Fitness of Anopheline Mosquitoes Expressing Transgenes That Inhibit Plasmodium Development

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ABSTRACT

One potential strategy for the control of malaria and other vector-borne diseases is the introduction into wild vector populations of genetic constructs that reduce vectorial capacity. An important caveat of this approach is that the genetic construct should have minimal fitness cost to the transformed vector. Previously, we produced transgenic *Anopheles stephensi* expressing either of two effector genes, a tetramer of the SM1 dodecapeptide or the phospholipase A2 gene (PLA2) from honeybee venom. Mosquitoes carrying either of these transgenes were impaired for *Plasmodium berghei* transmission. We have investigated the role of two effector genes for malaria parasite blockage in terms of the fitness imposed to the mosquito vector that expresses either molecule. By measuring mosquito survival, fecundity, fertility, and by running population cage experiments, we found that mosquitoes transformed with the SM1 construct showed no significant reduction in these fitness parameters relative to nontransgenic controls. The PLA2 transgenics, however, had reduced fitness that seemed to be independent of the insertion site of the transgene. We conclude that the fitness load imposed by refractory gene(s)-expressing mosquitoes depends on the effect of the transgenic protein produced in that mosquito. These results have important implications for implementation of malaria control via genetic modification of mosquitoes.

GREAT progress has been made during the past few years in the development of genetic engineering tools for mosquitoes. Both culicine (Coates *et al.* 1998; Jasinskiene *et al.* 1998; Allen *et al.* 2001) and anopheline (Catteruccia *et al.* 2000; Grossman *et al.* 2001) mosquitoes can now be transformed, and genetic constructs that block the development of malaria parasites in transformed mosquitoes have been produced (Ito *et al.* 2002; Moreira *et al.* 2002). While methods for driving such constructs into wild mosquito populations have not yet been developed, it will be important to use transgenes that impose the lowest possible fitness cost to the mosquito. Recently, the fitness of transgenic *Anopheles stephensi* expressing fluorescent protein markers from a ubiquitous actin promoter has been analyzed (Catteruccia *et al.* 2003). By performing cage experiments that started with equal numbers of homozygous transgenic and nontransgenic mosquitoes, the authors showed that four independently obtained transgenic lines had reduced fitness relative to the nontransformed population and that the transgenes disappeared from the cage populations after 5–15 generations. The most likely cause of the transgene loss was the reduced fitness of the inbred transgene lines relative to the more outbred parental population, but the experimental protocol could not rule out reduced fitness effects caused directly by the transgenic constructs.

Previously, we introduced into mosquitoes either of two genes that interfere with their ability to support parasite development. One encoded a tetramer of the SM1 peptide (Ghosh *et al.* 2001; Ito *et al.* 2002) and the other bee venom phospholipase A2 (PLA2; Moreira *et al.* 2002), both driven by the gut-specific and blood-inducible *A. gambiae* carboxypeptidase promoter (Edwards *et al.* 1997; Moreira *et al.* 2000). All transgenic mosquitoes also express an enhanced green fluorescent protein (GFP) mostly in eye tissues. Importantly, all transgenic lines were kept as heterozygotes for at least 16 generations by crossing in each generation, transgenic males with virgin females from the laboratory population cages. This strategy was adopted to avoid hitchhiking of any deleterious gene(s) residing near the point of transgene insertion. Here we report on experiments that compare fitness of these transgenic mosquitoes with their nontransgenic counterparts (Falconer and Mackay 1996).

MATERIALS AND METHODS

Life table experiments: Adult cages containing both transgenic (SM1 or PLA2 lines) and nontransgenic mosquitoes were fed on an anesthetized mouse for 30 min. Engorged females

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were selected from nonengorged females and placed individually into 0.5-liter mesh-covered cardboard cups (Sealright-Nestile, East Providence, RI) containing an egg pot. The egg pot consisted of a conical P5 filter paper (Fisherbrand, Pittsburgh) placed inside a P100 plastic cup (Solo, Urbana, IL) half-filled with tap water. Sucrose solution (10%) was provided with a wig of cotton placed on top of the mesh and covered with another P100 cup to decrease evaporation. Egg pots were collected on the third day and the eggs were counted with a dissecting microscope. The filter papers with eggs were then transferred to a 0.5-liter round plastic container (News Spring, East Newark, NJ), which contained a ring of filter paper (Fisherbrand) on the edge of the water to avoid attachment of eggs to the walls and desiccation. Larvae were fed with pellets of cat food (Friskies Senior, Glendale, CA). After 3–4 days the hatched larvae were sieved and transferred to ice-cold water to stop their movement and counted with a dissecting microscope.

Population cage experiments: Transgenic lines of both constructs (SM1 and PLA2) were maintained by crossing transgenic males with virgin nontransgenic females for at least 16 generations, yielding heterozygous mosquitoes carrying one copy of the transgene per genome. To select transgenics, mosquitoes were cold immobilized and screened with a UV dissecting microscope. Two crosses were set up for each experiment. For one, 250 virgin transgenic females and 250 nontransgenic males were placed in a 12 × 12 × 12-inch cage (Bioquip, Gardena, CA). The other cross was identical in design except that females were nontransgenic and males were transgenic. After 2 days, mosquitoes were fed on anesthetized mice and eggs were collected after another 2 days. Filter papers containing eggs were hatched in plastic trays with water and reared under standard laboratory conditions. About 100 fourth instar larvae were examined with a UV dissecting microscope to measure the proportion of transgenic (expressing GFP) and nontransgenic individuals. Adult mosquitoes were collected every day from each tray and kept segregated by sex until enough numbers (>250) had accumulated. Crosses for the next generation were performed as described above, with the important exception that male and female adults were chosen blindly, without consideration of whether they were transgenic or not. An additional 50 adult mosquitoes from each sex were scored for GFP expression and discarded. This process was repeated for 5 generations, always without selection, but always scoring 100 larvae and 100 adults of each generation for the expression of GFP.

Protein analysis: Total protein in midguts and ovaries was determined by use of the Bradford Assay (Bio-Rad, Hercules, CA). Wild-type and transgenic mosquitoes were fed on mice followed by dissection of guts and ovaries from five mosquitoes for each time point. Dissected tissues were placed into 100 μl of phosphate-buffered saline (PBS) and sonicated. Known amounts of bovine serum albumin were assayed at the same time as a quantification standard. The entire sample of guts before the blood meal was used for protein determination while only 10% of gut samples dissected just after and at 12, 24, and 48 hr after the blood meal were used because of the high protein content of those samples. For all ovary samples the 100-μl homogenate was diluted with 700 μl of PBS and 200 μl of the Bradford reagent was added. Samples were incubated 10 min at room temperature and the A^{595} was determined.

Statistical analysis: For the life table experiments the Mann-Whitney nonparametric test was used to compare means of both control and transgenic groups and constructs using the Statview v5.0.1 statistical software for the Macintosh. Data from population cage experiments were analyzed to check the deviation from the Hardy-Weinberg equilibria (Falconer and Mackay 1996) using the chi square ($\alpha = 5\%$; d.f. = 1).

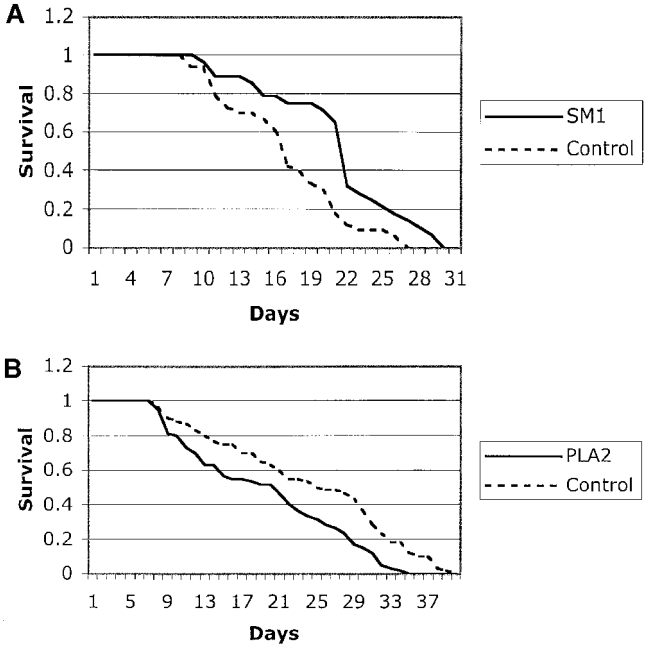

Figure 1.—Survival of wild-type and transgenic *A. stephensi* mosquitoes. Female mosquitoes were fed with mouse blood. Each female was placed in an individual cup and longevity was recorded every day until all died. Survival is plotted as the proportion of surviving adults in each day (100% survival = 1.0). (A) Transgenic SM1 mosquitoes compared to nontransgenic controls. (B) Transgenic PLA2 mosquitoes compared to nontransgenic controls. The graphs show the average of two independent experiments. These are the same mosquitoes used for the experiments in Table 1. The Mann-Whitney test indicated that SM1 transgenics lived significantly longer than nontransgenic controls, while there was no significant longevity difference between PLA2 transgenics and controls. Experiments with two other independently derived SM1 line(s) and two other PLA2 line(s) gave similar results.

RESULTS

Life span, fecundity (eggs laid per female), and fertility (proportion of eggs that hatched into larvae) of females expressing the SM1 tetramer (Ghosh *et al.* 2001; Ito *et al.* 2002) showed no significant differences when compared with their nontransgenic siblings (Figure 1A and Table 1). Transgenic mosquito fitness relative to wild type was also assessed with cage experiments (Table 2). The experiments started by crossing 250 heterozygous transgenic mosquitoes with an equal number of nontransgenic mosquitoes of the opposite sex. The transgene frequency in the parental population was therefore 0.25. If the transgene has no fitness cost, the expected frequency of GFP-positive individuals in the first generation is 0.50 (green fluorescence due to the transgene is dominant) and 44% GFP positive/56% GFP negative for subsequent generations (Hardy-Weinberg equilibrium; Falconer and Mackay 1996). No consistent deviation from the expected values was observed in two independent experiments (Table 2), indicating that the SM1 transgene did not impose a fitness load. Similar

TABLE 1

Life table for nontransgenic (control) and transgenic mosquitoes from two independent experiments for each transgene

Group	*n*	Mean no. eggs/ mosquito ± SD	Fertility (%)
Control	33	78.5 ± 62.3	65.2
SM1	28	69.9 ± 62.3	60.1
Control	60	146.5 ± 68.0	74.6
PLA2	60	39.6 ± 31.0[a]	83.8

Fertility is defined as the percentage of eggs that hatched into larvae; *n*, number of mosquitoes.

[a] Mean significantly different from its control according to the Mann-Whitney test ($P < 0.0001$).

results were obtained with another independently derived SM1 transgenic line (Ito *et al.* 2002; data not shown), indicating that the position of transgene insertion did not have an effect on fitness in either case.

In contrast to the SM1 transgene, mosquitoes carrying the PLA2 transgene had a significant fitness load. While survival was not significantly different from nontransgenic mosquitoes (Figure 1B), mosquitoes from two independently derived PLA2 transgenic lines (Moreira *et al.* 2002) laid fewer eggs (Table 1). Consistent with these observations, transgenic PLA2 mosquitoes ingested from 10 to 50% less blood and accumulated much less protein in their guts (Figure 2A) and ovaries (Figure 2B). The time course of blood digestion and protein accumulation in the ovary was not affected (Figure 2, A and B). The magnitude of the decrease in egg numbers was much higher than that expected from the decrease in amount of blood ingested (Table 1). Overall, these results are in agreement with the decreased fitness of the PLA2 mosquitoes in cage experiments (Table 3). The ratio of transgenic to nontransgenic in the first generation was 50%, as expected, but from the second generation onward the frequency of transgenic individuals rapidly decreased. PLA2 transgenics almost disappeared by the fifth generation. While the mechanism by which PLA2 inhibits parasite development merits further investigation, this transgene is clearly not appropriate for introduction into wild populations.

DISCUSSION

This report addresses for the first time with laboratory experiments the question of whether either of two transgenes that interfere with Plasmodium development affects mosquito fitness. This is an important issue because fitness is likely to determine the feasibility of using either of these constructs in a genetic control program. In contrast to previous results (Catteruccia *et al.* 2003), we find that SM1 expression in the gut and GFP expression in the eye (and a few other tissues) does not impose a fitness load. In this regard, it is important to consider mosquito genetic background and promoters used to drive transgene and marker expression. The transgenic mosquitoes used in this work were maintained by crossing at each generation to wild-type mosquitoes from lab population cages, thus avoiding "hitchhiking" of deleterious genes. Moreover, expression of both GFP and SM1 was limited to specific cell types (eye and posterior midgut epithelium, respectively), minimizing a possible load of a foreign protein strongly expressed in many tissues. Note that the SM1 protein does not accumulate in the midgut cells, but is secreted into the lumen. In previous experiments (Catteruccia *et al.* 2003), it is likely that deleterious genes residing near the point of transgene insertion caused an initial heavy decrease of the transgene, followed by loss due to drift.

TABLE 2

Distribution of SM1 transgenic and nontransgenic mosquitoes in five generations of cage experiments

	T females + NT males						T males + NT females					
			No. of adults						No. of adults			
	No. of larvae		Male		Female		No. of larvae		Male		Female	
Generation	NT	T	NT	T	NT	T	NT	T	NT	T	NT	T
F_1	50	50	49	51	58	42	57	43	56	44	66	54
F_2	54	46	52	48	62	38	55	45	70	30	60	40
F_3	59	41	54	46	63	37	58	42	64	36	66	34
F_4	69	31	60	40	58	42	81[a]	19	76[a]	24	68	32
F_5	71[a]	29	68	32	52	48	60	40	58	42	60	40

A total of 250 virgin females were crossed with 250 males and maintained for five generations without selection. The proportion of transgenic and nontransgenic individuals was recorded at each generation. T, SM1 transgenic; NT, nontransgenic.

[a] Significant deviation from the expected frequency of 50%:50% in F_1 and 56% NT and 44% T (Hardy-Weinberg) in subsequent generations, according to the chi-square test ($\alpha = 5\%$; d.f. = 1).

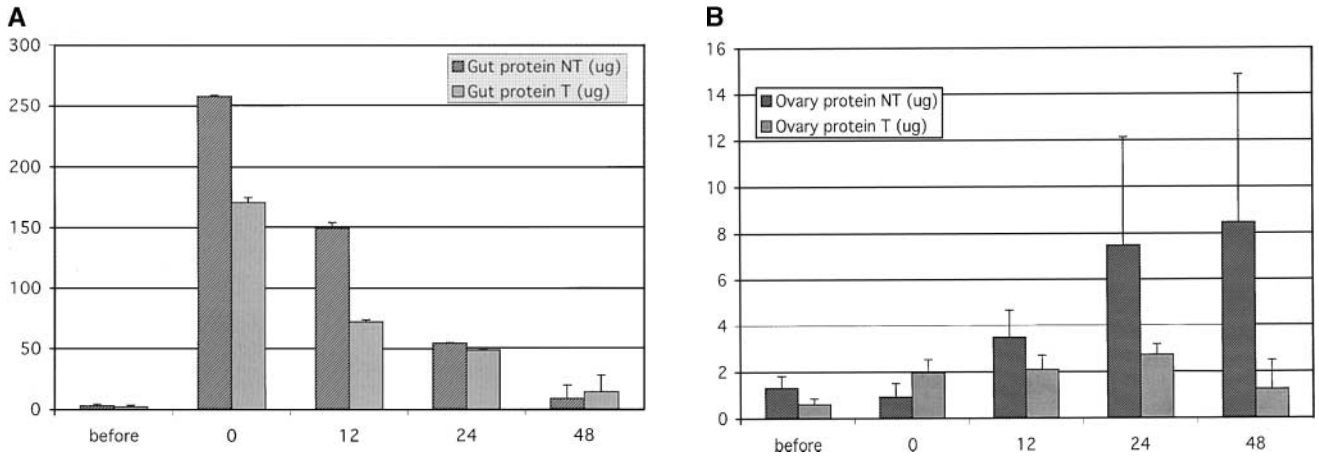

FIGURE 2.—Comparison of protein content of guts and ovaries from wild-type and AgCP-PLA2 transgenic mosquitoes. For each time point before or after a blood meal (in hours), five mosquitoes were dissected and the average protein content per organ (in micrograms) was determined. NT, nontransgenic mosquitoes; T, AgCP-PLA2 transgenic mosquitoes. (A) Protein content of guts. (B) Protein content of ovaries. Each graph shows the average of two independent experiments.

Losses due to the strong and generalized expression from the actin promoter of fluorescent proteins in many mosquito tissues may also have contributed to decreased fitness (LIU *et al.* 1999).

The caveat to the interpretation that SM1 transgene does not impose a fitness load is that fitness was measured in the laboratory. The possibility that other factors come into play when mosquitoes reproduce in the field cannot be ruled out. For other transgenes (such as PLA2), a significant load may be imposed, meaning that each transgene needs to be independently evaluated. Moreover, while for the limited number of SM1 lines tested we could not detect a load due to gene disruption by transgene insertion, in strategies where an active driving mechanism is used (*e.g.*, a functional transposase) insertional mutagenesis may impose significant load. Conversely, mosquitoes that harbor malaria parasites appear to be less fit, because they lay fewer eggs and may have a shorter life span (HOGG and HURD 1995; ANDERSON *et al.* 2000). Thus, in endemic areas, any fitness cost associated with a transgene that impairs malaria parasite transmission (such as those expressing SM1) may be countered to some degree by a fitness advantage associated with parasite refractoriness. Ongoing experiments in our laboratory address this hypothesis.

These experiments represent an initial step toward the much more ambitious goal of implementing a genetic strategy for the control of malaria. Much work remains to be done before releases in the field can be considered. Effective means of spreading genes through populations still need to be identified, population structure of vector species need to be better understood, and safety concerns

TABLE 3

Distribution of PLA2 transgenic and nontransgenic mosquitoes in five generations of cage experiments

	T females + NT males						T males + NT females					
			No. of adults						No. of adults			
	No. of larvae		Male		Female		No. of larvae		Male		Female	
Generation	NT	T	NT	T	NT	T	NT	T	NT	T	NT	T
F_1	43	57	53	47	61	39	59	41	52	48	64[a]	36
F_2	82[a]	18	80[a]	20	78[a]	22	70[a]	30	82[a]	18	80[a]	20
F_3	91[a]	9	82[a]	18	84[a]	16	89[a]	11	88[a]	12	94[a]	6
F_4	95[a]	5	92[a]	8	94[a]	6	95[a]	5	94[a]	6	94[a]	6
F_5	99[a]	1	98[a]	2	98[a]	2	95[a]	5	100[a]	0	98[a]	2

A total of 250 virgin females were crossed with 250 males and maintained for five generations without selection. The proportion of transgenic and nontransgenic individuals was recorded at each generation. T, PLA2 transgenic; NT, nontransgenic.

[a] Significant deviation from the expected frequency of 50%:50% in F_1 and 56% WT and 44% T (Hardy-Weinberg) in subsequent generations, according to the chi-square test ($\alpha = 5\%$; d.f. = 1).

need to be addressed. We are optimistic, however, that these concerns can be met and that an effective genetic control strategy for malaria can be achieved.

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Note added in proof: After this article was accepted for publication, Irvin *et al.* (N. Irvin, M. S. Hoddle, D. A. O'Brochta, B. Carey and P. W. Atkinson, 2004, Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. Proc. Natl. Acad. Sci. USA **101:** 891–896) reported that transgenic *Aedes aegypti* expressing GFP and transposase genes have a significant fitness load. However, as in the report of Catteruccia *et al.* (2003), the experiments were conducted with homozygous mosquitoes. Thus, it is not possible to determine whether the observed fitness load was due to the transgenes themselves or to hitchhiking effects of nearby deleterious genes.

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