/larvae/week (approximately 100 males). Because the mosquito population decreased over time in the treatment cages, this ratio correspondingly increased, from 8.5–10:1 OX3604C:target males at Week 0 PR to between 600:1 to 900:1 at population extinction (Fig. S2).

**Population Maintenance Post OX3604C Release.** After OX3604C male release was initiated at Week 13 (Week 0 PR), the number of larvae added back to each cage was adjusted to reflect any impact of OX3604C male release on egg and adult densities. The number of larvae returned to the three control cages remained constant at 200 second-instar larvae/week. However, the number of larvae added back to the treatment cages was changed to a rate proportional to egg densities in the control cages. For example, if a control cage produced 5,000 eggs one week and we returned 200 second-instar larvae to that cage, then we returned 4% (200/5000) of the eggs. If a treated cage in the same week produced 600 eggs, then 24 larvae ( $600 \times 0.04$ ) would be returned, or, if conversely, a treated cage the same week produced 15,000 eggs then 600 larvae ( $15,000 \times 0.04$ ) would be returned. Consistent with this

methodology, equal numbers of larvae were returned to each cage when egg densities were the same in treated and controls. If egg densities were lower in treated cages then fewer larvae were returned to the treated cage, and if egg densities were greater in treated cages (if for example, there was a greater survival of larvae due to density dependence), then more larvae were returned to the treated cage. If 200 larvae each week had been returned to the treated cages, the return rate would be artificially increased if egg densities in treated cages were low, and, conversely, artificially decreased the return rate when egg densities from treated cages were high.

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