Infertility resulting from transgenic I-Ppol male *Anopheles gambiae* in large cage trials

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Objectives: *Anopheles gambiae* is the primary vector of malaria in sub-Saharan Africa and is a potential target of genetic control programs. We determined the capacity of male *A. gambiae* created by germline transformation to introduce infertility into stable age-distribution populations. We also determined effects of the transgenes on life history.

Methods: Stable age-distribution populations of *A. gambiae* mosquitoes were established in large indoor cages. Male mosquitoes carrying an I-Ppol homing endonuclease gene were introduced at $\times 5$ and $\times 10$ release rates where they competed with target male mosquitoes for matings. Similar trials were conducted in small cages with an additional $\times 1$ release level.

Results: Infertility was successfully introduced into all target populations. In supporting experiments, complete female infertility was observed in all strains and species of the *A. gambiae* complex to which transgenic males were mated. Life history experiments demonstrated that reductions in I-Ppol male vigor exist in the form of reduced adult male emergence, longevity and competitiveness.

Discussion: *A. gambiae* I-Ppol males are capable of introducing high levels of infertility in target populations in indoor cage trials. This was accomplished despite losses of vigor resulting from the HEG transgene. These results motivate further trials of sexually I-Ppol *A. gambiae* in outdoor cage and field trials.

Keywords: GMO animals, Reproductive infertility, Biological pest control, Insect vectors, Malaria

Introduction

Methods to control the mosquitoes that are most important as vectors of human disease currently include environmental management such as land and container drainage, the use of insecticides and biopesticides as well as personal protection through systematic use of long-lasting insecticide treated nets. These are neither fully nor uniformly effective to prevent disease, and the mainstay, insecticides, is continually threatened by resistance.¹ As a consequence, several new methods are being investigated: both natural² and modified fungi,³ highly specific insecticides⁴ and several kinds of genetic control. Genetic control methods include release of *Wolbachia*-infected mosquitoes⁵ and those that create infertility and F1 mortality in target populations.⁶ The latter ones have similarities to sterile insect technique (SIT), which has been applied successfully and extensively against a variety of insect species including pink bollworm, screwworm and Mediterranean fruit flies.⁷ In spite of an early highly effective mosquito trial from releases of a total of 4.3 million chemo-sterilized

Anopheles albimanus males in El Salvador,⁸ an expanded program was not as successful, due at least in part to excessive immigration into the release site.⁹ Building upon the notable effectiveness against other insects and a history of attempts against mosquitoes,¹⁰ SIT is being developed against mosquitoes using both conventional¹¹ and transgenic approaches which may offer biological advantages over conventional SIT.¹²

Continual release of 'sterile' males can reduce populations by creating persistent infertility. In such programs, males are produced in factories and released from the ground and air. The term sterile is often applied to males that cause infertility among their mates regardless of the causal mechanism. The cause of infertility in conventional sterile insect technique is the creation of dominant lethals in gametes due to irradiation, but it can also result from gametes that are incapable of fertilizing eggs, for example, in hybrid male dysgenesis.¹³ In conventional SIT, dominant lethals are induced by irradiation with gamma-rays,¹⁵ but there is evidence that this procedure induces a level of somatic damage that impairs male mating competitiveness, a prime quality predictor for success. Factory produced irradiated males are seldom, if ever, fully competitive against wild

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males, and it is hoped that transgenic methods of creating infertility will mitigate such loss, at least that portion which is due to irradiation.¹⁶

Transgenic male-sterile *Anopheles gambiae* have been created by insertion of DNA encoding I-PpoI, a homing endonuclease (HEG) from a slime-mold (*Physarum polycephalum*) that specifically cleaves the rDNA repeats on the X chromosome.^{14,17} In these mosquitoes, I-PpoI expression is regulated by the beta2-tubulin promoter that is selectively transcribed in male mosquito testes during sperm cell differentiation.¹⁴ The production of I-PpoI induces the cleavage of the paternal X chromosome in differentiating spermatozoa, which was expected to eliminate holandric X chromosomes, thus causing male bias among the progeny. No progeny have been observed however;¹⁴ it is hypothesized that sufficient nuclease activity is transferred via the sperm to the zygote so that the maternal X chromosome is also cleaved, resulting in dominant embryo lethality. Transgenic I-PpoI mosquitoes combine a number of features that make them particularly suitable for SIT studies. These transgenic mosquitoes carry a visible fluorescent eye marker (RFP) and marked fluorescent spermatozoa thus facilitating the assessment of male competitiveness and survival. Before attempting field studies, we wanted to investigate life history parameters of I-PpoI mosquitoes as well as their ability to introduce infertility into target mosquito populations in large cage experiments.

We conducted a series of experiments in order to assess the value of male I-PpoI *A. gambiae* mosquitoes for SIT. We determined in large indoor cages whether infertility could be introduced into populations of mosquitoes when beta2-PpoI males were released at levels $\times 1$, $\times 5$, and $\times 10$ relative to the introduction rate of wild males. We also determined whether the infertility effects extended to additional species of the *A. gambiae* complex and effects of carrying the transgene on the development rate and eclosion of transgenic individuals.

Materials and Methods

Mosquito culture

All mosquito culture and experiments were conducted at the Centers for Disease Control and Prevention, Atlanta, GA, USA. Two strains of transgenic *A. gambiae*, beta2-Ppo1 and beta2-Ppo2, were obtained from the laboratory of Dr Andrea Crisanti at Imperial College, London. The two strains carried a single transgene insertion near the ends of chromosomes 2L and 3R, respectively.¹⁴ All other mosquito stocks were obtained from the Malaria Research and Reference Reagent Resource Center (MR4) in Atlanta, GA, USA, and are listed in Table 1. Larvae were provided a mixture of Argentinean beef liver powder (NOW®,

Bloomington, IL, USA, USA), tuna meal (T.C. Union Agrotech, Bangkok, Thailand), and Vanderzant vitamin mix (Bio-Serv Inc., Frenchtown, NJ, USA) in a weight ratio of 2:2:1. Large particles were removed by passing the powder through a 600 μ m sieve, after which it was mixed with water at 2% (w/v). After it had hydrated for at least 2 hours, it was again sieved through 600 μ m and the remaining large particles and heaviest sediment discarded. Food was provided daily based on visual estimation of the turbidity of the water and the stage in order to prevent complete depletion by the following day. All mosquitoes used for experiments except the release cages and mating competitions were maintained in an environmental room at 28°C, 70% relative humidity (RH). The light cycle began at midnight and consisted of 30-minute sunrise, 12-hour full light, 30-minute sunset followed by 1-hour 1% light level and 10-hour complete darkness. The air-conditioning system was turned off during sunrise (30 minutes) and for 3½ hours beginning at sunset to promote mating. Blood-feeding was performed on the forearm of a human volunteer (MQB) for 10 minutes, usually after sunset. Two days later, an egg collection dish was provided and removed the following day, at which time eggs were washed with 2% household bleach (in water) followed by water. Occasionally eggs were stored at this point at 15°C in order to delay hatching for release schedules. Adults were housed in Savage-Lowe cages¹⁸ (15 × 25 × 21 cm) covered with tube-gauze (No. 24-007; Lohmann & Rauscher International GmbH & Co., Topeka, KS, USA). Sugar water contained 10% sucrose and 0.2% (w/v) methylparaben (Acros 126965000, Geel, Belgium) to preserve its quality.¹⁹

PCR analysis of transgenic strains

To determine whether the two transgenic strains that were transferred to the facilities where the experiments were performed were distinct and to associate each with the published transgene genomic location, flanking genomic DNA and part of the insertion for beta2-Ppo1 and beta2-Ppo2 were amplified by the PCR. A crude DNA template was prepared by adding an adult mosquito to a solution of 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 0.1% Triton X-100 and incubating it at 95°C for 15 minutes. Two microliters of template DNA were used in a reaction which was otherwise by the manufacturer's recommendations (Promega, Madison, WI, USA). PCR cycling conditions were 95°C, 5 minutes — 35 × (95°C × 30 seconds, 54°C × 30 seconds, 72°C × 30 seconds) — 4°C. One transgene primer (5' to 3') was used to amplify both insertions: TGCCAAAGTTGTTTCTGACTG. Flanking genomic primers for beta2-Ppo1 and beta2-Ppo2 respectively were: AACACATCGCACCATTAGCA and CTGGTGAAGTGAGAGGAAACG.

Table 1 Hatching rates of eggs produced from crosses of I-Ppo1 males and females to wild-type A. gambiae s.l. strains and species

Female Species	Stock	Male	Non-transgenic male mate				Transgenic male mate			
			Egg hatch		Total eggs*	Mean hatch	Egg hatch		Total eggs*	Mean hatch
			Yes	No			Yes	No		
arabiensis	DONGOLA (856†)	Beta2-Ppo2	642	71	4329	90.0%	0	331	4541	0.0%
gambiae	Beta2-Ppo2 Beta2-Ppo1	G3	1043	128	1171	88.4%				
		G3	670	28	698	95.1%				
	AKRON (913) G3 (112) MOPTI (763) RSP (334)	Beta2-Ppo2	168	15	846	91.8%	0	350	406	0.0%
		Beta2-Ppo2	1078	433	1511	69.0%	0	2,823	3273	0.0%
		Beta2-Ppo2	250	10	4043	94.0%	0	894	1560	0.0%
		Beta2-Ppo2	71	32	722	68.9%	0	1056	1292	0.0%
		Subtotal	1567	490	7122		0	5123	6531	
merus	AKRON G3 MOPTI RSP	Beta2-Ppo1	137	36	790	79.2%	0	478	717	0.0%
		Beta2-Ppo1	1853	399	2252	80.6%	0	1031	1031	0.0%
		Beta2-Ppo1	323	39	380	82.8%	0	831	1178	0.0%
		Beta2-Ppo1	201	58	2021	77.6%	0	251	294	0.0%
		Subtotal	2514	532	5443		0	2591	3220	
	OPHANSI (803)	Beta2-Ppo2	101	134	235	48.4%	0	325	325	0.0%
quadiannulatus	SKUQUA (761)	Beta2-Ppo2	15	37	52	28.8%	0	1282	1282	0.0%

Note: *Total numbers of eggs may include eggs not examined microscopically for hatching as described in the section on 'Materials and methods'.
†Numbers are suffixes of 'MRA-' stock numbers used by the MR4.

Figure 1 Average temperatures of three 24-hour samples in different large cages (one of which was in a different room), humidities (95% CI of mean), and sample light level observed in large cages and within the resting shelters.

Infertility in HEG male outcrosses

As in all other crosses, virgin adults were obtained from the various stocks by separating male and female pupae. Hatching data were obtained either from individually egged females or *en masse*, the latter being more common. A sample of eggs was counted and/or the cup into which larvae might be found was examined in order to ensure that unexamined eggs had not hatched. Outcrosses were performed to various stocks using either transgenic beta2-Ppo2 and beta2-Ppo1 or their wild-type siblings. Because we first successfully amplified beta2-Ppo2 to the level necessary for experiments, this strain was chosen for releases, and the majority of crosses were performed with that stock. Typical crosses were performed using 25–50 females and males mated *en masse*. Eggs were counted directly and hatch determined using a stereo dissecting microscope, and at times, microscopic examination was supplemented by an estimate of the total number using scanned images as described below.

Large environmental room operation

Two large environmental rooms were programmed with identical light, temperature, and RH settings as described above. The light profile described above reached a maximum in the range of 330–350 Lux at the 100% setting and at 1–2 Lux at the 1% setting used during the dusk period in the large cages. The temperature profile was set so that the minimum temperature and maximum RH were reached in the night. Actual RH, temperature and light levels (Lux) in large cages were sampled (Fig. 1) at various heights with a HOBO® data logger (Model U12-012; Onset®, Pocasset, MA, USA).

Large and small cages

A total of nine greenhouses (1.8 × 2.4 × 2 m, vol. approx. 8.5 m³, Shelter Logic® model #70602, Watertown, CT, USA) were assembled and used as

large cages. Three cages were erected in one environmental room (5.7 × 11.7 × 3.6 m, *w* × *l* × *h*) and six in an adjacent environmental room (5.7 × 11.5 × 3.6 m, *w* × *l* × *h*). The reinforced plastic covers were secured to the floor with Gorilla® duct tape and 3M® transparent packing tape. PVC snap clamps (item #13; Circo Innovations, Grass Valley, CA, USA) were used to securely attach the cover to the metal pipe of the large cages. Each cage in the room containing three cages was randomly assigned a release rate of × 5, × 10, or control. Similarly in the second room, two large cages were randomly assigned each release rate.

On the door of each large cage, an opening was cut approximately 30 cm from the floor and lined with tubular gauze (product #081820; Alba Health, Rockwood, TN, USA) for the introduction of a blood meal, egg dish, and adult mosquitoes. Each large cage was provided with a resting shelter made from two clay tiles (30.5 × 30.5 cm) with two bricks supporting the top tile, all resting on top of a plastic flowerpot. A water soaked sponge was placed on the top of the resting shelter and covered with a metal pan to create a cool humid resting place. A window (28 × 13 cm) was cut at the same height as the resting shelter and covered with transparent plastic to allow viewing for a census of resting adults. The window was covered on the outside with a black foam door to keep the shelter dark when adults were not being observed. An artificial plant was strung along the bars inside the large cages as another resting option and to serve as a possible swarm marker. Small cages used for mating experiments were the same Savage–Lowe design (described above for stock keeping) as were the sugar feeders.

Release cage stocking

Strain G3 adults were used as the target population into which introduction of infertility was attempted and had been used to maintain the strains by backcrossing since their inception. Males and females were separated as pupae to ensure the adult females were virgins. I-Ppo1 males were selected as pupae under a Leica® MZ6 microscope (Leica, Wetzlar, Germany) with a BLS universal visualizing light source (BLS, Budapest, Hungary) for fluorescent stereomicroscopy to distinguish males expressing GFP in the testis. Transgenics were selected by RFP expression.

Blood feeding

Female mosquitoes in each cage were offered a blood meal for exactly 15 min twice/week from a human volunteer (MQB) by inserting a bare leg into the sleeve opening. Blood meals were offered beginning at late sunset. The volunteer used PVC tubing to blow breath into the large cages as an attractant

during the blood feed. The cage order and leg used for feeding was randomized.

Two days after the blood feed, an egg dish was placed in each cage and removed on the following morning. Large cage egg dishes consisted of bottoms of 150 mm polystyrene Petri dishes. The sides of the egg dishes for large cages were lined with 3 mm thick black foam 3 cm high. Oviposition water was prepared by filtering 200 ml of water from L4 cultures through a paper coffee filter and mixing it with 800 ml of pure water. A disk made of a terrycloth towel was placed in the bottom of the dish and a labeled filter paper was placed on top of this. Oviposition water was added until the total weight was 107 g. Egg dishes for small cages were similar except that the dish was a 90 mm polystyrene Petri dish and a sponge cloth disk was placed under the filter paper. Oviposition water was added until the total weight was 43 g. This amount of water resulted in a small amount of free water in the dish and was sufficient to keep it moist overnight. Once removed, adults sticking to the surface were picked off of the filters, recorded as dead, and the eggs were placed on moist paper towels in covered pans and allowed to incubate for 2 days before counting the hatch and number of eggs. After incubation, the hatch rate of a sample of 100–200 eggs was counted under a stereomicroscope and then scanned on a flat-bed scanner as a gray-scale image at 1200 dpi. The resulting image was modified by removing the filter label, frass, and other dark areas. The number of eggs was estimated by establishing a regression between the number of pixels with a gray value <150 (function ‘Histogram’) using (ImageJ v 1.43u²⁰). The resulting formula for estimating the egg number was $\text{number eggs} = \text{number pixels} \times 0.0064$ ($R^2 = 0.98$).

Releases into stable-age populations

We will refer to the populations into which we attempted to introduce infertility as ‘stable-age’ populations. By this we mean that the population size was determined by the rate of recruitment of new adults being equal to the mortality rate. Releases into the large cages began with progeny of the second backcross and terminated using backcross 4 progeny. Therefore, the degree of genetic similarity between the I-PpoI males and the strain G3 control males against which they were competing was high. In all cases, transgenic and wild males released on a particular day were closely matched for age among I-PpoI and G3 adults. Adults, usually 2-, but no more than 4-, day-old were released. Releases were performed into two different mating venues: large and small cages. In the large cages, five females and five males were recruited into populations three times per week, usually on Mon., Wed., and Fri. In the

small cages, two females and two males were recruited on the same schedule. The reduced number added to the small cages was selected so as to not create an excessively high density. Two indicators were used to determine when the populations had stabilized: (1) the number of adults resting in the masonry shelters of all large cages stabilized when counted upon first arriving on the mornings of Mon. Wed., and Fri.; and (2) the number of dead adults stabilized as determined on Wed. in both the large and small cages.

When we concluded that the populations had stabilized, we began releasing males at $\times 5$ and $\times 10$ rates in the large cages and at $\times 1$, $\times 5$, and $\times 10$ rates in the small cages. The male release rate was relative to the recruitment rate of G3 males which was continuous until the completion of the experiments. Eggs were collected *en masse* twice weekly and the hatching rate and total number was determined. When releases began, we discontinued collecting adult mortality data except in the control cages. Because the egg hatch rate of any one female was expected to be 0 or 1, samples of small numbers of ovipositions have intrinsically high variance. In order to reduce this, we excluded egg collections in which fewer than 360 eggs were obtained. This approximates ovipositions of four females as determined by individual-female ovipositions. Hatching rates were analyzed by a general linear model with ovipositions from cages treated as repeated measures (MiniTab®, State College, PA, USA) to determine whether mean hatching rates of the transgenic male release had a significant effect on hatch for the last five or six egg-batch collections in the large and small cages, respectively. Individual cages were nested within male release rates. This, as all other statistics, considered significance at $\alpha < 0.05$.

Competitive matings

Sex separation and handling was the same as for released mosquitoes as were the large cage operation including environmental conditions. I-PpoI males were competed against G3 males for virgin G3 females. Adults were usually 1, but occasionally 2, days old. Three competitions of beta2-Ppo2 were conducted simultaneously and two with beta2-Ppo1 simultaneously. Each of these contained 100 G3 females and 50 each of HEG and G3 males. Adults cohabited for 7 days. Mating status was determined by white light and UV fluorescence microscopy, typically at $\times 200$ magnification. It is difficult to distinguish fluorescence of individual sperm; therefore, we were unable to detect multiple matings of a single female by different types of males. To the extent that this occurs, it could bias the estimation of matings, likely in favor of I-PpoI males.

Figure 2 Average (95% CI of mean) adult censuses taken prior to each release into large cages (Δ) are plotted on the left axis: male and female dead in large cages ($\square$ and $\circ$, respectively) and small cages (filled $\square$ and $\circ$, respectively) are plotted on the right axis. No method for counting the number of resting adults in the small cages was available for estimating the number of living adults directly.

Development and survival of transgenics and wild siblings

Entire cohorts or samples of larvae being cultured in trays were analyzed for the proportion of transgenic and wild larvae, the number pupating each day, their sex, and the likelihood of emergence. Mosquitoes were cultured using the diet and densities described in the section on 'Materials and methods'. When most had reached the L4 stage, we examined and counted the number of transgenic and wild siblings, which were cultured separately thereafter.

Results

Infertility in *I-PpoI* male outcrosses

The identity of the two transgenic strains¹⁴ was confirmed by the PCR. Fragments consistent with the expected lengths of 444 and 556 bp were amplified from beta2-Ppo1 and beta2-Ppo2, respectively.

Males of these two strains conferred absolute infertility regardless of the strain or member of the *A. gambiae* complex to which they were crossed (Table 1). In dishes from which only a sample of eggs was examined microscopically and no hatching eggs were observed, the dishes were examined for hatched larvae but in no case were they observed. Consistent with the report describing the generation of these strains,¹⁴ as observed by the presence of larval eyes, apparently mature but unhatched embryos were observed at a frequency of approximately 1%.

Infertility in populations

Populations of wild-type mosquitoes with stable age distributions were created by introducing either five adults of each sex into nine large indoor cages or two adults of each sex into small laboratory cages three times per week. The process of establishing these populations was considered complete at 40 and 49 days for the large cages and small cages, respectively (Fig. 2), at which point releases of

Figure 3 Average weekly egg-hatching rates (95% CI of mean) of egg batches obtained from large cages into which HEG males were released at three levels: $\times 0$ (control), $\times 5$, and $\times 10$.

beta2-Ppo2 males began (except in controls) along with continued release of wild males and females at the previous level. The $\times 5$ and $\times 10$ release rates designate the relative proportion of *I-PpoI* males to wild males being introduced into the cages. These rates of males, plus a $\times 1$ level, were also introduced into the small cages.

Large cages

Eggs were collected *en masse* twice weekly (Tuesday and Sunday nights), and the hatching rate and total number of eggs were determined. Within 2 weeks, clear reductions in the egg hatching rates were observed in the $\times 5$ and $\times 10$ cages (Fig. 3). Male releases continued over a period of 51 days.

After 4 weeks of transgenic male releases, egg hatching rates reached a level that did not decline further. The near-zero-slope regression line (not shown) of the last five egg-collection dates of the releases demonstrated that no further reductions in fertility could be expected, and the releases were discontinued. The average hatching rates (std. dev.) of the final five egg batch collections for the control, $\times 5$ and $\times 10$ cages ($n=3$ in all cases) respectively were 0.69 (0.12), 0.13 (0.11), and 0.11 (0.11). Exclusion of ovipositions consisting of less than 360 eggs had little effect on these estimates: only three ovipositions out of a total of 70 were excluded. Egg-hatching rates were significantly affected by release rate (Table 2). Cage (within release rate) also had significant effects on hatching rates. In contrast, considering all ovipositions prior to the initiation of releases, there was no difference in the hatching rates of eggs obtained from control and release cages (25 ovipositions) when analyzed by the same statistical method.

Small cages

Wild mosquitoes have access to large spaces for mating, so an effect of cage size on these simulations

was explored. Similar to the design of the large cage experiments, releases began when small cage populations had stabilized and continued for 47 days. The hatching rate declined and stabilized over approximately 4 weeks, after which no further decline was observed (Fig. 4). Linear regression slopes of the hatching rates of the last six dates of egg collections were near zero (data not shown), demonstrating that no further reductions could be expected. Hatching rates in the small cages reached minimum averages (std. dev.) for the controls, $\times 1$, $\times 5$, and $\times 10$ release rates ($n=3$ in all cases) of 0.60 (0.17), 0.37 (0.15), 0.24 (0.11), and 0.09 (0.08). Exclusion of ovipositions consisting of less than 360 eggs had little effect on these estimates: only one oviposition of 110 was excluded. Male release and cage (within release rate) both affected egg-hatching rates (Table 2). When we considered all ovipositions ($n=56$) prior to the initiation of releases, there was no difference in the hatching rates of eggs from control and release cages (GLM).

We used the weekly mortality data from the small cages to determine the daily survival of G3 females and males. Over the first 7 weeks, average daily survival (95% CI) for females was 0.94 (± 0.05) and for males was 0.91 (± 0.06). This is consistent with typical observations of greater longevity of females.²¹ No attempt was made to estimate daily survival from the large cages as we had observed some mosquitoes outside of the cages that escaped through undetected routes. Such escapes would affect the rate of infertility observed only if there were differential rates of escape of females mated to wild versus transgenic males or of wild versus transgenic males.

Mating competitiveness of *I-Ppol* males

We performed typical mating competition experiments in which similar-age adults test males — in this case transgenic — and wild-type compete for virgin females for some fixed period of time. In these experiments, males and females cohabited for 7 days. Female mating status was determined by direct examination of spermathecae for the presence of sperm and the transgenic status of the origin of the sperm was determined by fluorescence. Values of

Figure 4 Average weekly egg-hatching rates (95% CI of mean) of egg batches obtained from small cages into which HEG males were released at four levels: $\times 0$ (control), $\times 1$, $\times 5$, and $\times 10$. Although the hatching rates in the controls appears to decline, we are confident that the reason is not accidental introduction of *I-Ppol* males but can offer no evidence-based explanation.

competitiveness typically range from 0 to 1, in which transgenic males that compete equally with wild males have competitiveness of 1,²² and males that mate no females have a value of zero. Competitiveness of *beta2-Ppo2* males ranged from 0.57 to 0.69 (Table 3): the average value was 0.64 (95% CI mean: 0.57–0.71). Two experiments with *beta2-Ppo1* resulted in much lower competitiveness values, 0.20 and 0.04. There was no difference in the number of the two types of males remaining at the end of the experiments.

We used two other methods to complement the standard competitiveness data above for the *beta2-Ppo2* strain. The first is based on the egg-hatching rate achieved in the large and small cage experiments: the difference between the theoretical egg-hatching minima (calculated based on control fertility and the male release rate) and the realized hatching rate of *en masse* egg collections can be used to estimate competitiveness. This calculation is possible if the effect of male type on two outcomes is known: (1) the number of eggs laid and (2) a female's likelihood of oviposition. We estimated these effects using egg-batches obtained from females removed from the

Table 2 GLM statistics of hatching rates for the last five and six egg-collection dates from the large and small cages, respectively

	Source	Degree of freedom	Adj. S.S.	F	P
Large cages	Release rate	2	2.57597	32.37	<0.0001
	Cage (release rate)	6	0.24118	3.79	0.005
	Error	34	0.36100		
	Total	42			
Small cages	Release rate	3	2.45855	19.61	<0.0001
	Cage (release rate)	8	0.33464	2.96	0.007
	Error	59	0.83302		
	Total	70			

large cages at the termination of the experiments: there was no difference (*t*-test) between the average number of eggs laid by females (mean, std. dev., sample size) that were mated to wild (92.50, 33.18, *n*=58, including controls) or transgenic (88.64, 30.44, *n*=98) males. Male type did affect the likelihood of egg-laying: not considering the control cages, females mated to transgenic males laid eggs 95% of the time (*n*=101), whereas 61% (*n*=28) of females mated to wild-type males laid eggs (contingency Chi-square test, 1 df, *P*<0.0005). These values were used to adjust the competitiveness estimates based on the average of the three cages at each release level of the last five egg collections. Five average realized rates of infertility for the $\times 5$ and $\times 10$ large cages respectively indicate adjusted competitiveness of 0.33 (std. dev.=0.066) and 0.35 (std. dev.=0.059). However, these values should be considered cautiously: (1) they differ greatly from the direct measures (Table 3) and (2) we do not know that the egg numbers and propensity to lay eggs obtained by isolated individual females accurately represents their behavior in large cages. If the adjustments for probability of eggs-laying are not applied, the competitiveness values for beta2-Ppo2 males were 0.52 (std. dev.=0.096) and 0.54 (std. dev.=0.094) for the $\times 5$ and $\times 10$ large cages, respectively. Similar egg-batch and laying probability was not available for the small cages because eggs were not obtained at the end of the releases, so equal numbers and probability of laying eggs was assumed. The estimated competitiveness values (std. dev.) based on the average fertility from the last six egg collections were 0.49 (0.393), 0.34 (0.190),

and 0.61 (0.259) for the $\times 1$, $\times 5$, and $\times 10$ release rate cages, respectively.

Comparison of development and survival of transgenics versus wild siblings

In experiments conducted separately from the releases of I-Ppo1 males and mating competitions, the ratio of transgenic to wild larvae was determined in the L4 stage in cohorts of larvae from *en masse* egg-collections. As observed previously,¹⁴ there was no significant deviation from the expected 1:1 ratio of transgenic to wild larvae. Transgenic pupae were rare on the first day of pupation (Fig. 4, Chi-square test by cohort, 2 df, maximum *P*=0.002). A difference between transgenic and wild individuals was also observed in the proportion of adults of both strains successfully eclosing (Table 4): transgenic male emergence (average, std. dev., *n*) for both $\beta 2$ -Ppo1 (0.68, 0.04, 3) and beta2-Ppo2 (0.63, 0.09, 6) was lower than that of transgenic females and non-transgenic mosquitoes, irrespective of the sex, which ranged from 0.81 to 0.89. Considering sex, strain, and the interaction of sex and stock, only sex had a significant effect on the eclosion rate of transgenics (MANOVA, 11 df, *F*=9.85, *P*=0.014), but there was no effect among non-transgenic siblings.

The number of adults remaining at the termination of the small and large cage experiments was determined and analyzed for the rates of adult survival (Table 5). In the small cages, there was a significant (GLM, *F*=5.14, 3 df, *P*=0.028) deficiency of females in the $\times 10$ release level relative to controls. The numbers of wild males did not differ across release levels. In contrast, particularly in cages with higher

Table 3 Competitive mating estimates (c) from experiments conducted in large cages

	Adults remaining at end of experiments							c
	Females	Wild males	Trans. males	Total* males	Trans. matings	Wild matings	Virgins	
Beta2-Ppo2 Cage								
D	73	29	31	71	18	26	22	0.69
F	77	20	20	66	20	35	18	0.57
G	94	36	28	53	21	32	30	0.65
				Total	59	93	70	
Beta2-Ppo1 Cage								
H	73	52	26	26	9	46	17	0.20
I	63	19	4	15	2	54	7	0.04
				Total	11	100	24	

Note: *Males were scored for transgenic status the following day after some mortality had occurred in the beta2-Ppo2 experiments. Therefore, 'total males' does not equal the sum of wild plus HEG males.

Table 4 Proportions of adults successfully eclosing (std. dev., number of cohorts)

Stock	Females		Males	
	Transgenic	Wild sibling	Transgenic	Wild sibling
Beta2-Ppo1	0.81 (na, <i>n</i> =1)	0.83 (na, <i>n</i> =1)	0.68 (0.04, <i>n</i> =3)	0.89 (0.01, <i>n</i> =3)
Beta2-Ppo2	0.82 (0.05, <i>n</i> =2)	0.88 (0.03, <i>n</i> =2)	0.63 (0.09, <i>n</i> =6)	0.85 (0.05, <i>n</i> =6)

stocking densities, significant deficiencies of beta2-Ppo2 males relative to non-transgenics were observed.

The numbers of adults remaining in the large cages were analyzed similarly. No significant difference between the numbers of females and wild males in the large cages by release level was observed (GLM); however, lack of significance may be due to the great variation in the number recovered. The total number of beta2-Ppo2 males in release large cages was expected to be 5 or 10 times the number of males in the controls for the $\times 5$ and $\times 10$ large cages, respectively. Gross deficiencies in the abundance of males were observed: in the $\times 5$ large cages, we observed 41% of the expected number (Chi-square, 2 df, $P=2^{-26}$) and in the $\times 10$ large cages 45% (Chi-square, 2 df, $P=2^{-44}$).

Discussion

The results show that I-PpoI males induce high levels of infertility in cage populations of stable-age-distribution mosquito populations. The experiments conducted in large cages particularly confirm the suitability of the beta2-Ppo2 strain for consideration in sterile insect technique programs. Several critical factors remain to be determined before they can be confirmed to be suitable for releases above that of small demonstration programs. Most problematic is that the strains are not pure-breeding. While a fluorescence sorter has been shown to be capable of facilitating this process,^{23,24} it remains to be determined whether this will

be practical for larger scales of production and for stock maintenance.

In a typical SIT program, declining recruitment rates of wild individuals resulting from infertility would result in even greater levels of infertility since the relative abundance of sterile males would increase. In our experimental system, we did not reduce the recruitment rate of wild males because adult mosquito population sizes are controlled in part by negative density dependent factors, particularly in the larval stage.^{25,26} Simply reducing the recruitment rate to the same extent as reduced egg-hatching was observed would therefore exaggerate the degree of population reduction that would be accomplished in a control program.

We explored the effect of the infertility that we observed on simulated mosquito populations (Fig. 6). A discrete-time deterministic population-dynamics-and-epidemiology model similar to that described by Deredec *et al.*²⁷ was built to study the consequences of introducing I-PpoI males into wild populations. It showed that regular releases of a constant number of I-PpoI males reduce the target population and will eliminate it if the release size exceeds a threshold (Fig. 6). The quantitative effect of releases depends on the longevity and mating competitiveness of released males. Assuming that mating likelihood is independent of age, reductions in competitiveness or longevity have the same effect on the level of population infertility. The minimum number of males to release

Table 5 Numbers and genotypes of adults removed from small cages at the end of releases

Small cages				Large cages		
	Females		Wild male	Females		Wild male
Control	23		14	35		23
	24		16	29		20
	25		17	38		23
Total	72		47	102		66
	Females	HEG male	Wild male			
$\times 1$	23	10	11			
	22	13	15			
	26	11	15			
Total	71	34	41			
	Females	HEG male*	Wild male	Females	HEG male	Wild male
$\times 5$	22	36	13	28	53	26
	24	52	26	31	49	6
	22	53	17	31	32	14
Total	68	141	56	90	134	46
	Females	HEG male†	Wild male	Females	HEG male	Wild male
$\times 10$	21	103	22	37	97	16
	19	110	23	31	92	12
	20	81	33	30	107	19
Total	60	294	78	98	296	47‡

Note: *The following numbers of males were collected, but mortality occurred before the next day when their phenotype was determined: 98, 71, and 53 from top to bottom rows. No Chi-square values were calculated since equal mortality of two male types was not known.
†Chi-square probabilities of 10:1 HEG to wild male by cage (1 df) top to bottom are 0.0009, 0.0010, <0.0001.
‡Chi-square probability 10:1 HEG to wild male (1 df) of total=0.003.

Figure 5 Proportion of transgenic pupae on first 3 days of pupation for six different cohorts. Actual numbers counted are overlaid on the bars.

daily in order to eliminate the population can be expressed as a fraction of the initial number of daily emerging vectors and depends on its R_m (the population intrinsic growth rate). We evaluated the threshold over a range of R_m for *A. gambiae* used previously.²⁷ We observed that a minimum of approximately $\times 5$ release rate of males with competitiveness of 0.5 would need to be maintained to eliminate target populations up to the maximum R_m modeled.

Similar experiments to those which we performed but using a RIDL® strain (Oxitec, Oxford, UK) of the dengue vector mosquito *Aedes aegypti* utilized extremely high levels of negative density dependence in the larval stage to stabilize populations to the carrying capacity of the larval containers.²⁸ However, the larval biology of *A. aegypti* is much more amenable to highly crowded larval culture than is *A. gambiae*. As an illustration of the conditions that would create the recruitment rate we utilized in the large cages, it would be have been necessary to create larval culture conditions in which the average number of hatching eggs (99.4) from each oviposition resulted in 10 adults. Such larval attrition rates for *A. gambiae* have not been observed in outdoor simulated larval habitats²⁵ and only in the most extreme density and diet levels in laboratory experiments with *A. arabiensis*.²⁹ Therefore, if we had applied the necessary larval conditions, the resulting adults would necessarily have been of very small size²⁹ and the wild males particularly would likely be of reduced competitiveness relative to the transgenic males which were cultured under relatively favorable conditions. This would artificially bias competitiveness in favor of transgenics and could result in a rate of population reduction that would be artificially high.

Life history analysis of the beta2-Ppo1 and beta2-Ppo2 lines revealed retarded development of the transgenic individuals and low adult eclosion in transgenic males. We hypothesize that this was due

Figure 6 Minimal release size (y axis, expressed as the ratio of the number of released I-Ppo1 males to the initial number of wild males recruited daily) in order to eliminate the target population as a function of the intrinsic population growth rate (R_m). Outcomes with three levels of mating competitiveness are shown (1) identical to wild (solid); (2) reduced by 10% (dotted), or by 50% (dashed), the case most similar to what we observed. Reduced longevity and reduced competitiveness require increased release ratios. The parameters and assumptions are the same as were used by Deredec *et al.*²⁷

to the transgene itself rather than to a position-dependent disruption of endogenous gene activity as both strains manifested a similar retardation and mortality. The strains were confirmed to be molecularly distinct; therefore, it seems unlikely that if that there were position effects that the same negative outcome would be caused by disruptions at two different chromosomal loci. With the exception of possibly lower competitiveness of beta2-Ppo1, we observed only one other distinguishing characteristic of that strain: the expression of the fluorescent markers appeared stronger in beta2-Ppo1, two characteristics that may be related.

The effect of transgenesis on various life-history characteristics of pest insects including mosquitoes has been reviewed by Scolari and colleagues.³⁰ They observe that due in part to founding, specific insertion sites, specific transgenes and strain manipulation, lines vary in their characteristics. They recommend the practice of developing numerous transgenic lines that are then screened for favorable characteristics or, alternatively, using host strains that contain docking sites, the performance of which can be predicted. *A. gambiae* docking strains are now available for this purpose.³¹ The difficulty of creating and purifying numerous anopheline strains and lack of standard assays of fitness has limited the extent to which such activities are performed. But as strains move from the lab to field testing, these methods should be routine, and developing simpler culture methods as well as testing candidate strains by focusing on characteristics relevant to field performance should be implemented.

A predicted advantage for transgenic approaches over irradiation for inducing sexual infertility is the reduction of somatic damage.¹⁶ The levels of irradiation that cause near complete infertility also cause severe loss of competitiveness;^{32,33} therefore, as a compromise, partly fertile males are usually released in SIT programs. The reduced competitiveness of I-PpoI males relative to wild-type in the release experiments compares favorably with that observed using radiation in both large and small cages³⁴ and reductions relative to wild comparators may be related to reduced daily survival observed when large cage experiments were terminated. However, there was no evidence of reduced survival of the I-PpoI males relative to the wild type in the large cage mating competitions in which the competitiveness estimates were only somewhat higher than those estimated from the release experiment hatching rates. The competitions lasted only 1 week and may have underestimated differential mortality that may be more evident at a later age. It also seems likely that even though most males were able to eclose that they shared some stress to which others in the cohort succumbed.

Mating competitiveness measures varied widely depending on the experimental method. Ultimately, these measures contribute to an assessment of whether these strains could be used for a SIT program. The effect of reductions in competitiveness translates directly into increased release rates necessary to accomplish the same level of population infertility. Assuming a 50% reduction in fitness similar to what we have observed means that twice as many males must be released to achieve the same infertility. This in turn translates into increased production and release costs. While any loss of competitiveness is undesirable, typical acceptable medfly values determined in laboratory experiments as quality control standards range from 0.2 to 0.4 for flies used in release programs.³⁵

We have demonstrated under a variety of laboratory conditions that the beta2-Ppo2 males provide good levels of competitiveness and reduced egg-hatching rates in target populations. The strain tested most extensively has no characteristics that preclude expanded testing in contained outdoor trials. After outcrossing the transgene to target population mosquitoes, it will be possible to further determine which characteristics are separable from the transgene and whether the performance we observed is confirmed in more demanding outdoor contained trials.

Acknowledgements

We appreciate the use of the environmental chambers and insectaries of the Centers for Disease Control and Prevention. The tuna meal was provided by Jeremie Gilles (International Atomic Energy Agency, Vienna,

Austria). The MR4 continues to be an essential source of support which provided many mosquito strains for these studies. This work is funded by a grant from the Foundation for the National Institutes of Health through the Vector-Based Control of Transmission: Discovery Research (VCTR) program of the Grand Challenges in Global Health initiative to AB.

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