8

Contained semi-field environments for ecological studies on transgenic African malaria vectors: benefits and constraints*

Bart G.J. Knols [1,2], Basilio N. Njiru[3], Richard W. Mukabana[2,3,4], Evan M. Mathenge[3,4] and Gerry F. Killeen[5,6]

Abstract

Recent successful genetic transformation of disease-transmitting insects has fuelled enthusiasm towards its potential application for disease control in the future. However, advances to date have been confined to laboratory settings and many questions relating to the fitness, behaviour, ecology and phenotypic characteristics of transformed insects remain unanswered. Spread of desired traits, such as refractoriness to *Plasmodium* infection, will depend on the reproductive fitness and manifestation of life-history behaviours, such as dispersal and mating, by engineered specimens. These should preferably be similar to those displayed by their wild con-specifics but may be compromised by genetic modification and difficult to assess realistically under standard laboratory conditions. Contained semi-field environments that mimic a near-natural environment and are exposed to ambient climatic conditions may serve to verify laboratory findings and yield valuable insights into transgene fixation processes prior to field releases of transgenic specimens into the wild. Here we describe the constraints and benefits of this approach with respect to containment stringency, facility design and operational guidelines for studies involving genetically-engineered malaria vectors. We also report on our initial success with such semi-field systems in West Kenya, using non-transgenic mosquitoes in a variety of behavioural and ecological studies. Successful completion of the *Anopheles gambiae* life cycle, and thus expression of all major life-history behaviours, occurred in three separate trials. However, our results show that the sustenance of successive and overlapping generations in such systems may be difficult. Considering the frequently expressed and explicit need for contained semi-field trials with engineered

[1] Entomology Unit, FAO/IAEA, Agency's Laboratories Seibersdorf, A-1400 Vienna, Austria. E-mail: B.Knols@iaea.org
[2] Laboratory of Entomology, Wageningen University & Research Centre, PO Box 8031, 6700 EH Wageningen, The Netherlands.
[3] International Centre of Insect Physiology and Ecology (ICIPE), Mbita Point Research & Training Centre, PO Box 30, Mbita Point, Kenya. Email: bnjiru@mbita.mimcom.net, emathenge@mbita.mimcom.net
[4] Department of Zoology, University of Nairobi, PO Box 30197, Nairobi, Kenya. E-mail: rmubakana@uonbi.ac.ke
[5] Swiss Tropical Institute, Department of Public Health and Epidemiology, Socinstrasse 57, CH 4002 Basel, Switzerland.
[6] Ifakara Health Research & Development Centre, PO Box 53, Ifakara, Tanzania. E-mail: gkilleen@ifakara.mimcom.net

insects prior to field releases, this calls for intensified development of improved semi-field systems, preferably in field sites earmarked for future releases.

Keywords: mosquito, malaria, genetic engineering, ecology, behaviour, containment, risk-benefit analysis

Introduction

The huge and intolerable burden of malarial disease, particularly in Africa (Breman 2001) warrants research efforts at all levels to contain further deterioration of an already desperate situation. Rampant drug resistance (e.g. Trape 2001; Trape et al. 2002) hinders effective case management and the cost of replacing first-line drugs undermines the activities by already under-funded ministries of Health. A strategy proven to reduce transmission and impact on disease morbidity and mortality is the use of insecticide-treated bed nets (Lengeler 1998). Whether this now widely advocated approach will avert a looming crisis in Africa remains to be seen as its effective implementation depends on a range of political, socio-economic and cultural factors. Moreover, insecticide resistance has been reported in several countries (e.g. Chandre et al. 1999) and will most likely intensify with increased exposure of vector populations to the insecticides currently used (see Zaim and Guillet 2002). Integrated disease control that combines the use of effective drugs with methods to reduce parasite transmission needs to be augmented with new tools directed at the parasite or the vector, and preferably both, if the ambitious targets of the Roll Back Malaria campaign are to be accomplished by 2010 (Nabarro and Tayler 1998; Killeen et al. 2000; Shiff 2002). Truly integrated programmes have been highly effective in the past, even in intensely malarious African settings (Utzinger, Tozan and Singer 2001), and it is currently argued that 'classical' approaches such as larval control can play an important role (Killeen, Fillinger and Knols 2002; Killeen et al. 2002; Utzinger et al. 2002).

New innovative strategies, with the aim to render vector populations less susceptible to infection with human pathogens by releasing genetically engineered mosquitoes have seen dramatic developments over the past few years (e.g. Catteruccia et al. 2000; Ito et al. 2002; Aultman, Beaty and Walker 2001). It is envisaged that, if transposable genetic elements can be used to drive genes coding for refractoriness into fixation in wild vector populations, this may substantially reduce transmission of disease. Genetic engineering may also find application in releases of insects carrying dominant lethals (RIDL technique, see Alphey and Andreasen 2002, Curtis see elsewhere in this volume) or the sterile-insect technique (SIT)(Benedict and Robinson (in press)). The above concepts have not extended beyond laboratory experimentation and have only recently started to focus on the vector/parasite systems found in Africa (e.g. *An. gambiae* germline transformation, see Grossman et al. 2001). A recent theoretical model concludes that efficient transposons will be able to drive genes conferring refractoriness into mosquito populations even if there is a substantial fitness cost of refractoriness, but also that a decrease in malaria prevalence can only be expected if refractoriness is nearly 100% effective (Boëte and Koella 2002, Koella see elsewhere in this volume). It was furthermore argued that environmental conditions, dietary history and age of the mosquito might negatively impact on the mosquito's immune response and thus impair efficiency of the system. Beyond the physiological level, concern has been raised over the ability of laboratory-reared transgenic mosquitoes to survive and compete with wild counterparts for mates upon

release (Catteruccia, Godfray and Crisanti 2003). Fitness of released specimens (in terms of their ability to survive and reproduce) probably needs to be at least 80% of that exhibited by wild-type insects, and gene fixation may then still require hundreds of generations (Kiszewski and Spielman 1998). Historically, attempts to use sterile hybrid males (in Burkina Faso) have failed due to an ethological mating barrier (Davidson et al. 1970), and a recent study with *An. gambiae s.s.* has shown that non-random mating exists, with strong competition amongst males for larger females (Okanda et al. 2002). On the other hand, size-independent mating success of male *An. gambiae s.s.* from Sao Tomé was recently reported (Charlwood et al. 2002), suggesting that size may not be an important determinant of gene flow. Given the likelihood of assortative mating, transgenic males and females may face strong competition upon release, which necessitates increased understanding of behavioural and ecological determinants of gene flow in wild mosquito populations (Donnelly, Simard and Lehmann 2002).

Clearly, it will be important to understand the consequences of releasing transgenic mosquitoes, and it was agreed during two recent meetings (London, September 2001 and Wageningen, June 2002 - see Enserink 2002) that field trials should only be conducted if the likelihood of achieving success in terms of public-health benefits can be maximized. It has furthermore been agreed that research designed to lead to field releases of transgenic mosquitoes should involve fully contained laboratory and semi-field systems (Scott et al. 2002). We describe here how the transition of laboratory studies to open field releases may be undertaken and highlight important safety issues associated with such an approach. How such systems may benefit research on genetically engineered mosquitoes is described and examples of how semi-field environments have been used in Kenya are presented.

From the bench to the field

The transition from baseline laboratory-based research to full application of insect-control technology in target areas typically encompasses four steps. First, the technology developed needs to be effective in the laboratory (using laboratory strains, cage environments, room studies). Second, appropriate support at both community and political level needs to be obtained from an area earmarked as suitable for a pilot trial. Third, given the necessary public/government support, a pilot *efficacy* trial is conducted in a well characterized setting so that potential impact is maximized while minimizing potential risks and side effects. Last, if the method is proven efficacious, a more widespread advocacy of the technique through appropriate channels, whilst monitoring and safeguarding its *efficiency*, is adopted. Several examples of how this process has been used to introduce and establish insect-control methods in Africa exist. The introduction of insecticide-treated bed nets has seen a decade of technology development in the laboratory (e.g. Lengeler, Cattani and De Savigny 1996), subsequent large-scale controlled field trials under the auspices of local governments and the World Health Organization, followed by initiatives to increase technology uptake (e.g. social marketing) (Armstrong-Schellenberg et al. 2001) and advocacy through national malaria-control programmes (NMCPs) and global initiatives like the Roll Back Malaria campaign. The use of the sterile-insect technique to eradicate the tsetse fly, *Glossina austeni* Newstead, from Zanzibar island followed a similar sequence (Msangi et al. 2000) and is currently being considered for more broad-scale application on mainland Africa (*Insect and Pest Control Newsletter no. 57* 2001).

The release of transgenic insects requires an additional step, intermediate between laboratory-based research and a pilot trial in a defined and isolated locality, which is the use of contained semi-field environments (i.e. large outdoor cages) for several important reasons raised in this volume. Perhaps the most important reason is the irreversibility of any release. If a transposon with an associated transgene does successfully drive itself into vector populations, like the P element did in the case of *Drosophila* (Engels 1992), it cannot be removed without eradicating the entire affected mosquito population. Thus the particular risk(s) associated with any transposon-driven genetic modification of wild populations is that it may not be repeatable, and any undesired effects, such as an unforeseen increase in vector competence or vectorial capacity, could be permanent. The primary objectives of semi-field studies on transgenic mosquitoes are two-fold: 1) to evaluate the efficacy of various drive systems or other mechanisms that can be used to propagate the spread of a genetic construct through (small) target populations, and 2) to evaluate the phenotypic expression of such constructs in terms of vector behaviour/ecology and *Plasmodium* susceptibility compared to non-transformed con-specifics.

Transgenic mosquitoes in contained semi-field environments

Benefits

The benefits of using contained semi-field environments are fourfold. First, in contrast with the controlled laboratory environment, exposure to ambient climate/light conditions and the relaxation of spatial contraints associated with relatively small cages may result in more natural behavioural interactions between the insects and between the insects and their environment. Second, such studies are much easier to control, reproduce and interpret than long-term longitudinal field studies, which are vulnerable to regular fluctuations in vector density, physiological background and habitat ecology. Third, recovery of released material is enhanced due to limited dispersal, which also allows for release of small numbers of insects and more direct interpretation of recapture results because mosquitoes do not enter or leave the experimental system. Last, such studies enable a careful safety assessment and evaluation of consequences of field releases of transgenic specimens because their transgenes are prevented from entering wild populations. The following list of topics, though not exhaustive, can be researched under semi-field conditions:

- *Drive systems*. The potential of available transposable elements to spread in small populations, individually or 'loaded' with a genetic construct can be studied. Various options for release (e.g. inundative release of males carrying a non-autonomous transposon) can be evaluated and seeding densities at which the system moves into fixation determined. The effects of ambient climate conditions on transposable-element biology and constructs, particularly the ability of the element to retain the transgene(s) can be evaluated.
- *Behaviour and ecology of the transgene phenotype*. The dominance and effectiveness of transgenic mosquitoes in relation to wild-type specimens needs evaluation. Assortative mating and competition with wild-type males is likely and understanding the extent to which this will affect the spread of desired traits is important. Colonization and mass rearing of mosquitoes have been shown to affect the mating competitiveness of subsequently released material (Reisen, see elsewhere in this volume) and the effects of germline transformation and

temporary laboratory colonization on transgene phenotype performance can be studied.

- Plasmodium *susceptibility*. It remains unknown to what extent parasite development can be reduced by transgenic females when exposed to ambient climate and other potential stress factors. Successive generations of transgenic mosquitoes can be challenged with different *P. falciparum* genotypes and refractoriness levels assessed.
- *Fitness evaluations*. Critical fitness parameters like longevity and fecundity and the influence of anti-parasitic traits on these can easily be studied under semi-field conditions and compared with performance of wild-type specimens.

Constraints

Perhaps the most significant obstacle towards moving research on transgenic mosquitoes from the laboratory to the semi-field environment is the absence of specific guidelines, appropriate evaluation boards (e.g. Institutional Biosafety Committees) and an Africa-wide network addressing regulatory issues (similar to the African Malaria Vaccine Testing Network, now incorporated in the African Malaria Network). Arthropod Containment Guidelines (*Arthropod Containment Guidelines, version 3.1* 2000) and general guidelines for biosafety of research involving recombinant-DNA molecules (*NIH guidelines for research involving recombinant DNA molecules* 1999) will need to be tailored to the African context and adherence to these ensured through an appropriate board of experts. Containment guidelines in existence focus predominantly on settings in laboratories and insectaries in developed countries and have not yet addressed the special circumstances encountered in tropical, disease-endemic settings. For instance, it may be just as important to focus on the *entrance* of wild mosquitoes in contained environments as on the *escape* of released insects. Potential transmission of malarial parasites by (semi-immune) researchers to vectors in contained settings requires specific guidelines not applicable to research environments in the North. Any semi-field experiment that requires the contained mosquitoes to reproduce will necessitate blood-feeding upon live hosts. For highly anthropophilic species such as *Anopheles gambiae* and *An. funestus* this inevitably means human exposure to mosquito bites so the accidental introduction of human malaria parasites into such experimental systems may have serious implications for the investigators.

Nevertheless, the most frequently expressed concern about semi-field research on genetically engineered insects is the scenario in which unwanted escape from the contained environment occurs, particularly of specimens not impaired (e.g. sterilized or non-viable mutants) and fully competent to disperse and settle in an environment occupied by wild con-specifics. Although such a risk can never be eliminated, it can be minimized through the construction of special facilities that maximize biological and physical containment. Suggested guidelines for the design and use of such a facility are described in Annex 1.

Research on transgenic mosquitoes in semi-field environments will require a stable population in order to study such issues as gene flow, transposon stability, the fate of genetic constructs etc. This also applies to studies in which various seeding rates of transgenics are used to determine thresholds above which the system proceeds to permanent fixation over realistically useful time periods. Limited trials in Kenya (see below) have so far shown that it may be difficult to produce successive and overlapping generations of *An. gambiae* in a contained semi-field environment, and suggestions for improvement are given in Annex 2.

With the ultimate goal to inhibit parasite development in the mosquito, it will be necessary to feed (transgenic) females inside semi-field systems on infectious blood meals. As it remains unknown whether blood-feeding by transgenic females poses a risk to humans it is unlikely that ethical clearance can be obtained to use a gametocyte carrier in the system. Rather, infections will have to be introduced using artificial blood-feeding systems (like membranes), which will lead to much reduced feeding rates in a large (greenhouse) environment. This inevitably means that females will need to be collected from the greenhouse and offered a membrane in a small cage. However, adaptation of strains to feeding on membrane feeders often requires several generations, thus meaning a further prolongation of laboratory colonization and an inevitable decline in genetic diversity of the transgenic strain.

Semi-field studies in Kenya

Semi-field environments for behavioural and ecological studies with wild and laboratory-reared *An. gambiae* have been developed in West Kenya at the Mbita Point Research and Training Centre (ICIPE) since February 2000. None of these have so far been used for studies with transgenic strains, but valuable insights into the benefits and constraints of such systems have been obtained. Seven existing greenhouses (7.1 x 11.4 m) have been modified for studies involving mosquitoes (Figure 1) and mosquito rearing (Figure 2). One of these has been designed to simulate a natural *An. gambiae* ecosystem, dubbed the 'Malariasphere' (Figure 3).

Figure 1. Greenhouse semi-field environment for studies with *An. gambiae*.

Figure 2. Semi-field based *An. gambiae* insectary (Photo: Peter Luethi)

Figure 3. Semi-field setup which simulates the *An. gambiae* ecosystem (note breeding site in the foreground)

We have used these greenhouses for a wide variety of studies including a) the evaluation of plants as repellents against *An. gambiae* either in potted form (Seyoum et al. 2002b) or through burning/thermal expulsion (Seyoum et al. 2002a); b) the development of new surveillance tools such as an exposure-free bed-net trap (Mathenge et al. 2002); c) research on factors affecting mating behaviour (Okanda et al. 2002); d) oviposition-site selection by gravid females (Fischer 2002); e) effects of diet (sugar, blood) on female survival (Okech et al. (in press)), f) sugar feeding and survival of mosquitoes on indigenous Kenyan plants (Impoinvil et al. submitted) and g) life-cycle studies in the Malariasphere (Knols et al. 2002, see Box 1), besides several other studies currently underway. All of the above studies received ethical clearance from the National Ethical Review Committee residing at the Kenya Medical Research Insititute (KEMRI; protocol KEMRI/RES/7/3/1).

There are several important advantages to this approach. Studies on the behavioural ecology of malaria vectors under field conditions are often difficult due to nocturnal habits of mosquitoes, the necessity to conduct (relatively expensive) PCR procedures to establish sibling species identity and the often dramatic variations in mosquito density within/between villages and over time (Smith et al. 1995; Lindsay et al. 1995). Age-grading through assessing the number of dilatations on the ovariole stalks in the ovaries (Detinova and Bertram 1962) or through pteridine fluorescence measurements of the head capsule (Wu and Lehane 1999) is complex, and methods to determine the physiological status of the insects require a well-equipped laboratory environment, frequently absent in remote areas where field research is undertaken. Furthermore, studies in which blood-feeding by vectors takes place is confronted with the risk of exposing human subjects to potentially infectious mosquito bites, which is difficult to justify in an era where drug resistance is rampant and on the increase. The power of semi-field systems therefore lies in the ability to fix the density, age and physiological status of the insects in experiments, whilst ensuring a transmission-free environment. A further advantage is the ability to consistently collect large amounts of data over short periods of time and at any time of the year, allowing for more powerful statistical analyses than are possible with data from the field.

Our work in the Malariasphere is of particular relevance to future studies with transgenic mosquitoes. This system consists of a screen-walled greenhouse (with gauze-covered walls and roof) inside which a local hut has been built, two breeding sites have been constructed with mud from natural larval habitats, and some 30 indigenous plant species have been introduced. Trials to assess whether life-cycle completion is feasible have been conducted on three separate occassions by introducing a) 100 bloodfed females, b) 1500 males and 500 virgin females, or c) 500 eggs in both breeding sites. A volunteer (BGJK or BNN) occupied the bed inside the hut during several nights of each trial as a blood source for host-seeking females. Details of the first trial are reported here (see Box 1), of the other ones elsewhere (Knols et al. 2002).

These three trials showed that all major life-history behaviours (mating, host seeking, plant feeding and oviposition) occurred in the Malariasphere. Even though we did not attempt to produce more generations, it was concluded that the size of the ‘inoculum’ may not have been sufficiently large to establish a population inside the system. The second trial (1500 males + 500 virgin females released) produced only 40 pupae, the third trial (500 eggs in each breeding site) only 9 eggs of the next generation. It remains unknown what the cause of this low reproductive rate was, but since larval/pupal survival was much higher than has been reported from field studies (Service 1977) (we estimated the average daily survival of the immature stages to be

Box 1. The introduction of 100 freshly blood-fed (15 min, on human forearm) *An. gambiae s.s.* females into the Malariasphere (Figure 3,4) resulted in the presence of eggs in the breeding sites on day 3 (2.5 days after release), and eggs continued to be observed in the sites until day 7. Larvae (from L1 to L4 stage) were seen feeding at the water surface until day 23, when the last L4 larva pupated. The first five pupae were seen in the breeding sites in the evening of day 10, meaning that the variation in maturation time from egg to pupa was 7- 20 days. In total, 57 pupae were counted in the breeding site in front (3.8 m) of the hut, versus 130 in the site (1.1 m) behind it. The first adults were seen on day 11, and continued to be present until the end of the experiment (on day 27). Starting in the morning of day 22, we observed new eggs in the breeding sites. From the above it can be deduced that specific behaviours of the adult insects occurred during certain times. Oviposition activity took place twice during this trial, meaning that females left the hut after having matured the eggs, successfully located a breeding site, accepted it for oviposition, and laid eggs. Other potential breeding sites, like the leaf axils of banana trees, were examined but did not harbour larvae. As the period for reaching sexual maturity for males may be at least one day and for females up to 60 hrs, mating may not have taken place until dusk on day 14. In spite of regular observations during dusk, we failed to see any swarming activity. As survival of newly emerged adults is <48 hrs without the availability of a carbohydrate source, mosquitoes must have supplemented their energy reserves with carbohydrates from the plants in the sphere for up to 6 days before they were offered a blood source. Some plants (like *Ricinus communis* L.) were flowering at the time of the experiment, and may have provided nectar sources. Within 15 min after entering the hut at night, the volunteer noticed the sound of mosquitoes and subsequently felt mosquito bites on his lower limbs that were exposed. This implies that females were receptive to host cues, entered the hut through the eaves, and successfully located and fed on the human host. At sunrise, several engorged females were seen resting on the walls, indicating successful blood-feeding and endophily (indoor resting), which is typical for this species. Following maturation of eggs, the second oviposition took place during the night of day 21, thus completing the life cycle. The experiment was terminated on day 27 (by no longer entering the greenhouse for one month afterwards).

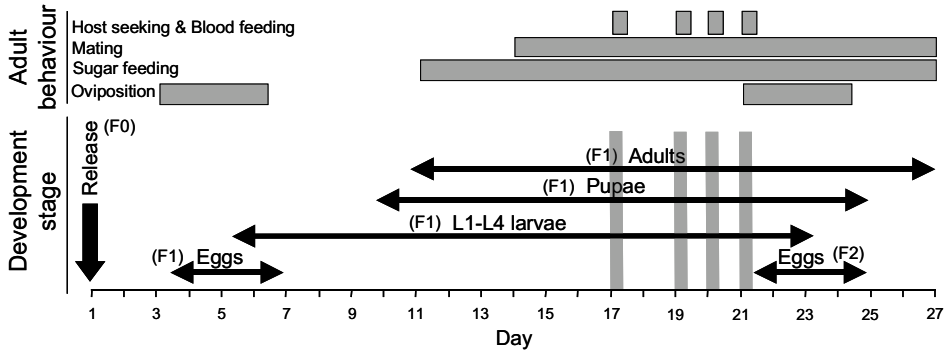

Figure 4

0.83 in the third trial), it is most likely the survival and reproductive success of the adults that was low. Predators, like Salticid spiders (Salticidae) and geckos (Geckonidae), were plentiful in the Malariasphere and may have reduced the size of the small population of adult mosquitoes.

The fact that the life cycle of this important malaria vector could be completed in a relatively small semi-field system (~80 m^2) is encouraging. Given a larger system (1800 m^2; see Annex 1), with more breeding sites, a larger 'inoculum' and better survival of adults (e.g. allowing no predators in the system) may be the way forward to establish a system in which a population of mosquitoes can be sustained over several generations.

Conclusions and recommendations

Routine genetic transformation of disease vectors belonging to all major genera has, owing to dramatic developments in the field of molecular biology, become possible. Although widely advocated and recommended, the next logical step to evaluate the fate of transgenic mosquitoes in contained semi-field systems, has hardly received attention. In fact, the studies described above are the first of its nature and need substantial expansion if the whole array of pertinent questions remaining to be answered are going to be addressed in earnest.

Considering the fact that development of transformation technology has primarily been a USA/Europe-led endeavour, it is of utmost and prime importance that capacity for research on genetic control strategies be developed in disease-endemic countries. Identification of suitable African partner institutions, coupled to a continent-wide network spearheaded by WHO-AFRO is a mandatory first step to initiate the myriad of questions raised in this volume. Clear containment and security guidelines for research on transgenic mosquitoes, adapted to semi-field studies in Africa, need to be developed. Although life-cycle completion has been observed in semi-field environments, it is the establishment of systems that can hold successive and overlapping generations of insects that is of immediate relevance to studying the effects of insect genetic transformation and therefore deserves priority.

References

Alphey, L. and Andreasen, M., 2002. Dominant lethality and insect population control. *Molecular and Biochemical Parasitology,* 121 (2), 173-178.

Andreasen, M.H., 1997. Sweaty socks are an aid to membrane feeding by mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 91, 250.

Armstrong-Schellenberg, J.R.M., Abdulla, S., Nathan, R., et al., 2001. Effect of large-scale social marketing of insecticide-treated nets on child survival in rural Tanzania. *Lancet,* 357 (9264), 1241-1247.

Arthropod Containment Guidelines, version 3.1, 2000. Amercian Committee of Medical Entomology. [http://www.astmh.org/subgroup/archive/ACGv31.pdf]

Aultman, K.S., Beaty, B.J. and Walker, E.D., 2001. Genetically manipulated vectors of human disease: a practical overview. *Trends in Parasitology,* 17 (11), 507-509.

Benedict, M.Q. and Robinson, A.S., (in press). The first releases of transgenic mosquitoes: an argument for the sterile insect technique. *Trends in Parasitology*.

Boëte, C. and Koella, J.C., 2002. A theoretical approach to predicting the success of genetic manipulation of malaria mosquitoes in malaria control. *Malaria Journal,* 1 (1), 1-7. [http://www.malariajournal.com/content/1/1/3]

Breman, J.G., 2001. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *American Journal of Tropical Medicine and Hygiene,* 64 (1-2 Suppl), 1-11.

Catteruccia, F., Godfray, H.C.J. and Crisanti, A., 2003. Impact of genetic manipulation on the fitness of *Anopheles stephensi* mosquitoes. *Science,* 299 (5610), 1225-1227.

Catteruccia, F., Nolan, T., Loukeris, T.G., et al., 2000. Stable germline transformation of the malaria mosquito *Anopheles stephensi*. *Nature,* 405 (6789), 959-962.

Chandre, F., Darrier, F., Manga, L., et al., 1999. Status of pyrethroid resistance in *Anopheles gambiae sensu lato*. *Bulletin of the World Health Organization,* 77 (3), 230-234.

Charlwood, J.D., Pinto, J., Sousa, C.A., et al., 2002. The swarming and mating behaviour of *Anopheles gambiae s.s.* (Diptera : Culicidae) from São Tomé Island. *Journal of Vector Ecology,* 27 (2), 178-183.

Davidson, G., Odetoyinbo, J.A., Colussa, B., et al., 1970. Field attempt to assess the mating competitiveness of sterile males produced by crossing 2 member species of the *Anopheles gambiae* complex. *Bulletin of the World Health Organization,* 42 (1), 55-67.

Detinova, T.S. and Bertram, D.S., 1962. *Age-grouping methods in Diptera of medical importance: with special reference to some vectors of Malaria.* World Health Organization, Geneva. World Health Organization monograph series no. 47.

Donnelly, M.J., Simard, F. and Lehmann, T., 2002. Evolutionary studies of malaria vectors. *Trends in Parasitology,* 18 (2), 75-80.

Engels, W.R., 1992. The origin of P elements in *Drosophila melanogaster*. *BioEssays,* 14 (10), 681-686.

Enserink, M., 2002. Malaria : ecologists see flaws in transgenic mosquito. *Science,* 297 (5578), 30-31.

Fillinger, U., Knols, B.G.J. and Becker, N., 2003. Efficacy and efficiency of new *Bacillus thuringiensis* var *israelensis* and *Bacillus sphaericus* formulations against Afrotropical anophelines in Western Kenya. *Tropical Medicine and International Health,* 8 (1), 37-47.

Fischer, E., 2002. *Oviposition site selection of Anopheles gambiae s.s. Giles*. MSc thesis, Wageningen University Research Centre, The Netherlands.

Grossman, G.L., Rafferty, C.S., Clayton, J.R., et al., 2001. Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. *Insect Molecular Biology,* 10 (6), 597-604.

Healy, T.P., Copland, M.J.W., Cork, A., et al., 2002. Landing responses of *Anopheles gambiae* elicited by oxocarboxylic acids. *Medical and Veterinary Entomology,* 16 (2), 126-132.

Impoinvil, D.E., Kongere, J.D., Foster, W.A., et al., submitted. Survival of the malaria mosquito *Anopheles gambiae* on plants from Mbita, Western Kenya. *Medical and Veterinary Entomology*.

Insect and Pest Control Newsletter no. 57, 2001. Available: [http://www.iaea.or.at/programmes/nafa/d4/public/newsletter-jul01.pdf] (13 Jun 2003).

Ito, J., Ghosh, A., Moreira, L.A., et al., 2002. Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature,* 417 (6887), 452-455.

Killeen, G.F., Fillinger, U., Kiche, I., et al., 2002. Eradication of *Anopheles gambiae* from Brazil: lessons for malaria control in Africa? *Lancet Infectious Diseases,* 2 (10), 618-627.

Killeen, G.F., Fillinger, U. and Knols, B.G.J., 2002. Advantages of larval control for African malaria vectors: low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. *Malaria Journal,* 1 (8), 1-7. [http://www.malariajournal.com/content/1/1/8]

Killeen, G.F., McKenzie, F.E., Foy, B.D., et al., 2000. The potential impact of integrated malaria transmission control on entomologic inoculation rate in highly endemic areas. *American Journal of Tropical Medicine and Hygiene,* 62 (5), 545-551.

Kiszewski, A.E. and Spielman, A., 1998. Spatially explicit model of transposon-based genetic drive mechanisms for displacing fluctuating populations of anopheline vector mosquitoes. *Journal of Medical Entomology,* 35 (4), 584-590.

Knols, B.G.J., Njiru, B.N., Mathenge, E.M., et al., 2002. Malariasphere: a greenhouse-enclosed simulation of a natural *Anopheles gambiae* ecosystem. *Malaria Journal,* 1 (19), 1-13. [http://www.malariajournal.com/content/1/1/19]

Lengeler, C., 1998. Insecticide treated bednets and curtains for malaria control (Cochrane review). *The Cochrane Library* (3).

Lengeler, C., Cattani, J. and De Savigny, D. (eds.), 1996. *Net gain: a new method of preventing malaria deaths*. International Development Research Centre, Ottawa. [http://www.idrc.ca/books/792.html]

Lindsay, S.W., Armstrong-Schellenberg, J.R., Zeiler, H.A., et al., 1995. Exposure of Gambian children to *Anopheles gambiae* malaria vectors in an irrigated rice production area. *Medical and Veterinary Entomolgy,* 9 (1), 50-58.

Marchand, R.P., 1984. Field observations on swarming and mating in *Anopheles gambiae* mosquitoes in Tanzania. *Netherlands Journal of Zoology,* 34, 367-387.

Mathenge, E.M., Killeen, G.F., Oulo, D.O., et al., 2002. Development of an exposure-free bednet trap for sampling Afrotropical malaria vectors. *Medical and Veterinary Entomology,* 16 (1), 67-74.

Msangi, A.R., Saleh, K.M., Kiwia, N., et al., 2000. Success in Zanzibar: eradication of tsetse. *In:* Tan, K.-H. ed. *Area-wide control of fruit flies and other insect pests*. Penerbit Universiti Sains Malaysia, Pulau Penang, 57-66.

Nabarro, D.N. and Tayler, E.M., 1998. The "roll back malaria" campaign. *Science,* 280 (5372), 2067-2068.

NIH guidelines for research involving recombinant DNA molecules, 1999. Available: [http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html].

Okanda, F., Dao, A., Njiru, B., et al., 2002. Behavioural determinants of gene flow in malaria vector populations: *Anopheles gambiae* males select large females as mates. *Malaria Journal,* 1 (10), 1-7. [http://www.malariajournal.com/content/1/1/10]

Okech, B.A., Gouagna, L.C., Killeen, G.F., et al., (in press). Influence of sugar availability and indoor microclimate on survival of Anopheles gambiae under semi-field conditions in western Kenya. *Journal of Medical Entomology*.

Scott, T.W., Takken, W., Knols, B.G.J., et al., 2002. The ecology of genetically modified mosquitoes. *Science,* 298 (5591), 117-119.

Service, M.W., 1977. Mortalities of the immature stages of species B of the *Anopheles gambiae* complex in Kenya: comparison between rice fields and temporary pools, identification of predators, and effects of insecticidal spraying. *Journal of Medical Entomology,* 13, 535-545.

Seyoum, A., Kabiru, E.W., Lwande, W., et al., 2002a. Repellency of live potted plants against *Anopheles gambiae* from human baits in semi-field experimental huts. *American Journal of Tropical Medicine and Hygiene,* 67 (2), 191-195.

Seyoum, A., Pålsson, K., Kung'a, S., et al., 2002b. Traditional use of mosquito-repellent plants in western Kenya and their evaluation in semi-field experimental huts against *Anopheles gambiae*: ethnobotanical studies and application by thermal expulsion and direct burning. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 96 (3), 225-231.

Shiff, C., 2002. Integrated approach to malaria control. *Clinical Microbiology Reviews,* 15 (2), 278-293.

Smith, T., Charlwood, J.D., Takken, W., et al., 1995. Mapping the densities of malaria vectors within a single village. *Acta Tropica,* 59, 1-18.

Trape, J.F., 2001. The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene,* 64 (1-2 Suppl), 12-17.

Trape, J.F., Pison, G., Spiegel, A., et al., 2002. Combating malaria in Africa. *Trends in Parasitology,* 18 (5), 224-230.

Utzinger, J., Tanner, M., Kammen, D.M., et al., 2002. Integrated programme is key to malaria control. *Nature,* 419 (6906), 431.

Utzinger, J., Tozan, Y. and Singer, B.H., 2001. Efficacy and cost-effectiveness of environmental management for malaria control. *Tropical Medicine and International Health,* 6 (9), 677-687.

Wu, D. and Lehane, M.J., 1999. Pteridine fluorescence for age determination of *Anopheles* mosquitoes. *Medical and Veterinary Entomology,* 13 (1), 48-52.

Zaim, M. and Guillet, P., 2002. Alternative insecticides: an urgent need. *Trends in Parasitology,* 18 (4), 161-163.

Annex 1. Containment guidelines for semi-field studies with transgenic African malaria vectors

Guidelines for the handling of and research on transgenic insects in the laboratory are available (*Arthropod Containment Guidelines, version 3.1* 2000) as well as general guidelines for 'Biosafety in Microbiological and Biomedical Laboratories' (BMBL), but these are limited in advice for semi-field research. Though not exhaustive, this section summarizes those guidelines from the perspective of semi-field studies in contained environments in disease endemic environments. Failure of containment, i.e. the escape of transgenic specimens from an enclosure, poses several important questions: 1) Will there be harmful effects on humans, other organisms and the environment at large?; 2) what are the consequences of genetic introgression of the transgene into wild populations?; 3) will horizontal transfer of the transgene occur and what will be the consequences of this? These, amongst other questions, necessitate stringent physical and biological containment procedures combined with implementation of safety practices.

Facility location: The locations where contained semi-field systems can be built in Africa are limited and obviously linked to the presence of well-equipped laboratories. In addition, it may be the objective of field-release studies that determine where such facilities will be located (e.g. on an off-shore island or an area where only the target species is involved in disease transmission). Political stability at country and research-environment level is important, and will ensure governmental support. A facility should not be constructed in areas prone to earthquakes, flooding, adverse weather conditions or other natural disasters. The presence of a good medical facility (hospital) is desirable.

Physical containment and security: The best physical containment is the location of a facility in an ecological or physical island, thus limiting the possibility of spread after escape. As for the facility itself, containment increases with the number of physical barriers separating the insects from the outdoor environment. This can be achieved by using small cages and containers as primary and double-screened facilities with double (pressurized) door systems as secondary barriers. However, each additional layer further inhibits airflow, and may lead to stress for the insects in hot

climates. Attractive devices (such as bug-zappers) can be used to trap insects passing into entrance/exit chambers. Strict rules should apply to the biological material entering or leaving the facility. For instance, only dead adult insects should be allowed to leave the facility; live material in the form of eggs poses a much lower escape risk. Access by other insects (like ants) can be prevented with external barriers (such as water ant traps around facilities). Restricted access by humans can be secured using intrusion alarms, the presence of guards, and the use of doors with electronic or keypad locks.

Safety practices and calamity control: Restricted access by personnel fully trained in arthropod handling procedures and biosafety guidelines is mandatory. They also need to be conversant with procedures to control calamities (e.g. by using insecticide fogging). Inspection of the structure and all screening, doors and pressurized systems, and collection of specimens from trapping devices should be undertaken at regular intervals to ensure maximum containment. Procedures and guidelines to be followed in the event of accidental escape of transgenic mosquitoes in the environment need to be defined and practised (resource mobilization, control operations, informing the public/government/press, etc.).

Biological containment: The level of biological containment that can be applied is dependent on the type of experiments planned and can focus on either the insect or the genetic construct. Irradiation of insects, rendering them sterile, or use of insects carrying dominant lethals will not lead to offspring if mating with wild females occurs. Research on transgenic mosquitoes may initially focus on the use of non-autonomous drive systems and innocuous markers (e.g. fluorescent marker genes). A combination of methods affecting the fitness of the vector and use of harmless constructs will substantially reduce the risks upon escape, but may yield valuable information prior to experiments with insects carrying constructs expressing antiparasitic genes.

Based on the above guidelines, our experience with semi-field systems in Kenya and after consultation with experts from an international firm that constructs greenhouses we propose a semi-field system for work on transgenic mosquitoes as shown in Figure 5. This is a compartmentalized system (120 x 60 m; four compartments of 60 x 30 m), consisting of a double-screened (mosquito gauze) primary barrier (a), with a single entrance through a double-door pressurized system that contains bug-zappers (b), exiting in an air-conditioned working area (c) where handling of insects can take place (incubators, freezers, dissection benches etc.). A single double-door pressurized system provides entrance/exit to the entire facility (d). A secondary barrier (e) is encompassing the primary structure and consists of shade netting walls, and the roof consists of transparent roofing sheets. The secondary structure is surrounded by an ant-trap (f) and fence. The facility is constructed on concrete slabs/walls (g) that protrude above ground level. An initial cost estimate for the primary (inner) structure and the adjacent working area (excluding pressurized door systems) amounted to ca. 150,000 US dollars (Irrico International, Nairobi, Kenya; August 2001). Including the secondary structure and additional facilities will increase the cost to a current estimate of 500,000-600,000 US dollars.

The system described here serves as an example of what a (triple-layer) contained semi-field structure could look like, but may have to be adjusted to local conditions.

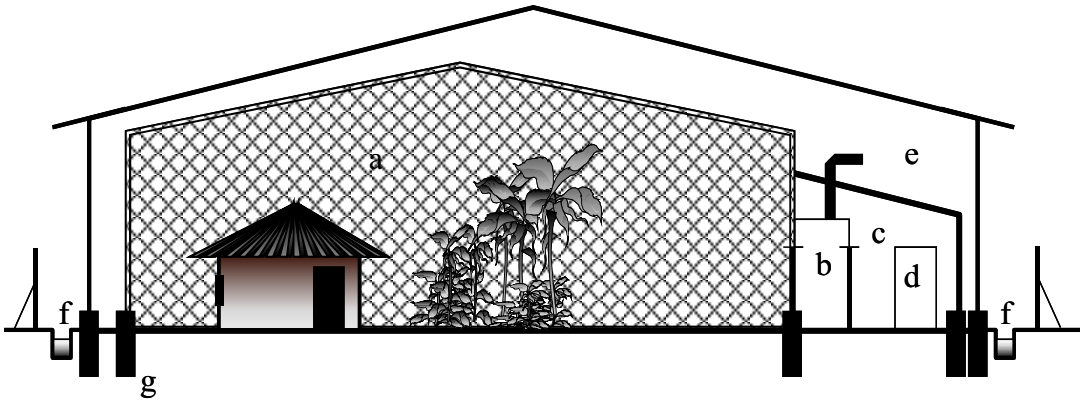

Figure 5. A contained semi-field environment for studies on transgenic mosquitoes (details see text).

Annex 2. Maintenance of mosquito populations in contained semi-field system

Experience with the release of *An. gambiae* mosquitoes in semi-field systems in Kenya has yielded valuable insight on how the survival and reproductive success of the insects can be improved. With the aim to establish a self-perpetuating population of insects that can be maintained on an artificial blood source we suggest the following:

Breeding sites: Survival of the immature stages in the trials has been high, and modification of the breeding sites may not be necessary. The use of plastic (60 cm diameter) containers filled with suitable water (e.g. distilled or rain water), supplemented with dried and sieved soil from natural habitats is adequate. We have used such systems in open-field settings, which resulted in wild females ovipositing in them and offspring surviving well until emergence (Fillinger, Knols and Becker 2003).

Plants and refuges: It may not be necessary to use a wide variety of plants, particularly if adult feeding sources are provided. Plants also hinder easy recovery of released specimens. However, they do provide refuge for mosquitoes and feeding on them may supplement their energy reserves. Extra refuges can be constructed by digging pits that provide a cool dark environment for resting mosquitoes. Using plants in potted form will facilitate their removal and positioning in the system. Plants may also serve as swarm markers (Marchand 1984) and may thus play an important role in mating behaviour. Nevertheless, our studies on survival of *An. gambiae* on a variety of indigenous Kenyan plants have shown dramatic differences between plant species (Impoinvil et al. submitted), and further research on plant feeding is warranted.

House: The use of a local hut made of local materials inside the system has the disadvantage that recovery of insects is difficult (especially with a grass-thatched roof). It seems therefore better to construct an experimental hut with white-coloured inner walls and a black roof (see Seyoum et al. 2002b).

Other organisms: In our initial trials we allowed other organisms inside the Malariasphere. However, as predation may have been intense (by spiders, geckos etc.), we suggest a system without any other organisms.

Sugar/Blood sources: Adult survival in the experiments was not very high, which may have been caused by predation (see above), or the lack of access to sugar and/or blood sources. Depending on the number and species of plants that will be used, it may be necessary to provide additional carbohydrate sources in the form of glucose/sucrose solutions. A ring of honey droplets around the source may enhance its attractiveness (W. Foster, pers. comm.). If humans cannot be used as a blood source for host-seeking females, it will be necessary to enhance the attractiveness of and feeding on artificial feeders. With heat as the sole (physical) stimulus it may be difficult to obtain high feeding rates in an experimental hut inside a large greenhouse. However, recent studies have shown excellent attraction of *An. gambiae* to a combination of carbon dioxide (400 cc/min) and human foot odour (worn socks)(Njiru et al., unpublished data). It has also been found that feeding on membranes is enhanced and increases fecundity in *An. gambiae* in the presence of foot odour (Andreasen 1997). Application of compounds that enhance alighting responses may further increase the feeding rate (Healy et al. 2002).

---

[*] Annex 1 is partially based on a presentation by Mark Q. Benedict and Bart G.J. Knols titled: 'Containment strategies for greenhouse studies', which was presented in London, September 14, 2001 during a meeting titled: 'Genetically engineered arthropod vectors of human infectious diseases: a meeting to consider benefits and risks'.