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Measuring public-health outcomes of release of transgenic mosquitoes

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Abstract

The transgenic RIDL method could ensure that male mosquitos can be released without biting females and that the males would have no female progeny after mating to wild females. Urban *Anopheles* or *Aedes* vector populations, surrounded by different species in rural areas, would seem to be the most appropriate targets for such releases, aiming at eradication. In urban areas intensity of transmission is generally not very high and the public-health outcomes of such urban programmes could be monitored by passive surveillance through health facilities or by active surveillance for infections with or without associated symptoms.

The alternative use of transgenic mosquitos would be to produce strains refractory to infection by pathogens such as *Plasmodium* and to drive such genes into wild populations. In theory, in contrast to sterile-male eradication, such a procedure could "resist" a limited level of immigration and could open up the possibility of using the method against African rural malaria. However, in practice it would seem extremely difficult or impossible to ensure the necessary complete linkage of the refractoriness genes to the driving system. If this problem could be overcome one could monitor the impact of the spreading of the refractoriness genes by its impact on (i) the sporozoite rate in the wild population; (ii) the incidence of re-infection after clearing existing infections with an appropriate drug treatment; (iii) active surveillance for prevalence of malaria fever and anaemia in children; (iv) attacks of severe malaria and deaths monitored though hospitals and village reporters.

Keywords: transgenic mosquitoes; sterile males; urban vectors; malaria surveillance; dengue surveillance; malaria refractoriness; gene driving systems; sporozoite rate; malaria incidence; malaria morbidity

Introduction

It has been suggested that transgenic mosquitoes might be used for:

(a) production of sterile males without the need for treatment of the males for release and ensuring that no females are released with them (the so-called RIDL system, see Figure 1) (Thomas et al. 2000; Heinrich and Scott 2000; Alphey and Andreasen 2002)

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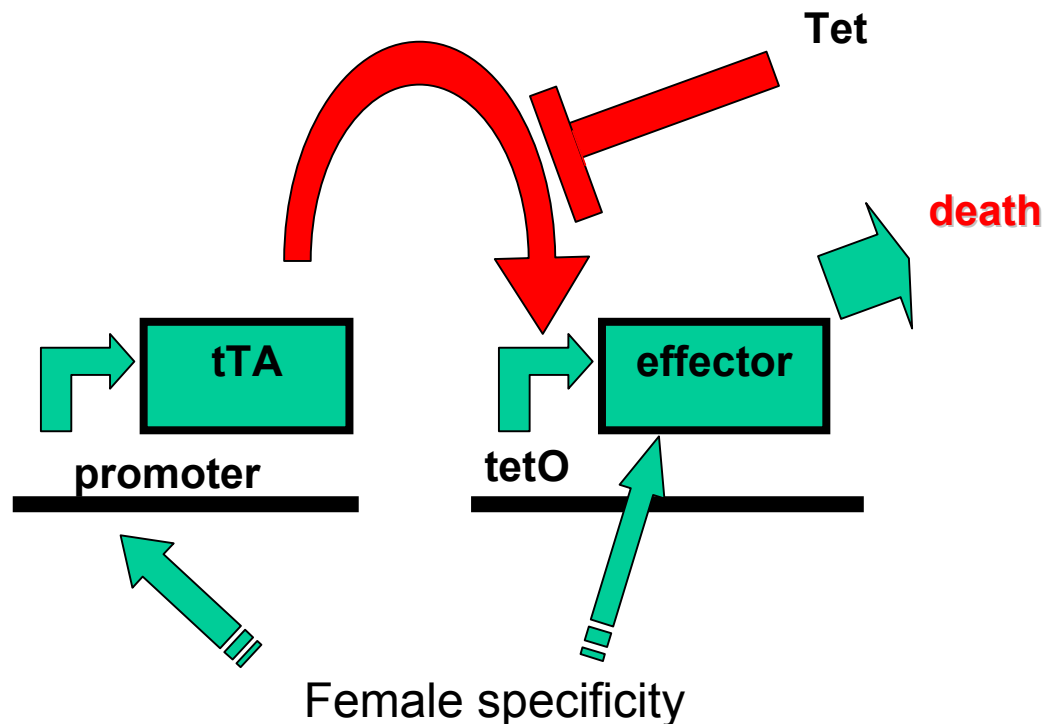
Figure 1. Diagram kindly supplied by Alphey and Andreasen (2002) of repressible, female specific dominant lethality. Removal of tetracycline from the larval medium releases female-killing mechanism by unblocking teracycline transactivator

production of pathogen-refractory mosquitoes and attachment of the gene(s) concerned to a genetic system for driving them to high frequency in a wild population (Curtis 1968; Davis, Bax and Grewe 2001; Boëte and Koella 2002; Ito et al. 2002).

Possibilities of eradication by sterile males

Male insects are adapted for searching out females even if they are rare. Therefore, in appropriate circumstances, sterile males can eradicate wild populations. Radiation-sterilized *Glossina austeni* eradicated this species from Zanzibar (Msangi et al. 2000) where immigration was geographically impossible. Mass release on a rolling front, of radiation-sterilized New World Screw Worm Flies (*Cochliomyia hominovorax*) has eradicated this species all the way from the southern USA to Panama (Wyss 2000).

The RIDL system leads to the activation of a dominant female-killing gene as soon as a dietary supplement of tetracycline, which is supplied to the laboratory or factory colony, is removed. It could have the advantages of:

1. avoiding the need for radiation which is harmful to the competitiveness of mosquitoes, especially if given to the more easily handled pupae, rather than adults (Smittle and Patterson 1974, Andreasen, M. in prep.);
2. avoiding the need for chemosterilants such as bisazir, which Lofgren et al. (1974) found to give sterile male *Anopheles albimanus* with good competitiveness after release. However, such alkylating agents are mutagenic and would be unlikely to be allowed for treatment of insects for release nowadays;
3. activation of a RIDL system has been shown to give 100% killing of females in *Drosophila* (Thomas et al. 2000) and may well be more reliable for sex separation

in *Anopheles* than has been achievable with Y-chromosome translocations of insecticide-resistance genes (Seawright et al. 1978) or on the basis of pupal size in *Aedes aegypti* (Ansari et al. 1977).

It would be nice to think that one could attack the world's major malaria problem in African villages by setting up a rolling front of releases of sterile male *An. gambiae s.s* and/or *An. funestus*. However, funding for protection of the health of poor people is much less generously available than for protection of profitable cash crops such as citrus fruit or cattle. Therefore a rolling front of sterile male *An. gambiae* or *funestus,* comparable to that of Screw Worm Flies, seems almost inconceivable. The population of *An. arabiensis* on the island of Réunion is being considered as a target for a demonstration project with sterile males. Malaria has already been eradicated from the island, but the French government spends considerable sums on precautions against re-infestation of this island with malaria. An area surrounded by desert has been suggested as an alternative site but, after all the effort of setting up such a project, one might finish up only benefiting three men and a camel! Such sites are sometimes advocated for "proof of principle", but a demonstration eradication in such sites may not tell us much about application of the method against other more worthwhile targets such as "urban islands", i.e.where there is one vector species in an urban environment and another in the surrounding countryside (Curtis and Andreasen 2000), as appears to be more or less true for:

(a) southern Nigerian cities with urban *An. arabiensis* surrounded by rural *An. gambiae s.s* (Coluzzi et al. 1979; Kristan et al. 2002);
(b) Indian cities with urban *An. stephensi stephensi* surrounded by rural *An. culicifacies* (Ramachandra Rao 1984);
(c) urbanised Singapore with mainly *Aedes aegypti* vectoring resurgent dengue (Goh 1995) and *Ae. albopictus* as a less efficient vector in more rural habitats and with a causeway across about 1 km of water separating Singapore from Malaysia.

One advantage of concentrating on urban areas is that, if the programme was successful, a large human population would benefit from the resources and effort expended. One might think that if entomological data confirmed that eradication of populations of the above mentioned urban vectors had indeed been achieved, then eradication of the disease would inevitably follow; so disease monitoring would only be a public-relations exercise. However, unfortunately in all three cases mentioned above there is not a completely clear-cut division between the urban and rural vectors so the possibility of continued disease transmission by predominantly rural species infiltrating into the urban area would need to be checked. In the case of Singapore, it appears that as the *Ae. aegypti* infestation level has been reduced over the last 30 years by legally enforced source reduction, the amount of serious disease has increased (Goh 1995; Ooi et al. 2001). This is an apparent example of a "rebound", of the type that has been much discussed in relation to control of African malaria vectors, resulting from reduced exposure of infants to acquiring immunity and more serious disease outcomes if non-immune older people are infected (Coleman, Perry and Woolhouse 2001). One would have to consider whether successful eradication of *Ae. aegypti* might be counter-productive if a reduced, but definite, amount of transmission due to *Ae. albopictus* remained, with virtually no acquired immunity in the human population. It might be necessary also to eradicate the latter species and to eradicate dengue vectors in southern Malaysia to minimize the chances of re-invasion

via vehicle traffic. In the sterile-male campaign to prevent invasion of Los Angeles by Mediterranean fruit fly, there have been so many cases of re-invasion, as a result of people illegally carrying in infested fruit from Central America, that a permanent low level of sterile male release is now continued as a precaution (Dowell et al. 2000). Perhaps the same would be needed if populations of any of the above mentioned three vectors showed initial success of an eradication attempt. However, if this proved necessary it would cancel out the great "selling point" of eradication: that expenditure is time-limited and the high cost of eradication can justifiably be spread over a time period far into the future.

Strong supporters of the above mentioned "rebound" idea oppose attempts at vector control in the African rural lowlands where malaria is holo-endemic and acquired immunity is very important (Trape et al. 2002). They propose urban environments as more hopeful targets for malaria-vector control. If they were prepared to consider sterile males at all, they would certainly, and quite rightly, press for maximum initial efforts at conventional source reduction with the sterile males as a "mopping up operation".

In urban areas it ought to be possible to monitor progress against the diseases, as a result of urban sterile-male release or other forms of vector control, by individual case detection via passive surveillance through the public-health system. In tropical countries there is a tendency to ascribe any fever to "malaria". Therefore complaints of fever would need to be backed up by a quality controlled system of slide reading or use of test kits for detecting malaria infection, or immunological testing for dengue infection.

Even in areas of moderate transmission, symptomless malaria infections occur. Mass blood surveys for prevalence of infection, regardless of occurrence of fever, give information about the impact of vector control in addition to that from passive surveillance for malaria cases (e.g. Yapabandara et al. 2001).

In India it would be customary to monitor progress of malaria-vector control (including control by release of sterile male *An. stephensi*) through active surveillance by house-to-house enquiries by inspectors about fever cases and the taking of slides to check whether the cause is malaria. All urban areas, but especially those in India, attract immigrant workers from poverty-stricken rural areas. Thus many cases of vector-borne disease found in urban areas have been acquired elsewhere. Thus, for disease statistics to reflect properly the results of urban vector suppression, it would be necessary for careful enquiries to be made to identify, as far as possible, cases of imported disease. In the case of Nigerian cities, a preliminary study might show that so much "urban malaria" is imported that the above suggestion of urban vector eradication there might be pointless.

Driving of refractoriness genes into rural vector populations

As a first step any malaria-refractory strain should be checked by laboratory feeding experiments to ensure that there are no unexpected increases in susceptibilty to viral or filarial pathogens.

Boëte and Koella (2002) emphasized the need for malaria-refractoriness genes to be 100% effective if they are to have a beneficial impact in areas of intense transmission. They have also modelled effects of refractoriness genes in lowering fitness in the majority of mosquitoes which never encounter *Plasmodium,* or of raising fitness by allowing those mosquitoes which encounter infection to avoid its damaging effects. It seems very unlikely that the latter effect could be strong enough to drive an

introduced refractoriness gene to fixation in a wild population and a special driving system would seem to be required for this.

The most hopeful possibilities for gene-driving systems seem to be:
1. Mobile transposons which tend to copy themselves without deleting the "original" (Curtis and Graves 1988; Kidwell and Ribeiro 1992; Hager 2002). These would have to be checked before any releases are made to ensure that they cannot undergo possibly dangerous horizontal transfer into other species.
2. *Wolbachia* (Curtis and Sinkins 1998), which is maternally inherited and causes unidirectional sterility when *Wolbachia*-infected males mate to uninfected females. Thus *Wolbachia* infection is favoured by selection because infected females make no sterile matings and thus always transmit their infected cytoplasm.
3. Constructs consisting of two transgenic unlinked lethals with each one tightly linked to a suppressor of the other lethal (Davis, Bax and Grewe 2001 and Figure 2). This system provides for two independent refractoriness genes to be simultaneously selected. This could have the advantage of acting like multi-drug therapy in hindering the evolution in *Plasmodium* of resistance to (or evasion of) the refractoriness.

If one of these driving systems was completely linked to a gene or genes for refractoriness to *Plasmodium* and the system functioned perfectly, a relatively small "seeding" release should initiate a process of spreading which, unlike sterile males, would have some ability to overcome the effects of immigration. Thus it would open up the possibility of using transgenic mosquitoes against rural African malaria transmitted by *An. gambiae s.s.* and/or *An. funestus.* The problem of complete linkage of the driving system and the gene to be driven was raised by Curtis (1968) and has still not been solved. If there is the likelihood that somewhere or at some time the driving system would detach from its "load" of refractoriness genes, the unloaded driving system would most probably be at a selective advantage and would move to fixation. This is likely to prevent any further use of that driving system against the same population. In view of the large wild populations and long time periods involved, it is difficult to see how one could even study the question of whether the system is proof against occasional, but fatal, recombination. If expert molecular geneticists cannot confidently suggest a solution, one should seriously consider abandoning the whole refractoriness enterprise. If it is really not conceivable that the system could work, it is misguided to continue to use scarce resources from the malaria-research budget for activities which could not control malaria but could only produce NPS (*Nature*-paper synthetase). However, if expert opinion considers that absolutely unbreakable linkage is achievable then one has to consider how to monitor the effects on malaria transmission of a driver-refractoriness system. The first problem is that the system is supposed to spread. Thus setting up a conventional replicated pattern of treated and control villages would not be appropriate because villages not deliberately seeded with the refractory strain are intended to steadily acquire it over time as a result of mosquito immigration backed up by selection for the driver. Thus one would have to monitor progress in the spreading of the refractoriness gene and test for a negative correlation of this with appropriate malariological parameters.

Engineered allele β

Suppressor G

Promoter F

Lethal gene

Refractoriness gene B

Engineered allele α

SuppressorF
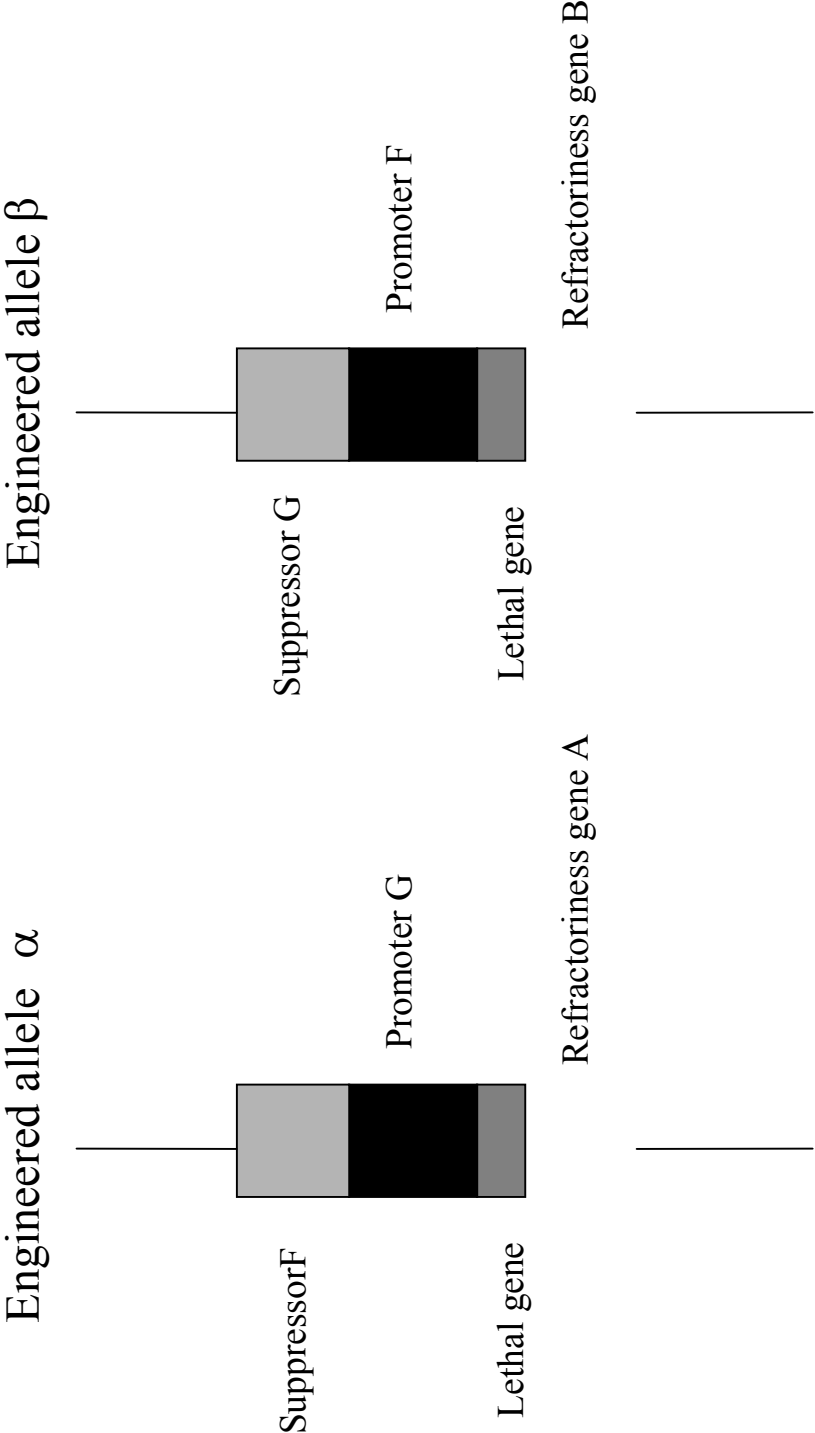
Promoter G

Lethal gene

Refractoriness gene A

Figure 2. Mechanism for transgenic underdominance for fitness proposed by Davis, Bax and Grewe (2001). Suppressor on one chromosome suppresses the promoter of the lethal on the other. A mosquito with neither construct or both is viable, but having only one is lethal. If enough with both constructs are released, selection will favour them and displace the wild type which has neither construct. Constructs could be on homologous or non-homologous chromosomes.

The refractoriness genes are intended to reduce the proportion of mosquitoes that can become infective. Use of ELISA (Burkot, Williams and Schneider 1984) for large-scale testing for sporozoites is now routine. In Tanzania we have collected mosquitoes for such tests, as well as for monitoring mosquito population densities, using light traps set indoors beside occupied untreated bed nets (Lines et al. 1991) and pyrethrum spray catches plus exit traps on windows. These methods are a far more efficient use of manpower, and less ethically objectionable in areas of multi-drug-resistant malaria, than human biting catches. We hope to confirm very soon that the bed net trap devised by Mathenge et al. (2002) is an even more efficient way of monitoring the biting population without getting bitten.

Our collections with light traps in cool highlands and warm lowlands, with or without vector control by community-wide use of pyrethroid treated nets or house spraying (Curtis and Sinkins 1998; Maxwell, Carneiro and Curtis (submitted)), showed that at both altitudes these interventions reduced both village vector-population density (*An. gambiae s.s., An. funestus* and *An. marshallii s.l.*) and sporozoite rate (see Table 1). Hence they greatly reduced the infective biting rate per person per year (i.e. the entomological inoculation rate – EIR). One should remember that introduction of refractoriness genes would only impact upon the sporozoite rate.

Table 1. Data on malaria vectors *(An. gambiae s.s., An. funestus* and *An. marshallii s.l.*) and malaria transmission in relation to altitude in Tanzania and community-wide vector control

	HIGHLAND (c.1200m)		LOWLAND (c..200m)		
	No nets	Treated nets	No nets	Treated nets	House spray[a]
Bites/person/year	282	116	3339	1374	2036
Sporozoite +ve	3.68%	2.74%	4.45%	2.73%	1.02%
EIR/year	10.4	3.2	148.6	37.5	20.8
Re-infections/ Child/ week	0.220	no data	0.423	0.100[a]	0.122

Data mainly from Maxwell, Carneiro and Curtis ((submitted)); [a]data from Curtis and Sinkins (1998); tests of statistical significance of differences are shown in these papers.

In areas of intense transmission one cannot easily monitor incidence of infection because people are frequently re-infected and most people carry some malaria parasites most of the time. It was customary to monitor very young babies for occurrence of their first infection and thus to obtain an infant parasite conversion rate as a measure of incidence. In Tanzania we have avoided this method (a) because we do not think mothers would like to have the fingers of their tiny babies repeatedly pricked, (b) it would require a great deal of travelling along bad roads to accumulate an adequate cohort of babies of a narrow age range. Instead we have used the short-half-life drug combination chlorproguanil-dapsone ("lap-dap"), which is effective where sulfadoxine-pyrimethamine fails due to resistance. We use this drug combination to clear existing infections from a group of children and then monitor them weekly for rate of re-infection per child week at risk (e.g. Maxwell et al. 1999; Maxwell, Carneiro and Curtis (submitted)). We realized that any recrudescence of incompletely cured infections could give misleading results. To check on this we have used the method of molecular matching of polymorphic alleles present before treatment and after recurrence of infection (Curtis et al. 2002). However, more reliably, we also took a group of lap-dap-treated children (with their mothers) from the lowlands to spend 6 weeks at 1700 metres altitude, at which there was no

transmission, as evidenced by lack of any infection in local highland children. The results confirmed that recrudescence could occur but did so rarely (3 out of 41 children in 6 weeks, Maxwell et al. 1999). In the lowlands without vector control, 20-40% of children showed recurrence of parasitaemia each week; this result can be corrected by our measured rate of recrudescence of parasitaemia as well as the possibility of two infections occurring in the same week. Reductions in EIR, due to (a) increase of altitude from 200 to 1200 metres, (b) installation of insecticide-treated nets or (c) indoor residual spraying, were all reflected in reductions in incidence of re-infection after clearance of pre-existing infections with lap-dap (Curtis and Sinkins 1998; Maxwell, Carneiro and Curtis (submitted), and Table 1). However it is notable that in all cases the scale of the reduction in incidence is less than the change in EIR. We interpret this as a result of adaptation of the immune status to the current infective biting rate and have some evidence for differences in measured antibody levels (Askjaer et al. 2001 and Table 2). One can expect the same phenomenon if reduction of infective biting was due to driving of refractoriness genes into the population. Thus, though it would be worthwhile to measure the sporozoite rate after release of mosquitoes carrying refractoriness genes, the incidence of re-infection would be a more realistic measure of how much impact one was achieving on malaria transmission. Incidence measured this way is not immediately convertible to public-health benefit against malaria morbidity. However, morbidity cannot be reduced unless incidence is reduced and one of our incidence trials takes 6-8 weeks to complete and can be done with quite a small field team.

Table 2. Active surveillance for mild malaria related morbidity in relation to altitude in Tanzania and community wide use of treated nets

Prevalence of morbidity	Child's age	HIGHLAND(c1200m)		LOWLAND (c.200m)		
		No nets	New nets	No nets	New nets	3-4 yr old nets
% malaria fever[a]	<2	8.6	2.3	18.3	7.4	2.9
	2-5	5.5	2.1	9.5	4.0	5.4
% Hb < 8g/dl	<2	20.4	4.3	38.7	16.2	18.3
	2-5	4.4	1.9	8.4	3.5	8.7
Mean antibody level at age 4 against VSA (arbitrary units)[b]	No data			1150	No data	990

[a] fever reported in last 2 days and/or temperature >37.4°C with >4000 parasites/μl
(Data mainly from Maxwell et al. 2002; Maxwell, Carneiro and Curtis (submitted)); [b](Askjaer et al. 2001); tests of statistical significance of differences are shown in these papers.

We have monitored whether conventional vector control (house spraying or insecticide-treated bed nets) in rural Tanzania sustainably reduces mild malaria morbidity in children (Curtis and Sinkins 1998; Maxwell et al. 1999; Maxwell, Carneiro and Curtis (submitted)). We have a routine of monthly active surveillance of children from treated and untreated villages, based on samples whose names have been picked randomly from our census lists from each village and whose mothers are asked to bring them along to a surveillance session. These methods would presumably be applicable to where the transmission control was via the action of refractoriness genes, whose progress into each village's vector population would presumably be measurable.

It is important to establish each child's age as accurately as possible because, in an area of holo-endemic malaria and acquisition of strong immunity, prevalence of malaria-related morbidity is strongly age-related. We then take (a) a blood slide to determine presence and density of *P. falciparum* parasitaemia, (b) the core body temperature with a quick reading Thermoscan thermometer which reads radiant heat from the ear drum, (c) the haemoglobin concentration with a Hemocue machine. Many aspects of these data (and other data which are taken on these village visits) are of interest, but we have found that the data can usefully be summarized into age groups <2, 2-5 and 6-12. For each we present (i) prevalence of malaria fever defined as temperature $>37.4^{\mathrm{o}}$C and/or a fever reported in the last two days with a parasitaemia $>4000/\mu l$, (ii) haemoglobin <8 g/dl as a measure of anaemia. The data in Table 2 indicate that morbidity is less (a) in the 2-5-year age group than in infants, (b) at the higher altitude and (c) in villages with treated nets, including ones that have been in use for 3-4 years. This was found despite the evidence that where treated nets had been in use for several years there was a decline in antibody levels (Askjaer et al. 2001, Table 2). The lower morbidity observed with age and with treated nets at each altitude argues against the rebound idea and also against the idea expressed, for example, by Touré and Coluzzi (2000) that in the African lowlands transmission is so intense that trying to reduce it is hopeless and that one should therefore concentrate vector-control efforts at higher altitude in epidemic-prone (not holo-endemic) areas. This suggests that if a refractoriness gene were driven into any vector population it would at least not make the malaria morbidity in the area paradoxically worse, contrary to more extreme versions of the "rebound" hypothesis. Ellman et al. (1998) earlier obtained similar results in the same highland and lowland areas without vector control, but pointed out that, though there appeared to be no rebound for relatively mild effects of malaria such as those shown in Table 2, there might be such paradoxical effects for more severe consequences of malaria.

Short monthly visits such as those used for our active surveillance procedure would miss nearly all of the incidents of severe malaria attacks and deaths. We have tried to obtain measures of these by stationing members of our research team at the nearby District Hospital to record the arrival of children from our study villages and the outcomes of their hospital visits. The likelihood of a child being brought to the hospital depends not only on the mother's estimate of the seriousness of the attack, but also the difficulty of reaching hospital from villages with poor or no bus services. Therefore we have also appointed literate, resident village reporters to record deaths and occurrences of what, in their estimate, constitutes a malaria attack. The data are problematic because, although there is a clear understanding among literate villagers of mild malaria, its cause and treatment, for more severe cases with coma etc., evil spirits may be blamed and traditional healers consulted. Table 3 shows some data collected at the hospital and from village reporters. We have instituted various types of cross checks on such data and hope to be able to present a "cleaner" version of such data in the near future. However, there are no signs so far of a paradoxical "rebound".

The populations in our studies have not been large enough to obtain reliable data on mortality. In a number of large trials of residual house spraying in the past (see Curtis and Mnzava 2000) and of treated nets more recently (Lengeler 1998) mortality data have been collected by the taking of censuses at regular intervals. Attempts have been made at "verbal autopsies" to attribute cause of death. However, it now seems generally agreed that these are unreliable and it is best to stick to all cause mortality.

Table 3. Attempts to detect severe malaria cases via village reporters and visits to District Hospital

Attacks reported/ census population	Child's age	HIGHLAND(c1200m)		LOWLAND (c.200m)		
		No nets	New nets	No nets	New nets	3-4 yr old nets
Village reporters	<2	1.12	0.88	2.07	1.11	0.86
	2-5	0.67	0.52	1.57	0.94	0.51
Hospital visits	<2	no data		0.81	0.56	0.61
	2-5			0.086	0.051	0.18

(Data from Maxwell et al. 2002; Maxwell, Carneiro and Curtis (submitted))

Malaria is such an important cause of child mortality in rural Africa that effective vector control has a strong impact on this and it has been concluded that 6 lives are saved annually per 1000 children provided with treated nets (Lengeler 1998). If transgenic refractory mosquitoes are really to have a useful impact on the worst effect of malaria they will ultimately have to be evaluated by their large-scale impact on child mortality. Furthermore, they will have to be rated for cost effectiveness in saving lives, in comparison with the reasonably effective methods of vector control which we already have.

Acknowledgements

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