

MalariaSphere: A greenhouse-enclosed simulation of a natural *Anopheles gambiae* (Diptera: Culicidae) ecosystem in western Kenya

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Abstract

Background: The development and implementation of innovative vector control strategies for malaria control in Africa requires in-depth ecological studies in contained semi-field environments. This particularly applies to the development and release of genetically-engineered vectors that are refractory to *Plasmodium* infection. Here we describe a modified greenhouse, designed to simulate a natural *Anopheles gambiae* Giles ecosystem, and the first successful trials to complete the life-cycle of this mosquito vector therein.

Methods: We constructed a local house, planted crops and created breeding sites to simulate the natural ecosystem of this vector in a screen-walled greenhouse, exposed to ambient climate conditions, in western Kenya. Using three different starting points for release (blood-fed females, virgin females and males, or eggs), we allowed subsequent stages of the life-cycle to proceed under close observation until one cycle was completed.

Results: Completion of the life-cycle was observed in all three trials, indicating that the major life-history behaviours (mating, sugar feeding, oviposition and host seeking) occurred successfully.

Conclusion: The system described can be used to study the behavioural ecology of laboratory-reared and wild mosquitoes, and lends itself to contained studies on the stability of transgenes, fitness effects and phenotypic characteristics of genetically-engineered disease vectors. The extension of this approach, to enable continuous maintenance of successive and overlapping insect generations, should be prioritised. Semi-field systems represent a promising means to significantly enhance our understanding of the behavioural and evolutionary ecology of African malaria vectors and our ability to develop and evaluate innovative control strategies. With regard to genetically-modified mosquitoes, development of such systems is an essential prerequisite to full field releases.

Background

Two proven vector control strategies are currently advocat-

ed to reduce transmission of malarial disease in Africa, namely indoor residual spraying (IRS) [1-5] and

insecticide-treated bednets (ITNs) [6–9]. Both methods are based on the use of residual insecticides in the intradomestic domain and target mosquito vectors either before (ITNs) or after host-feeding (IRS). Impressive reductions in childhood morbidity and mortality have been demonstrated in a variety of epidemiological settings [6], and it can be expected that IRS/ITNs will remain in the forefront of malaria vector control for at least the remainder of this decade. In spite of their proven effectiveness [5–9], both methods have some drawbacks and limitations, such as insecticide resistance [10–13], environmental or human health concerns [14,15] and socio-economic or cultural acceptance by communities. It is also clear that these powerful tools are not sufficient on their own to eliminate or drastically reduce the malaria burden from the most intensely endemic regions of the Tropics, notably sub-Saharan Africa [16,17]. An expansion of this limited arsenal of vector-control tools, with new strategies to reduce human exposure, the size of mosquito populations [18], or transmissibility of disease, is therefore needed, and preferably appropriate for use in an integrated fashion with IRS/ITNs [19–21].

New innovative strategies, involving the release of genetically-engineered mosquitoes, aimed at rendering vector populations less susceptible to infection by human pathogens have seen enormous developments over the past few years [22–27]. If transposable genetic elements can be used to drive genes coding for refractoriness into fixation in wild vector populations, a substantial reduction in disease transmission may result. 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 [27–29]. The spread of desired traits, such as refractoriness to *Plasmodium* infection, will depend on the reproductive fitness, evolutionary cost of the introduced trait [30,31], and manifestation of life-history behaviours, such as dispersal and mating [32], by engineered specimens. For instance, given the likelihood of assortative mating, transgenic males and females may face strong competition upon release, which necessitates an increased understanding of the behavioural and ecological determinants of gene flow in mosquito populations [33]. The characteristics of genetically-engineered mosquitoes should preferably be similar to those of their wild conspecifics but may be compromised by genetic modification, selection for specific traits or routine laboratory maintenance and difficult to assess realistically under standard laboratory conditions [32,34].

Many of the ecological and population biology issues thus remain serious challenges to the application of genetically-engineered mosquitoes [28]. Moreover, until such time that the probability of potential public health benefits can

be maximised, it will be unlikely that approval for release can and will be granted [27]. The use of large contained field-based research settings is now widely advocated to face the shortfalls in our understanding of the behaviour and ecology of genetically-engineered vectors, prior to their release in the wild [23,27,28,35,36].

Contained semi-field systems have been used for a variety of studies on mosquitoes, albeit outside Africa [37–40]. In Kenya, we have recently rejuvenated this approach and developed semi-field systems to study the behavioural ecology of malaria vectors [32,41–44]. By doing so we have generated a wealth of information on the behaviour and ecology of *An. gambiae* s.s. in such confined settings. In this article we present the first attempts to complete the life-cycle of this important vector in such systems, as a first step towards studies involving genetically-engineered specimens.

Methods

Design

We transformed an existing greenhouse (Cambridge Glass House Co. Ltd., UK), measuring 11.4 × 7.1 m (Fig. 1A,1B,1C,1D) into a 'MalariaSphere' (an enclosed environment with all components of a natural *Anopheles* ecosystem) by replacing all glass parts with dark-green shade netting (density 90%) permitting airflow (wind) and precipitation to enter the system. Consequently, climatic conditions (T, RH) are similar to ambient. A sliding door provides entrance to the sphere, after passing a double layer of similar shade netting to prevent escape of released mosquitoes and entry of wild ones. The sphere is located on the shores of Lake Victoria, West Kenya at the Mbita Point Research & Training Centre (00°25'S, 34°13'E) of the International Centre of Insect Physiology and Ecology (ICIPE). The area experiences two rainy seasons; the long rains from mid-March through June and short rains from October through December. Annual rainfall ranges from 700–1200 mm. Relatively high temperatures prevail throughout the year, ranging from 16°C to 34°C. During the rainy season, there are ample breeding sites in the Mbita area for *An. gambiae* s.s., *An. arabiensis* Patton and *An. funestus* Giles, all of which are important vectors [45,46]. Consequently malarial disease is holoendemic [47]. Suba District is inhabited by some 156,000 people, mostly belonging to the Luo ethnic group, who practice mainly fishing and subsistence farming.

Inside the sphere, a traditional Luo house (3.2 × 2.8 × 1.7 m, Fig. 1A,1B) was built from walls of a clay/soil mixture. A mixture of wood ash and clay was used for plastering and smoothing the wall surfaces. The roof (2.8 m at its apex) was made of grass thatch mounted on a wooden frame. The house has a single door and no windows, which is typical for a local 'simba' house. Eaves (height 15

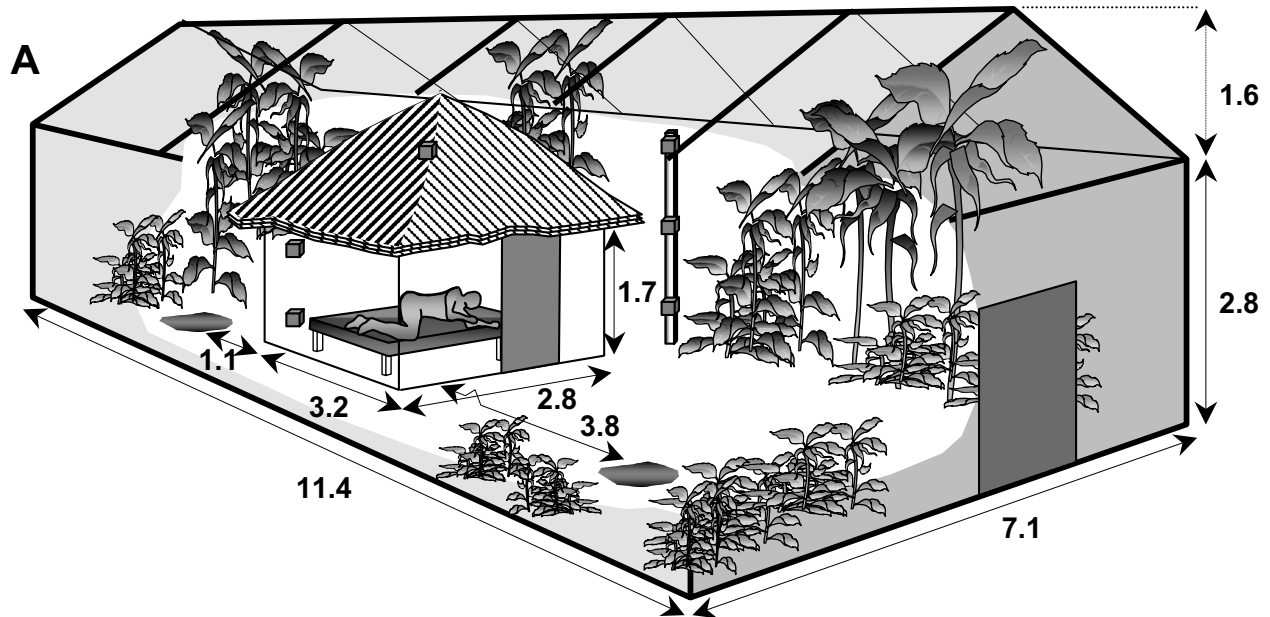


Figure 1
 The MalariaSphere. A schematic drawing (A, dimensions in m; data-loggers are shown as grey cubes) and photographs of the hut (B, note the white arrow showing the breeding site in front of the hut (C,D). Further details see text.

Table 1: Plants in the MalariaSphere. Species marked with an asterisk have been planted, all other species occurred naturally. Popular names are shown in brackets.

<i>Ageratum conyzoides</i> L. (Goat weed)
<i>Amaranthus hybridus</i> L.* (Smooth pigweed)
<i>Asystasia schimperi</i> T. Anders
<i>Bidens pilosa</i> L. (Black jack)
<i>Brassica oleracea</i> sp.* (Wild cabbage)
<i>Colocasia esculenta</i> L.* (Coco yam)
<i>Commelina benghalensis</i> L.
<i>Corchorus tridens</i> L.
<i>Cynodon dactylon</i> L. (Bermuda grass)
<i>Digitaria velutina</i> Forsk. (Velvet fingergrass)
<i>Eichornia crassipes</i> Mart. (Water hyacinth)
<i>Euphorbia</i> sp.
<i>Hibiscus fuscus</i> Garcke
<i>Hoslundia opposita</i> Vahl.
<i>Indigofera</i> sp. (Indigo)
<i>Ipomoea batatas</i> L. (Sweet potato)
<i>Ipomoea cairica</i> L. (Messina creeper)
<i>Kyllinga macrocephala</i> L.
<i>Lagascea mollis</i> Cav. (Acuate)
<i>Ipomoea batatas</i> L.* (Sweet potato)
<i>Lantana camara</i> L. (Wild sage)
<i>Manihot esculenta</i> Crantz* (Cassava)
<i>Musa paradisiaca</i> * (Plantain banana)
<i>Panicum maximum</i> Jacq. (Guinea grass)
<i>Pennisetum purpureum</i> Schum. (Napier grass)
<i>Ricinus communis</i> L. (Castorbean)
<i>Sechium edule</i> Jacq. (Vegetable pear)
<i>Senna hirsuta</i> L. (Hairy senna)
<i>Senna occidentalis</i> L. (Negro coffee)
<i>Sesbania sesban</i> L. (Egyptian sesban)
<i>Vigna schimperi</i> Baker
<i>Vigna unguiculata</i> L.* (Cowpea)

cm) all around provide ventilation and serve as the predominant entry point for mosquitoes [48]. The hut contains a single bed for a volunteer to occupy during experiments.

Two breeding sites (diameter ca. 30 cm; Fig. 1C,1D) were constructed, by burying plastic containers below ground surface level. These were partially filled with soil collected from known *An. gambiae* breeding sites in the area. We maintained the depth of the site at 15 cm by replenishing it with water collected from Lake Victoria. In each site we suspended a HOBO® Optic Stowaway Tidbit data logger just below the water surface, and recorded the temperature at 30 min intervals.

In order to monitor climatic conditions inside the system, we fitted six more HOBO® H8 data loggers, three inside the hut (at 0.5, 1.5 and 2.5 m from ground level) and three outside the hut on a pole at similar heights (Fig. 1A). These recorded temperature and relative humidity at 15

min intervals. Climatic data were collected in February (peak summer) and June (onset of the cold season).

We allowed plants to emerge from seeds present in the soil brought into the sphere, and in addition to this we planted a variety of food crops normally found around local homesteads (Table 1). Prior to the release of mosquitoes and during construction of the house and planting of crops, a wide variety of other organisms entered the sphere, including some known mosquito predators such as ants (Formicidae), spiders (e.g. Salticidae) and geckos (Geckonidae).

Mosquitoes

The strain of *An. gambiae* s.s. mosquitoes used in the experiments originates from Njage village (70 km from Ifakara), south-east Tanzania, and has been maintained under laboratory conditions since April 1996. Adult insects were kept in standard 30 × 30 × 30 cm netting cages and offered 6% glucose as a carbohydrate source. The cages were kept under ambient climatic conditions and

females given the opportunity to feed on an arm of a volunteer for 10 min three times per week. Eggs were collected on wet filter paper disks (9 cm diameter) and transferred to plastic containers containing water from Lake Victoria. Larvae were fed daily on Tetramin® fish food. Upon pupation, insects were transferred to cages for emergence.

Life-cycle completion

In order to assess whether all major life-history behaviours (i.e. mating, sugar feeding, host seeking and oviposition) occurred successfully in the sphere, we attempted to complete the life-cycle during three separate experiments by introducing i) a group of 100 blood-fed females, ii) groups of 500 virgin females and 1500 males or iii) batches of 500 eggs in both breeding sites:

i) In the first experiment we introduced 100 three-day-old females (F0), which had been held in cages with males since the time of emergence. They were blood fed (for the first time) on the forearm of a volunteer for 15 min and subsequently released (at 21.30 hrs) from a paper cup placed on the bed inside the hut. We then monitored the presence and development of eggs, larvae and pupae by inspecting the breeding sites at daily intervals. Following emergence of the first adults, we deliberately waited for six days before entering the greenhouse at night, in order to assess whether mosquitoes would successfully mate and survive/feed on the plants in the system. On day 17, 19, 20 and 21 following the introduction of females, a volunteer slept inside the hut from 21.30 hrs until 07.00 the following day, which allowed the F1 population, and any of their parents that had survived, to feed on human blood. We subsequently searched the breeding sites daily for newly oviposited eggs until day 27.

ii) As some of the parental (F0) females could have survived until day 17, it needed to be ascertained that virgin (newly emerged) insects survived, mated and blood-fed successfully too. We therefore introduced 500, 3 to 5 day-old virgin females, which had emerged from the pupa individually in glass vials, together with 1500, 5 to 7 day-old males at 21.00 hrs. Starting three days afterwards, a volunteer slept in the hut for 5 consecutive nights. We observed daily whether eggs were laid in the breeding sites to ensure that insemination, blood feeding and oviposition had taken place for two weeks following the release. All pupae were collected from the breeding sites as they appeared so that assessments of survival by the F0 generation would not be confounded by the emergence of an overlapping F1 generation.

iii) A third experiment was started by introducing 500 eggs at night (22.30 hrs) in each of the two breeding sites. Concurrently we reared one thousand eggs from the same

batch under standard laboratory conditions described above. This enabled us to determine the sex ratio and thus the number of males and females released. A volunteer occupied the hut for four consecutive nights, starting on day 22 after the start of the experiment. Thereafter, the breeding sites were monitored daily for the presence of eggs/larvae until day 32.

Ethical considerations

A research protocol for the above experiments was submitted to the Kenya National Ethical Review Committee, based at the Kenya Medical Research Institute (KEMRI), in which the discomfort and potential risks of (non-infectious) mosquito bites to volunteers was explained. Ethical clearance was subsequently granted (protocol KEMRI/RES/7/3/1). BNN, BGJK and GFK were involved in the experiments, and do not object to their names being revealed for publication. A parasite-free environment was ensured through a) regular screening of the volunteers' peripheral blood for *Plasmodium* parasites and b) non-occupancy of the sphere beyond 5 days after the mosquitoes were given the first opportunity to obtain a blood meal. As malaria infections in mosquitoes take at least 10 days to reach the sporozoite-infective stage [49], this procedure minimised risks of volunteers being infected within the experimental set up.

Results and Discussion

Microclimate

Figure 2 shows the climatic conditions recorded in the greenhouse over a 3-week period in June 2000, coinciding with the time of the third experiment (onset cold dry season). Daily temperature fluctuations in the breeding sites (Fig. 2A) were highly consistent and water temperature averaged 22.5°C (range 19.0–24.3°C). Similar data sets for February 2000 (peak of the main hot and dry season) showed an average temperature at the water surface of 24.0°C (range 20.0–29.8°C). Other studies have recorded slightly lower average temperatures and larger ranges over which these fluctuate, both in artificially constructed and natural sites. Haddow [50] recorded a range of 19.0–34.5°C in pans of similar size that were supplemented with soil and had water of similar depth near Kisumu (80 km from Mbita Point). Gimnig *et al.* [51] recorded an average of 26.4 ± 0.7°C from natural sites between March and August 1998 in that same area, as did Koenraadt *et al.* (pers. comm.), with 25.8 ± 4.2°C, in two subsequent years. The lower temperatures recorded from sites in the sphere were probably caused by reduced infiltration of sunlight through the roof's shade netting.

Air temperature conditions (for June, Fig. 2A) inside the hut at various heights fluctuated much more considerably but nevertheless remained relatively consistent throughout the observational periods. Average temperatures

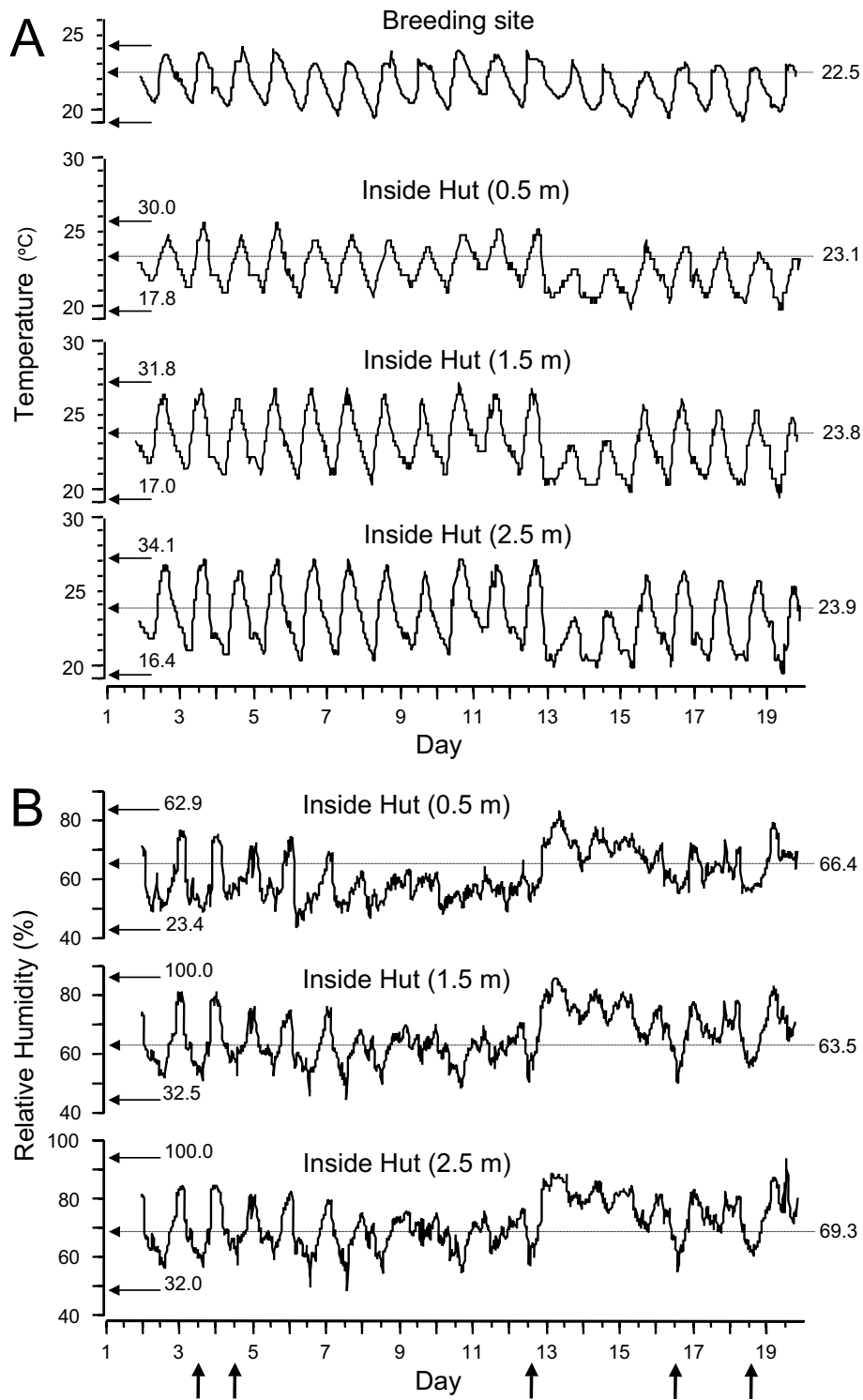


Figure 2

Temperature (A) and Relative Humidity (B) data recorded in one of the two the breeding sites and different heights (0.5, 1.5 and 2.5 m) inside the hut over a 3-week period in June 2000. Arrows on y-axis show maximum and minimum recorded and accompanying figures show the same data for data-loggers outside the hut at those same heights. Arrowed lines show averages (data on the right). Vertical arrows on x-axis show days with rainfall.

increased both inside (23.1, 23.8 and 23.9°C for 0.5, 1.5 and 2.5 m respectively) and outside the hut with height as did the range over which these fluctuated daily. Corresponding data for February inside the hut showed higher averages (24.8, 25.1 and 25.2°C for 0.5, 1.5 and 2.5 m respectively). Between the seasons, maximum variation between temperatures was found outside the hut at 2.5 m, from 37.0°C (February-maximum) to 16.4°C (June-minimum). The smallest variation was observed inside the hut at 0.5 m, from 28.7°C (February-maximum) to 19.7°C (June-minimum). As such, the range over which temperatures fluctuated between seasons was 2.3 times larger outside (2.5 m) than inside (0.5 m) the hut. Haddow [52] recorded mean temperatures between October and December in local huts (at 1 m height) in the Kisumu area and found an average temperature of 24.2°C (range 21.0–27.0°C). Our own measurements inside the hut in the sphere between 18 October and 15 November (2000) at 1.5 m height gave values of $24.0 \pm 1.76^\circ\text{C}$ (range 19.8–29.1°C) whereas measurements inside 4 local houses in Mbita of similar design during that same period gave higher average values (0.5–0.8°C) and absolute maxima (0.8°C). Ambient conditions inside local houses are more constant than outdoor climatic conditions (this study and [52]) and it would seem that the sphere itself exerts a similar, albeit small, insulating effect: slightly lower temperatures and smaller ranges over which these fluctuate, both inside and outside the hut.

Relative humidity (RH) data (Fig. 2B) were collected during the same periods. Being close to Lake Victoria, relative humidity is fairly constant and averages inside the hut during June ranged from 63.5% (1.0 m) to 69.3% (2.5 m). Minimum values were always higher inside the hut than outside, providing more suitable microclimatic conditions for resting mosquitoes. Rainfall, as expected, increased the RH, sometimes for several days. Measurements in October/November, both in the sphere and a local house of similar design in Mbita Point, showed slightly higher average RH values outdoors in both settings than indoors with minimal differences between the sphere and the village hut. Overall, as with temperatures, the range over which the RH fluctuated was smaller inside than outside the hut and minima inside the sphere were always 3 to 4 % higher than those measured in village huts.

Although small, these climatic differences may affect development of immature stages and survival of adults, and research findings from experiments inside the sphere should be compared with field conditions at slightly higher altitudes.

Life-cycle completion

Blood-fed females

The introduction of blood-fed females into the greenhouse 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 (Fig. 3). 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 spite of having conditions with higher larval density and smaller water surface area, Gimnig *et al.* [53] recorded much reduced periods to pupation, from 5 to 12 days. In total, 57 pupae were counted in the breeding site 3.8 m in front of the hut and 130 in the site 1.1 m behind it, and on average they harboured only 0.08 and 0.18 larva/cm², respectively. These densities are lower than those observed in natural habitats [53], and given the fact that we observed algal growth, considered important for larval growth [53], it seems that the prolonged time to pupation in this trial may be attributed to the relatively small range over which temperatures fluctuate. Alternatively, re-introducing mosquitoes that had been maintained under laboratory conditions for several years in a more natural setting may have caused these effects, and poor adaptation to these conditions may have stunted their development.

The first adults were seen inside the hut on day 11, and continued to be present until the end of the experiment (day 27). Starting in the morning of day 22, we observed new eggs in the breeding sites and subsequent larval development.

From the above it can be deduced that specific behaviours of the adult insects occurred during certain time periods (Fig. 3). Oviposition activity took place twice during this trial, meaning that females survived until eggs were mature, that they successfully located a breeding site, accepted it for oviposition, and laid eggs. Other potential breeding sites, like the leaf axils of the banana trees in the sphere, were examined but were not found to harbour eggs, larvae or pupae. The period for reaching sexual maturity for males may be at least one day and for females up to 60 hrs [54], so mating of the F1 generation may not have taken place until dusk on day 14. In spite of regular observations during dusk, we did not see any swarming activity typically associated with mating in *An. gambiae* [55–57]. This is a particularly interesting point, because in contrast with other settings (e.g. [56]), where mating swarms were frequently observed we failed to do so over a 3-year period in the Mbita area as did Charlwood (pers.comm.) who observed only one anopheline swarm during 4 years in the Kilombero valley of Tanzania, raising the question as to whether swarming is an obligate

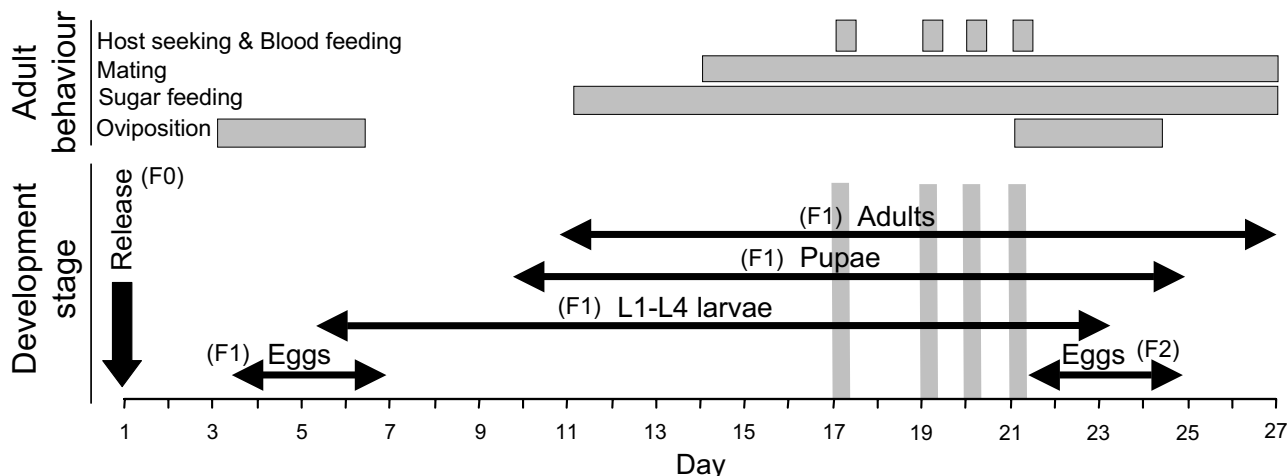


Figure 3
 Completion of the *Anopheles gambiae* life-cycle in the greenhouse over a 27-day period. Blood-fed females were released on day 1, and arrows show time periods during which the various developmental stages were observed. Grey vertical bars show times when a volunteer slept in the sphere. Horizontal bars show periods during which certain behaviours can be deduced to have occurred.

component of the *An. gambiae* life-cycle or a facultative adaptation to local ecological and/or seasonal conditions. Alternatively, maintenance of mosquitoes in laboratory cages for several years forced this strain to become stenogamous (i.e. the ability to mate in small cages), and this adaptation may have interfered with its ability to swarm when introduced into the sphere.

As newly emerged adults rarely survive for more than 48 hrs without the availability of an energy source [58], mosquitoes must have supplemented their energy reserves with carbohydrates from the plants in the sphere (Table 1) for up to 6 days before they were allowed access to a blood source, in the form of a human volunteer sleeping in the hut. Some plants like Castorbean (*Ricinus communis* L.) were flowering at the time of the experiment, and may have provided nectar sources. In cage experiments (Impoinvil et al., in prep.) we have observed a mean survival time of 7.0 ± 0.2 days on this plant, which is comparable to mosquitoes given 6% glucose (8.7 ± 0.2 days). However, given the fact that *An. gambiae* mosquitoes emerge from the pupal stage with a deficit not only in carbohydrates, but also lipid and protein [59,60], which usually is compensated for by consuming a (small) blood meal within the first few days of adult life [61], it is likely that mortality during the 6-day post-emergence period in the current trial was too high to have a good number of the

80–90 females that emerged survive long enough to obtain their first blood meal.

Within 15 min of entering the hut at night, the volunteer noticed the sound of mosquitoes and subsequently felt mosquito bites on his exposed lower limbs. This implies that females were receptive to host cues, entered the hut, probably through the eaves [48], and then 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 [62]. Following maturation of eggs, the second oviposition began during the night of day 21, thus completing the life-cycle. We continued to observe both immature and adult insects (presumably mostly from the F1 generation) until day 27, when the experiment was terminated (by refraining from entering the sphere for about 1 month).

Males and virgin females

The second experiment, in which we released 500 virgin females together with 1500 males demonstrated that mating does occur in a relatively small, semi-field system. After the third night that a volunteer had slept in the hut, we observed eggs in the breeding sites. The production of offspring, though, was low, and we only collected 40 pupae by the end of the trial period. This may have been caused by heavy rainfall during three consecutive nights

(day 2–4), which may have affected the survival of the adults and/or larvae or washed away the larvae from the breeding sites due to overflow. Since we observed few mosquitoes, we decided to conduct a human landing catch during two nights inside the hut, starting two nights after sleeping in the hut had ended. Apparently no host-seeking females were present, as no mosquitoes were collected. Nevertheless, the life-cycle was completed, as manifested by the harvested pupae, which were removed from the breeding sites to prevent emergence of the F1 generation which would have compromised interpretation of survival of the F0 generation.

Eggs

The third experiment started by introducing 500 eggs into each of the breeding sites, whilst 1000 eggs (from the same original batch) were reared under laboratory conditions. In the laboratory, larvae developed at the same rate and most reached maturity by day 10, when the first pupae were observed (Fig. 4). This was similar for the breeding sites in the sphere, except that development was highly asynchronous, i.e. some larvae pupated by day 10, whereas others took up to day 24 before pupation. These times to pupation are similar to those observed in the first trial, but are again in contrast with other studies [53]. On day 7 we counted all larvae and observed 887 in the insectary, as opposed to 652 in the sphere. Overall, the laboratory batch yielded 804 pupae, versus 495 from the breeding sites. On the basis of these data, the average daily survival was 0.90 for the laboratory larvae, and 0.83 for the larvae in the sphere. With nearly half the larvae surviving to the adult stage, these results contrast sharply with much higher mortalities (up to 90%) observed in the Kisumu area [63,64] and São Tome (Charlwood, pers. comm.). The sex ratio of emerging adults in the laboratory was 2:3, which translated into a female population in the sphere of 297, on the assumption that no insects died during emergence. On day 28, after the release of eggs in the breeding sites, we observed that eggs had been laid by the F1 generation, but with only 6 and 3 eggs in the two breeding sites respectively.

Our results have shown that by starting either at the post-blood feeding, pre-mating, or egg stage, a new generation of insects can be reared under these semi-field conditions, and that all life-history behaviours were successfully completed to a lesser or greater extent. This therefore represents the first and promising step towards continuous maintenance of parasite-free *An. gambiae* populations under semi-natural conditions that can be experimentally manipulated in studies of malaria vector ecology and transmission control. Russell and Rao [38] used a large outdoor cage, based on a design by Hackett and Bates [37], to study swarming and oviposition behaviour of *An. culicifacies* Giles in India, and showed that such systems

can be used to unravel aspects of the behavioural ecology of anophelines. Our study shows that such systems can now also be developed for studies on African malaria vectors in order to start filling the gaps in our understanding of the behavioural ecology of these insects [65].

This system has obvious advantages over natural outdoor conditions. First and foremost, it provides a suitable intermediate between laboratory-based studies addressing mosquito behaviour and ecology, and the field situation. Too often, conclusions are drawn from results obtained under laboratory conditions that necessitate speculation as to what may or may not happen in the field. Fixed climatic conditions, cage-experiments, olfactometers and windtunnels, in which the mosquito strains used have been laboratory-reared for sometimes decades, may readily distort behavioural and ecological phenomena. Here we have shown that, beyond introducing F1 generation malaria-free mosquitoes from wild populations, it may be possible to rear vectors *in situ* within a semi-natural system that may minimize such artefacts. Conversely, the advantage of using insectary-reared mosquitoes is in the level of control that may be exerted that would not otherwise be possible: Experiments can be conducted all-year-round, with fixed numbers of insects, of known age and physiological status, in a malaria parasite-free environment under ambient climatic conditions. This enables more direct inferences to be drawn from data analysis as compared to longitudinal field studies, because of constant conditions and simplified experimental design.

We have recently evaluated the efficacy of several plants traditionally used by the Luo community as repellents in a similar semi-field set-up, and simple logistic regression, on data collected during four nights per plant, yielded significant results. Within a year of nearly continuous experimentation, the repellency of 8 plant species and 3 combinations thereof was evaluated through thermal expulsion or direct burning [41], and 9 species and 2 combinations thereof were tested in potted form [43]. Such studies would have taken several years under field conditions, and would be limited to times when mosquito densities would have been sufficiently high to permit meaningful experiments. Another recent study [44] focused on the survival of *An. gambiae* maintained on a variety of diets (blood or sugar alone, or a combination of both) and revealed similar results to those obtained under laboratory conditions [66]. Within one year of starting studies on the behaviour of mosquitoes around bednets in a semi-field setup, we transformed a regular conical bednet into a trap that may catch up to 70% of the females released [42]. Recent field evaluation of this trap shows it to be a promising replacement for the human landing catch (Mathenge *et al.*, in preparation.). With the trap now being considered for commercial manufacturing (it took a

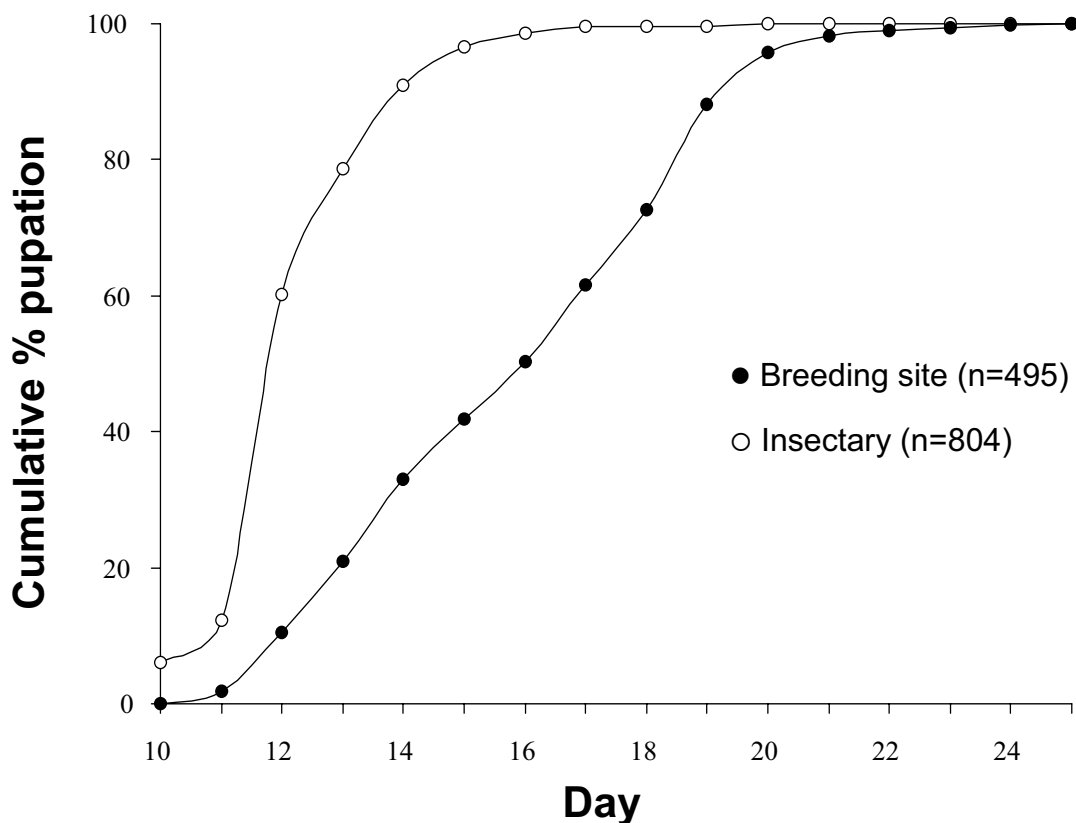


Figure 4

Cumulative percentage of pupation of eggs introduced in the two breeding sites inside the greenhouse (●) or under insectary conditions (○).

mere two years to reach this stage), this would have been impossible without the availability of a semi-field set-up in which continuous experimentation ensured rapid progress towards product development.

Even though our system resembles more closely the field situation, it remains to be ascertained to what extent. Our current study was mainly qualitative in design and focused on life-cycle completion. Various observations were made that have been reported before from field studies. For instance, observation of eggs in the breeding sites in the morning of day 22 during the first trial implies that these originated from females that fed once on day 19, as those that fed on day 17 should have laid before. However, it is likely that these females fed twice, on day 17 and day 19, and should be classified as pre-gravids [61,67], before fully maturing a batch of eggs. Furthermore, in the ab-

sence of a human host, mosquitoes survived up to six days after emergence, confirming field results that feeding on plant sugars does occur and may be an important feature of the life-cycle in this species [68] (Foster and Knols, unpublished data). Obviously, there are limitations associated with these studies. Some phenomena, like dispersal, cannot be studied. There may be other, yet unknown, factors that affect the behavioural ecology of the insects in such systems. Or, as Bates [69] wrote, following his outdoor cage studies in Albania: "One still cannot be sure that the reactions of the mosquito are 'natural' because there is always the barrier of wire liable to be encountered on extended flights; and when the flight of a mosquito has been interrupted by this wire barrier its further activity may be definitely unnatural". Rightly so, and even though we did not observe any obvious distorted behaviours, it is

imperative that findings from semi-field studies be verified under natural outdoor conditions.

Additional studies in which the release and performance of field-collected, blood-fed mosquitoes in the sphere is compared with that of laboratory specimens in terms of egg-recovery, developmental periods and important behavioural characteristics (like swarming) will provide further insight to what extent such systems mimic the natural *Anopheles* environment and colony adaptation impairs natural behaviours.

Nevertheless, since Bates' days, advances in science merit a renewed impetus towards semi-field studies in contained near-natural environments, particularly with respect to transgenic mosquitoes. Fitness evaluations of engineered strains of vectors are mandatory for transformation technology to become an established disease control tool in Africa. Perhaps this alone, is ample justification for more intensive sphere studies, hopefully not only in Kenya, but also in other African countries likely to be involved in this endeavour. Studies on gene flow, mating behaviour and reproductive fitness, combined with studies on the effects of laboratory maintenance on the genetic make-up of transformed strains to be released, can be conducted in semi-field systems [28,36]. Such systems, particularly when used to study genetically-engineered mosquitoes will require more advanced containment levels than the system described here. Guidelines for facility location, physical and biological containment, safety practices and calamity control need to be developed and adapted from existing arthropod containment guidelines [36,70].

There are several good reasons to further such studies in disease-endemic settings. Under such conditions it will be possible to transform offspring from wild mosquitoes, conduct experiments under local ambient climatic conditions and evaluate transgene spread and fixation in offspring from field-collected gravid females that emerge in a semi-field setup. Last, but not least, it will enable scientists from developing countries to become more directly involved in evaluating the potential use and application of transgenic mosquitoes for future malarial disease control.

Authors contributions

BGJK conceived of the study, and developed the system and experiments together with BNN, EMM and WRM. BNN, GFK and BGJK served as volunteers during the experiments. JCB and GFK actively contributed to the interpretation of the findings and drafting of the final manuscript.

Competing interests

BGJK and EMM are engaged in commercialising the Mbita bednet trap, developed in semi-field systems similar in nature to that described in this article, in collaboration with the Vestergaard Frandsen Group (Denmark).

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