A New Larval Tray and Rack System for Improved Mosquito Mass Rearing

F. BALESTRINO,[1,2,3] M. Q. BENEDICT,[1,4] AND J.R.L. GILLES[1]

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ABSTRACT The requirement for efficient mosquito mass rearing technology has been one of the major obstacles preventing the large scale application of the Sterile Insect Technique against mosquitoes. At the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Insect Pest Control Laboratories we developed a larval rearing unit based on the use of a stainless steel rack that operates 50 thermoformed ABS plastic trays and is expected to be able to successfully rear 140,000–175,000 *Anopheles arabiensis* (Patton) adult mosquitoes per rack. The mechanized rearing unit is simple to handle, maintains minimal water temperature variation and negligible water evaporation and allows normal larval development. The mosquito mass-rearing tray was designed to provide a large surface area of shallow water that would closely mimic natural breeding sites. The trays stack into a dedicated rack structure and filling and draining were easily performed. The close stacking of the trays in the rack and the possibility to tightly line up several racks makes this rearing unit a valid solution for maximal use of the space thus reducing construction, heating, and cooling costs. The low amount of labor required to operate the system also reduces labor costs that represent one of the main expenditures in any mass rearing facility operation. Preliminary experiments performed on *Aedes albopictus* (Skuse) also confirm the possibility of successfully extending the use of this technology to other mosquito species. Our larval rearing unit could enhance any mosquito control strategy in which large-scale releases of mosquitoes are needed to suppress or replace natural populations.

KEY WORDS mosquito mass rearing technology, Sterile Insect Technique (SIT), area-wide control

The Sterile Insect Technique (SIT) is a pest control method using area-wide inundative releases of sterile insects to reduce reproduction in a field population of the same species. The use of SIT as a tool for area-wide integrated pest management (AW-IPM) programs has been successfully demonstrated and applied for several insect pests over the past 50 yr (Dyck et al. 2005). The feasibility of a practical application of the SIT for controlling mosquitoes of public health importance has been established by several research activities performed from the mid-1950s to the mid-1980s (Klassen and Curtis 2005, Dame et al. 2009). Despite the evidence supporting SITs efficacy, no subsequent projects were suitably supported financially to reach operational level for several decades. More recently, because of the sparcity of adequate public health measures and sustainable means of mosquito vector control, the use of SIT's technology has been receiving renewed interest as a possible method to support and enhance existing control and prevention efforts.

In addition to the fundamental information collected by feasibility studies on mosquito SIT applications in the past, several topics need to be addressed to move this technology toward a large-scale operational level. Among these topics, the development and the optimization of a mass rearing technology are considered one of the major priorities (Benedict et al. 2009, Dame et al. 2009). The availability of a mechanized and efficient mass rearing system able to support continuous production of large numbers of insects at low cost and with standardized quality has always been of primary importance and a critical point in any AW-IPM program with an SIT component (Parker 2005). In common with SIT, recent vector control approaches aiming at the suppression or replacement of natural populations through large-scale releases of modified mosquitoes strains (e.g., RIDL, cytoplasmic incompatibility CI, vector-incompetence) rely on the availability on effective mass production structures (WHO/TDR 2010).

Mass rearing of mosquitoes could be defined as a large, continuous production of mosquitoes on a regular schedule resulting in adults that are comparable in specific ways to the wild mosquitoes. A mass rearing system implies the application of mechanization and

[1] Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Wagramerstrasse 5, A-1400 Vienna, Austria.

[2] Current address: Medical and Veterinary Entomology Department, Centro Agricoltura Ambiente "G.Nicoli", Via Argini Nord 3351, 40014 Crevalcore, Italy.

[3] Corresponding author, e-mail: fabriziobalestrino@hotmail.com.

[4] Current address: Universitá degli Studi di Perugia, Via del Giochetto, 06126 Perugia, Italy.

standardized rearing procedures to produce, on an industrial scale, adequate numbers of insects by an efficient, controlled, and economical process. Merely increasing the number of typical laboratory culture units to the level of a mass rearing facility becomes cumbersome, expensive, and often ineffective for the production of large quantities of defined-quality insects on a sustained basis (Singh et al. 1975).

The production of large numbers of sterile male anopheline mosquitoes has been one of the major problems retarding the operational use of SIT for control of malaria vectors (Savage et al. 1980) and, except for the improvements introduced by Morlan (1963) with *Aedes aegypti* (L.) and further technical innovation done by Singh (1975) and Bailey et al. (1980) with *Culex pipens fatigans* (Wiedemann) and *Anopheles albimanus* (Wiedemann), respectively, little new equipment has been proposed for the implementation of mosquito mass rearing technology. Morlan (1963) introduced the idea to create large larval trays with an outlet tube to facilitate draining procedures and in the studies of Singh (1975) this idea was implemented by introducing a trough system for filling, feeding, and collecting the immature stages. In both cases the procedure for collection needed to be operated on every single tray by manually opening a drain valve. Despite the availability of this technology, only some technical solutions were successfully adopted in the rearing module of pilot field program evaluating the possible integration of SIT for controlling mosquitoes.

One of the most-cited SIT feasibility projects that achieved measurable reductions in the mosquito population size was performed in El Salvador in the 1970s to control *An. albimanus*, one of the most important vectors of malaria in Central and South America (Lofgren et al. 1974, Lowe et al. 1980, Benedict and Robinson 2003, Curtis 2006, Dame et al. 2009). In this program, a great increase of the mass rearing productivity was achieved mainly by the introduction of a genetic sexing strain (MACHO, with pesticide resistant males) rather than by major innovations in mosquito mass-rearing equipment. In fact, the use of this strain permitted the two-fold increase in rearing capacity thanks to selective and early elimination of susceptible females by treating the eggs with propoxur (o-isopropoxyphenylmethylcarbamate) (Bailey et al. 1980). The introduction of this strain also reduced problems connected with accidental release of females that, even after sterilization, maintain their vector capacity.

In 2004, the Tropical Medicine Research Institute (TMRI) in Khartoum, Sudan, initiated a feasibility study for the application of the SIT on *Anopheles arabiensis* (Patton) in collaboration with the Joint Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) Division of Nuclear Techniques in Food and Agriculture and the Department of Technical Cooperation of the International Atomic Energy Agency (Vienna, Austria). In addition to the creation of a classical *An. arabiensis* genetic sexing strain where the gene for dieldrin resistance has been artificially male-linked (J.R.L.G., personal communication), another fundamental part of this project is the establishment of a mass rearing system able to mechanize the production of large numbers of *An. arabiensis* males.

At IAEA, as a part of this program, we designed, produced, and tested a larval rearing unit based on the use of thermoformed plastic trays stacked in a stainless steel, rolling, and tilting rack system. This unit, fully loaded, contains 50 trays with a total expected production of 140,000–175,000 adults and requires minimal rearing space and manpower.

Materials and Methods

Mosquito Stocks and Rearing Methods. The *An. arabiensis* Dongola strain (available from the Malaria Research and Reference Reagent Resource Center, MR4, as MRA-856) was used in all experiments. The strain originated from the Northern State of Sudan and has been in the colony since 2005. Adult colonies were kept in a climate-controlled room operating at 27 ± 1°C, 70 ± 10% RH with a 12:12 h (light:dark) photocycle that included periods of dusk (1 h) and dawn (1 h). Rack and tray experiments described in this paper were all conducted in a larger climatic-controlled room (88 m^3) where temperature and humidity were maintained at 30 ± 1°C, 70 ± 10% RH.

Mass-Rearing Tray Design Concept and Prototype. A prototype tray made of 5 mm thick thermoformed ABS (acrilonitrile butadiene styrene) plastic was fabricated. The overall dimensions of the tray are 100 × 60 × 3 cm with a flat bottom with two long ridges (each 32 cm long, 2.5 cm high) running along the major axis (Fig. 1).

The ridges were conceived to provide additional structural stability and to increase the edge resting space available to the larvae. In addition, by cutting a slot of a specific depth in the center of each ridge, a water overflow system was created. With this modification, a stack of trays can be filled using a water inlet above the top tray and allowing the water to cascade to the tray below when the upper one becomes full. To avoid a direct flow of water from tray to tray before the upper tray is completely full, plastic plates were glued to the tray bottom beneath the overflow openings to guide the overflowing water away from the slots of the tray below (Fig. 1). The flat area between the ridges encourages uniform water circulation and food distribution inside the tray.

The tray was designed to fully drain by tilting the end side 14 degrees. This inclination raises a short end of the tray ≈25 cm. Two slopes of 14 degree angle were therefore created in both short sides of the tray and equipped at their extremity with dedicated lips to direct and facilitate drainage. The trays are symmetrical on both sides and ends (Fig. 1).

The fluid capacity of this tray is adjustable between 4 and 7 liters depending on the depth of the drainage slot cut into the ridges (from 1.5 to 0.7 cm). The water depth is uniform over most of the bottom of the tray and variable from 1.0 to 1.8 cm according to the water volume. However, the water level at the short sides

Fig. 1. Mass rearing tray. The ridges which create the overflow system and increase larval resting space availability are visible on the lower tray. The underside of the tray shows the two plastic plates which deflect the overflowing water to avoid water falling straight down. (Online figure in color.)

decreases to zero because of the slope created. Regardless of the water capacity, the water surface area in the tray is ≈5,000 cm^2. In accordance with the thickness of the plastic chosen (5 mm), the weight of each tray is ≈4.5 kg. For these experiments, trays with a 6 liter capacity in black, white, and light gray ABS plastic were produced and tested. The FAO/IAEA technical design of the tray was thermoformed and assembled by GLIMBERGER GmbH (Voesendorf, Austria).

Rack Design Concept and Prototype. A dedicated tilting and rolling rack structure was designed to support, tilt, and collect the larvae and pupae from 50 mosquito trays described above (Fig. 2).

To accommodate the trays, the distance between each shelf in the rack is 3 cm. Using a larval density of 0.8–1.0 larvae per cm^2 of surface area, every rack has a production potential equal to 140,000–175,000 adult mosquitoes. The total height of the rack is 210 cm and the trays are stacked in the structure between 50 and

Fig. 2. Rack unit. (Left) Front view of the rack structure fully stacked with trays in tilted position. (Center) Detail of the endless screw jack system and its hand wheel controller. (Right) Back view of the bottom frame of the rack with the plastic curtain, the metallic slide and the basket collector. (Online figure in color.)

200 cm from the floor level. The rack structure is made of stainless steel (type AISI 304), which is highly durable and rust resistant. The rack is mounted on four swivel casters equipped with brakes, 200 mm diameter (SUPERLANMD/123RX-FA, FIR, Modena, Italy) that allow easy handling when empty trays are loaded in the rack. The weight of the empty rack structure is around 185 kg. The weight of the total structure when all trays are filled with water is around 710 kg. The rack and trays are intended to be positioned where larval culture will occur and filled with water and mosquitoes without further repositioning.

An endless screw jack (SJ10Mod.A; Servomech, Bologna, Italy) fixed on the rack structure (Fig. 2) permits the vertical tilting of an inner movable frame that is connected to all the shelves on the front side. This mechanism, operated by a 200 mm diameter hand wheel (VR200, Elesa, Monza, Italy), assures an easy, slow, and controlled tilting of the trays without moving the main rack structure. The complete tilting of the rack is accomplished in 132 revolutions of the hand wheel. The trays can therefore be simultaneously lifted on the front side to drain the mosquitoes from the opposite end.

A transparent, corrugated, acrylic roofing sheet (Guttacryl 76/18, Guttal, Dolany, Czech Republic) is mounted on the drainage side of the rack to direct and ease the course of water containing larvae and pupae as the rack is tilted. Cascading water falling from the stacked trays converges through a metallic slide toward a stainless steel collector basket beneath the trays. The collector basket is supported by the rack frame at the bottom of the structure (Fig. 2). Complete removal of larvae and pupae from tilted trays is not expected, so an operator will need to spray the rack with water to remove the remaining individuals.

The collector basket ($60 \times 40 \times 10$ cm) has a solid bottom and three filtering sides made of expanded stainless steel mesh with round openings (0.70 mm diameter). Three handles are present on the basket to permit easy handling from the side. In preparation for pupa/larva separation, those collected in the basket are concentrated and filtered from the culture water. The basket maintains a small amount of water to not stress the insects more than is necessary.

The rack occupies a floor space equal to ≈ 1 m^2 and the same space is needed at the front side of the rack to remove the larval and pupal mixture contained in the collector basket after emptying and rolling the rack forward. The FAO/IAEA technical design of the rack was created and assembled by INOX-FER s.r.l. (Borzano di Albinea, Italy).

Shadow Effect on Mosquito Development. When stacked inside the dedicated rack structure, trays, and the larvae therein receive reduced light exposure in comparison to standard laboratory-rearing conditions. To evaluate the effect of continuous rearing under reduced-light conditions, polystyrene petri dishes (9 cm diameter) each containing 32 ml of dechlorinated water and 32 first instar larvae (L1) at a density of 0.5 larva/cm^2 with a water depth of 0.5 cm were placed between either white, gray, or black rearing trays with an additional five petri dishes placed on a white plastic tray under normal light conditions as a control. Each treatment was composed of five petri dishes (duplicates) covered with lids throughout the experiment.

The shadow treatments were positioned inside the rack between 70 and 90 cm from the floor level in such a way that each treatment was subjected to a water temperature of 27 ± 0.5°C. The control petri dishes were placed on a table at 80 cm from the floor and were subjected to the same water temperature. The water temperature inside each petri dish was recorded daily to detect any fluctuation between treatments and replications. The light intensity received by the petri dishes was measured on a data logger (HOBO U-12 Onset Computer Corp., Pocasset, MA).

The petri dishes received 640 μl of liquid diet (0.2 mg/larva/d) composed of a 1:1:1 mixture of bovine liver powder, tuna meal, and vitamin mix (1% wt:vol) daily until all the larvae had pupated. The diet composition and feeding schedule were developed at FAO/IAEA IPCL for mosquito rearing purpose and further details will be reported elsewhere (J.R.L. Gilles, personal communication).

The petri dishes were checked daily for pupae at 9:00, 12:00, and 15:00, at which time pupae were removed, counted, and placed in a square plastic cup ($11 \times 11 \times 7$ cm) filled with 200 ml of dechlorinated water and sealed with a net. Adult emergence time was recorded by monitoring the cups during the daily pupa checks. Twenty-four hours after emergence, adults were collected, sexed, and killed by freezing at −20°C for 24 h. The left wing (or right if the left was deteriorated or lost) was removed on a sample of 30 adults (1:1 sex ratio) from each treatment for measurement. Wings were measured from the distal edge of the alula to the end of the radius vein (excluding fringe scales). A digital image of the wing was taken using a CC-12 camera mounted on a stereomicroscope, and measurements were performed with analySIS B software (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Mass Rearing Tray Test. We evaluated the feasibility of larval rearing in the tray designed for mass rearing. Only light gray trays were used. Three replicates were performed using a larval density of 0.8 larva/cm^2. The trays were filled with 6 liters of dechlorinated water into which 4,000 first instar larvae (L1) were manually counted.

To simulate conditions when trays are stacked closely together in the rack, the trays were laid on a table at 80 cm from the floor level and covered with identical trays elevated 3 cm over them using plastic spacers. This experimental design was adopted because of the difficulties in removing and handling full trays from the rack, preventing larval and water loss through spillage. The water temperature in the trays was equal to 27 ± 0.5°C and its fluctuation was recorded daily during the experiment using a digital thermometer (Ama-digit ad 15 th, Buddeberg GmbH, Mannheim, Germany; range −40 to +120°C, resolution 0.1°C) with stainless steel water probe. The water level in each tray was marked and adjusted as necessary to compensate for water lost through evaporation.

The diet was administered to the trays during the first 9 d of the experiment. In the first 2 d (L1–L2 stage), the daily food dose per tray was equal to 40 ml. At day 6 and 7 (L4 stage) the dose was increased to 120 ml while the remaining days trays received daily 80 ml of diet. The total mean amount of diet used in this experiment was therefore equal to 0.2 mg/larva. Trays were monitored as described in the previous trial.

Rack Tests

Water Temperature Variation and Evaporation Rate. The variation of water temperature in the trays when stacked in the rack was evaluated using two identical models of the rack described above. One rack was fully loaded with 50 trays (stacked trays, ST) whereas in the second rack six trays were placed, starting from the bottom level (50 cm from the floor) at 30 cm intervals, ending at the top (200 cm) (open trays, OT). Each tray was filled with 6 liters of dechlorinated water using the cascade filling system created by water outlets placed over the racks. The initial temperature of the water added was equal to 25°C. Times for filling the racks were recorded in all tests. Water temperature in the trays and ambient temperature just outside of the trays were recorded using a digital thermometer. All measurements were recorded three times a day (9:00, 12:00, and 15:00) for 8 d after filling. In these experiments we used black trays for both racks.

The rate of evaporation was evaluated for the two rack configurations by measuring the percentage of water volume remaining in the trays 8 d after filling. The different trays were removed individually and poured into a graduated plastic bucket to determine the volume of water remaining. The period for the trial was chosen based on the mean pupation time observed in previous tests. In this test we measured the volume only of the trays placed at 50, 80, 110, 140, 170, and 200 cm. For the fully loaded rack, the tray at 197 cm was also evaluated to better understand the trend of evaporation at the top of the rack. This experiment was replicated three times.

Effect of Draining on Larvae (L4) and Pupae. To evaluate stress on larvae and pupae during the collection procedure, tests were conducted by draining mixtures of 100 fourth-instar larvae (L4) and 100 pupae (P) from the lowest and highest trays within the rack (50 and 200 cm). These two points represent the highest and lowest pouring heights experienced by insects during collection. During the test, the rack was completely filled and every tray, including those with the pupae/larvae mixture, was filled with 6 liters of water for a total of 300 liters. Using the tilting mechanism, trays were drained and the pupae and larvae were collected in the bottom collector basket. Tilting of the trays was accomplished by rotating the handwheel controlled endless screw jack at a rate of ≈30 revolutions per minute and was completed after ≈4.5 min. After draining, the rack was rinsed for 2 min by spraying water from the top and between each tray level. Larvae and pupae were manually counted and separated, placed in square plastic cups (11 × 11 × 7 cm) filled with 200 ml of dechlorinated water and sealed with a net. Cups were observed to evaluate mortality and variation in metamorphosis rate at 24 h from treatment. Three replicates were performed using either the top or the lower shelf of the rack. For each replicate, samples of 100 larvae (L4) and 100 pupae were collected from the same cohort as those processed and observed as untreated control.

Statistical Analysis. All statistical analyses were performed using MiniTab (MiniTab Inc., State College, PA).

Shadow Effect on Mosquito Development. The effect of shading on the larval duration until pupation and adult stages and wing length were evaluated by a generalized linear model (GLM). Data on larval survival until pupal and adult stages were analyzed with GLM after angular (arcsinsqrt) transformation of the data expressed in percent.

Mass Rearing Tray Test. Data collected for larval survival and duration until pupal and adult stages and wing length were described and compared with results obtained in the previous experiment at the same water temperature.

Rack Tests

Water Temperature Variation and Evaporation Rate. Linear regression analyses were performed for the evaluation of the relation between the water temperature and evaporation rate in comparison to the height of the trays in both the rack's setting.

Effect of Draining on Larvae (L4) and Pupae. The larval and pupal mortality and the metamorphosis rate (pupation and emergence rate) were evaluated in comparison with the untreated control. The percentage of larvae and pupae recollected from the top and bottom tray was also compared. The analyses were all performed using GLM after angular (arcsinsqrt) transformation of the data in percent.

Results

Shadow Effect on Mosquito Development. The maximum light intensity received by the petri dishes on white, gray and black trays was 0.1, 0.05, and 0.0 lux, respectively, and 5.0 lux for the control. The shading because of trays of different color did not change rearing temperatures or larval development. The daily water temperature recorded in all treatments fluctuated inside the expected range of 27 ± 0.5°C with an overall mean value (±SD) equal to 27.2°C (0.39).

The values recorded for larval survival and duration until pupation and adult emergence were not statistically different between the different treatments (Table 1, PETRI DISHES). Overall pupation occurred between day 6 and day 11 from L1 introduction. At day 8 from L1 introduction (third day of pupation) we had collected a cumulative mean (±SE) of 83.54% (2.41) of all the pupae produced.

Wing lengths did not differ between treatments (Table 1, PETRI DISHES). As expected, male wing lengths were significantly smaller than females ($F_{1,118}$ =

Table 1. Larval survival and duration until pupal and adult stages and adult wing length in petri dishes under shade and light condition

	Petri dishes													MR tray		
	Light			Shade										Shade		
	White			Grey			Black			White				Grey		
	N	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	*F*	*p*	*N*	Mean	SE
Survival (% on L1 introduced)																	
Pupa	5	75.6	4.2	5	75.0	1.4	5	69.4	6.7	5	68.1	5.5	0.61	0.617	3	78.3	5.1
Adult	5	65.6	3.8	5	69.4	3.7	5	61.9	3.9	5	61.0	3.9	0.97	0.432	3	74.2	2.5
Duration (Day from L1)																	
Pupa	5	7.4	0.1	5	7.2	0.2	5	7.3	0.1	5	7.3	0.1	0.79	0.515	3	6.9	0.0
Adult	5	8.9	0.2	5	8.5	0.3	5	8.6	0.3	5	8.4	0.6	1.74	0.199	3	8.4	0.1
Wing length (micrometer)																	
Male	15	2719	38	15	2,681	37	15	2,682	25	15	2,739	19	0.85	0.471	15	2,796	23
Female	15	2870	32	15	2,941	32	15	2,960	28	15	2,957	31	1.91	0.139	15	2,942	46

Data on the same parameters are reported for the mass rearing tray (MR tray) exp performed under shade condition.
White grey and black refer to the color of the tray used in the different tests.
Statistical data refer to the comparison among Petri dishes treatments and have to be read along the rows.

106.67; $P < 0.001$). For all treatments we obtained 412 adults, with 193 females and 219 males.

Mass Rearing Tray Test. No loss of water by evaporation was observed during this test. The mean water temperature (±SD) registered along the experiment was equal to 27.7 (0.4).

Larval survival and duration until pupa and adult stages and wing length measurements recorded for this experiment showed values comparable with the values observed in the previous test (Table 1, MR TRAY). Similar to the previous tests, at day 8 from L1 introduction we had collected a cumulative mean (±SE) of 81.26% (1.32) of the total pupal production. Wing length measurements for adult males and females fell within the range described in the previous tests showing again a difference in relation to the sex ($F_{1,28} = 7.79$; $P < 0.01$). In this experiment we produced a total of 8,904 adults with 4,401 females and 4,503 males.

Rack Tests

Water Temperature Variation and Evaporation Rate. The variations of the air temperature (AIR) and the water temperature recorded in a fully stacked rack (ST) and in a rack with trays placed 30 cm apart (OT) are graphically described in Fig. 3 as function of the different trays' heights. Linear regression analyses were performed and statistical parameters are reported in the Table 2 considering all the values observed over the 8 d of measurement.

When trays were stacked a distance of 30 cm from each other, the mean value (±SD) of the water temperature was equal to 27.72°C (0.61) and its variation trend suggest a direct correlation with the air temperature (Fig. 3). The slopes calculated for the daily regression lines in this rack setting ranged around similar values without showing a relation with the time from filling (data not shown). The water temperature reached its final temperature at 1 d after filling and maintained during the test a mean (±SD) range variation (maximum temperature minus minimum temperature) of 1.73°C (0.20). The mean temperature difference (±SD) between the air and the water calculated for each height of the rack was equal to 2.18°C (0.30).

The water temperature in trays fully stacked showed a more gradual warming and a progressive equalization along the test. In Fig. 4A are described the mean values of the daily water temperature (mean of three observations) registered in trays fully stacked

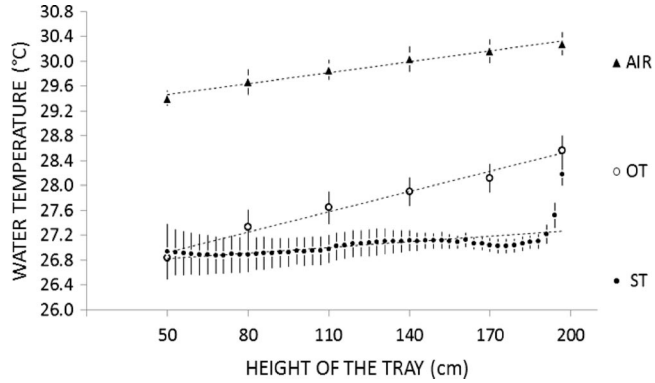

Fig. 3. Water temperature values (mean ± SD) registered during 8-d observations in trays fully stacked in the rack (ST) and stacked at 30-cm intervals (OT). Air temperature variation (AIR) registered at the different rack heights is also described. Linear regression lines are described and the relative statistical parameters are reported in Table 2.

Table 2. Equation parameters and statistical analysis of the linear regression lines of Figs 3, 4, and 5

Figure		Statistics			Coefficients		ANOVA		
No.	Legend	Obsv.	R^2	SE	Intc. (SE)	Slope (SE)	df	F	p
3	AIR	144	0.7337	0.1795	291.765 (0.0487)	0.0058 (0.0004)	1,142	259.01	0.00000
3	OT	144	0.8246	0.2559	263.727 (0.0579)	0.0109 (0.0004)	1,142	639.58	0.00000
3	ST	1200	0.1878	0.2669	266.788 (0.0233)	0.0030 (0.0002)	1,1198	277.04	0.00000
4	ST-1	150	0.7719	0.2000	257.660 (0.0494)	0.0084 (0.0004)	1,148	500.82	0.00000
4	ST-2	150	0.6468	0.1600	262.553 (0.0395)	0.0050 (0.0003)	1,148	271.02	0.00000
4	ST-3	150	0.4054	0.1689	265.274 (0.0417)	0.0032 (0.0003)	1,148	100.89	0.00000
4	ST-4	150	0.3271	0.1937	266.500 (0.0478)	0.0031 (0.0004)	1,148	71.95	0.00000
4	ST-5	150	0.3294	0.1325	268.562 (0.0327)	0.0021 (0.0002)	1,148	72.69	0.00000
4	ST-6	150	0.1043	0.2008	268.638 (0.0496)	0.0016 (0.0004)	1,148	17.23	0.00006
4	ST-7	150	0.0782	0.1522	271.091 (0.0376)	0.0010 (0.0003)	1,148	12.55	0.00053
4	ST-8	150	0.0233	0.2071	274.025 (0.0511)	−0.0007 (0.0004)	1,148	3.52	0.06249
5	OT	18	0.602	0.0766	0.7032 (0.0480)	−0.0018 (0.0004)	1,16	24.2	0.00015
5	ST	21	0.3934	0.1042	1.0519 (0.0623)	−0.0015 (0.0004)	1,19	12.32	0.00234
5	ST	18	0.6954	0.0170	0.9595 (0.0108)	−0.0005 (0.0001)	1,16	36.53	0.00002

in the rack during the 8 d after filling. Linear regression lines are shown in Fig. 4B and their statistical parameters are reported in the Table 2 for each day after filling. The slopes of the regression equations (Table 2, from ST-1 to ST-8) decreased daily until zero when there was no correlation between the water temperature and the height. It is noteworthy that, for all the observations recorded, the major source of water temperature variability in the full stack setting is attributed to the top two trays (197 and 200 cm) that show the highest registered temperatures (Figs. 3 and 4A). The water temperature in this rack setting was more constant and less affected by the height in comparison with the trays stacked at 30 cm distance. The mean value (±SD) of water temperature recorded in trays fully stacked was equal to 27.04°C (0.29) with a temperature range variation of 1.41 (0.34). Excluding the top two trays from the data set the mean value (±SD) of water temperature becomes 27.01 (0.24) and the temperature range variation dropped drastically to 0.54 (0.33).

The maximum water flow rate of the overflow system during filling was recorded as 8 liters per minute. Using a water cascade of this rate, the time (±SD) for filling the trays in a fully stacked rack setting was 45.6 ± 5.6 min while the filling of the six trays placed at 30 cm distance in the rack was complete after 5.5 ± 0.1 min. Higher variability in the time needed for

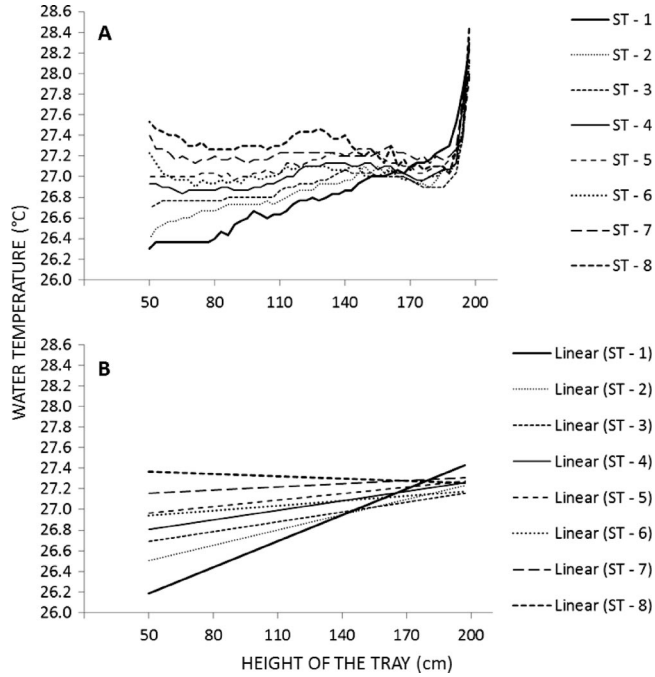

Fig. 4. Water temperature registered in trays fully stacked in the rack (ST) during 8 d after filling. (A) Mean daily water temperature trend. (B) Linear regression lines for each day after filling. Linear regression analysis and statistical parameters for each day after filling are reported in Table 2.

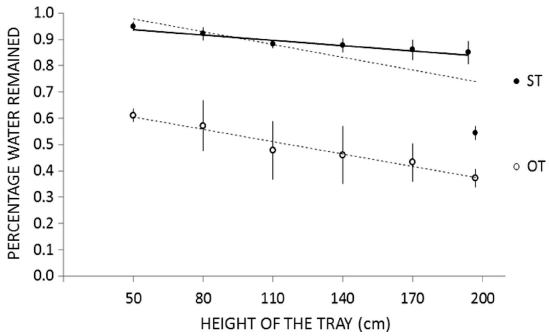

Fig. 5. Mean (± SD) percentage of water remaining in trays at 8 d after filling in trays fully stacked in the rack (ST) and stacked at 30-cm distance (OT). Linear regression lines are shown in the figure and the relative statistical parameters are reported in Table 2. The linear regression line (solid line) and the relative statistical parameters in bold (Table 2) refer to the relation between the height and the water remaining in the fully stacked trays when the evaporation data of the top tray were removed from the data set.

filling the fully stacked rack was because of the difficulties in keeping the water flow rate consistent from the dechlorinated water system.

The different rack settings evaluated showed a different trend in the water evaporation rate from the various trays. The percentage of water remaining in trays 8 d after filling is shown in Fig. 5. Linear regression and statistical parameters calculated for the relation between the percentage of water remaining in trays and the height of the rack show a height correlation coefficient (Table 2) when trays are stacked 30 cm apart. In this open setting (OT) the percentage of water remaining in trays decreases linearly as the height of the rack increases. This linear relation is also observed in the fully stacked rack setting (ST) but with minimal loss of water for evaporation (Fig. 5; Table 2). The linear regression analysis between the percentage of water remaining in trays and the height of the rack for this setting (ST) show a strong correlation only when the data for evaporation from the top tray were removed from the analysis (Fig. 5 and Table 2, solid regression line and statistical parameters in bold). The top tray placed in the fully stacked setting had a mean percentage (±SD) of evaporation rate equal to 54.44% (2.55), similar to the evaporation rate obtained from the top tray in the open tray setting.

Effect of Draining on Larvae (L4) and Pupae. No statistical differences were observed in the larval and pupal mortality recorded in samples after pouring in comparison with the unprocessed control. In addition, no significant differences were observed between the larval and pupal metamorphosis rates in comparison with the control (Table 3).

The difference in recovery rates of a mixture of larvae and pupae collected from the top tray and the bottom tray was significant ($F_{1,8} = 52.28$; $P < 0.001$). Recovery of larvae and pupae was the same when poured from the same height ($F_{1,8} = 2.37$; $P = 0.163$) and no interaction was observed between the stage processed and the height ($F_{1,8} = 2.86$; $P = 0.129$) (Table 3).

Discussion

To create an appropriate rearing system for *An. arabiensis*, the ecology and the natural behavior of the immature stages of this species was considered and balanced against the need to create an efficient and mechanized rearing system capable of exploiting the facility's space at its maximum capacity (Benedict et al. 2009).

Natural larval sites provide some guidance for mass-production of larvae. In nature, larvae of *An. arabiensis* are associated with shallow, small, and temporary habitats such as foot or hoof prints, rain pools, puddles, tire tracks, and garden wells. These environments typically contain algae and bacteria exploited by the larvae as primary source of food (Gimnig et al. 2002, Kaufman et al. 2006) and are often exposed directly to sunlight (Minakawa et al. 1999, Gimnig et al. 2001). However, the intensity of light and turbidity of the water are not always determinants for larval distribution and abundance (Le Suer and Sharp 1988, Shililu et al. 2003, Ye-Ebiyo et al. 2003). The density of anopheline larvae in their natural habitats is extremely difficult to evaluate (Gimnig et al. 2002); seasonal fluctuation of the habitat size, food availability, presence of predators, water temperature (Shelton 1973, Bayoh and Lindsay

Table 3. Percent of mortality, metamorphosis and recovery rate for larvae and pupae discharged from the top and the bottom side of the rack

	Control			Top (200 cm)			Bottom (50 cm)			*F*	*p*
	N	Mean	SE	*N*	Mean	SE	*N*	Mean	SE		
Mortality											
Larva	3	7.7	1.9	3	7.2	0.7	3	6.4	0.9	0.28	0.768
Pupa	3	9.8	2.2	3	10.3	2.2	3	9.0	1.5	0.10	0.906
Metamorphosis											
Larva	3	50.3	10.4	3	57.1	11.2	3	51.7	11.8	0.11	0.903
Pupa	3	90.2	2.2	3	89.8	2.2	3	91.0	1.5	0.10	0.906
Recovery rate											
Larva				3	87.3	0.9	3	99.3	0.7	117.8	< 0.001
Pupa				3	80.3	4.2	3	99.7	0.3	21.29	< 0.01

Statistical data refer to the comparison among the different treatments and the unprocessed control (when applicable) and have to be read along the rows.

2003) and variation in the physicochemical characteristics of the breeding site can affect larval survival, abundance and subsequently adult fitness (Robert et al. 1998, Minakawa et al. 1999, Gimnig et al. 2001).

Because of the parallel necessity to obtain an adequate synchronization in the larval development and pupation time, we integrated the information on the natural breeding sites characteristics with rearing parameters suggested in different manuals and published papers on mosquito rearing and culture technique to determine the equipment required for production of anopheline pupae. The majority of these studies indicate a water temperature of 26–28°C as optimal temperature for larval development for several mosquito species (Lyimo et al. 1992, Gerberg et al. 1994, Robert et al. 1998, Bayoh and Lindsay 2003). For anophelines, this temperature appears appropriate as does an initial larval density between 0.5–2 larvae per centimeter square. This density is generally higher compared with that observed in the natural larval sites which typically contain predators or inadequate food. Larval density has been reported to affect the development of larvae especially in case of paucity of food (Lyimo et al. 1992, Gimnig et al. 2002, Ye-Ebiyo et al. 2003).

The vacuum thermoforming technique used to mold the tray's shape produced a shallow container with uniform depth and extensive side space for larval and pupal resting. Moreover, additional resting surfaces have been integrated in the tray structure with the creation of the ridges. The two long ridges running across the tray also create an unattended filling system that guarantees a uniform and controlled volume of water in each tray when stacked in the rack structure. This fabrication method allows rapid and economical prototyping and is consistent with the shape of tray chosen.

The use of trays with low water depth (≈2 cm) and large water surface area is typical for anopheline rearing (Dame et al. 1974) but is also adequate for other mosquito species (Gerberg et al. 1994, Timmerman and Briegel 1999). Furthermore, the diving behavior of mosquito larvae and pupae associated with foraging and alarm reaction in shallow trays is limited, possibly minimizing the energy lost through this activity (Shuey et al. 1987, Tuno et al. 2004).

During our tray experiments no aerators or water agitators were used. These procedures were often reported in large scale and laboratory procedure for rearing mosquitoes to avoid scum formation on water surface (Singh et al. 1975, Ansari et al. 1977, Fritz et al. 1989). The formation of a persistent scum could block the access to the air-water interface and cause high mortality especially in anophelines. The mosquito liquid diet used in these experiments and its schedule administration did not promote scum formation, and mortality from overfeeding was not observed. Although feeding the larvae once a day could be kept as an acceptable procedure (Fritz et al. 1989) other experiments conducted in our laboratory demonstrate that feeding twice as much food on alternate days give indistinguishable results (J.R.L. Gilles, personal communication).

In contrast to the fully sunlit conditions typical of natural larval sites for this species, the rearing of *An. arabiensis* in shadows is probably the most artificial larval rearing condition realized by the system we report. Experiments conducted under sun and shadow exposure on *Anopheles gambiaes.s.* (Giles) have demonstrated that, when larval food was added in both rearing environments, no significant differences were observed in larval survival, pupation rate and time to pupation (Kaufman et al. 2006). While under feeding conditions a normal development in complete darkness has also been demonstrated in *Culiseta incidens* (Thomson) (Lee 1972), *Culex pipiens* (Linnaeus), *Culex pipiens fatigans* (Wiedemann), and *Aedes argenteus* (Poiret) (Jobling 1937) and a marked negative phototropism observed in several other species (Callot 1965) could suggest that darkness does not affect larval development. Light entrainment for the timing of pupation, emergence and mating still must be achieved for this system. Our results obtained in petri dishes and in covered mass rearing trays confirm the possibility of a normal development of immature stages of *An. arabiensis* under artificial feeding regime with strongly reduced light exposure. At any rate, a small space between the rack shelves and a consequent degree of shading for the larval trays is presumably permissible based on previous mass rearing methods described for mosquitoes (Morlan et al. 1963, Dame et al. 1974, Sigh et al. 1975, Ansari et al. 1977, Gerberg et al. 1994).

The tray described herein has also undergone preliminary testing for rearing feasibility with *Aedes albopictus* (Skuse). The tests performed using a density of 12,000 L1/tray, covered and fed a liquid diet, showed negligible water loss through evaporation and a survival rate of over 95% of L1 introduced. Aggregation of larvae and pupae, as seen in trays exposed to artificial light, was strongly reduced when covered with another tray, with the insects then showing a tendency to exploit all available space. Pupation started 6 d after L1 introduction and pupal production was comparable with results obtained using standard rearing procedures (R. Bellini, personal communication).

Temperature and humidity controls are probably the most important factors in the successful rearing of mosquitoes (Gerber et al. 1994). Keeping constant humidity and minimal air thermal stratification in a climatic-controlled room is a difficult and expensive operation especially if performed in such a large space as a mass-rearing facility (Singh et al. 1975). In the past mass rearing experiences with mosquitoes, the same problems were solved by covering the rearing units with plastic to maintain a more constant temperature, to diminish the water evaporation and to protect larvae from air currents (Trembley 1944) and by the use of electric heating tapes placed underneath the trays (Dame et al. 1978). When our rack was fully loaded the water temperature was surprisingly uniform from top to bottom in the trays. We suspect that the close

proximity of the trays creates a mutual thermal influence between the different rack levels and minimizes the evaporation rate from the tray. The proximity between the trays also permits larval rearing in a climatically controlled room with high ventilation without severely affecting the larval development. In fact, direct air currents over the water surface could strongly affect the larval development by inducing imperceptible but continuous motion (Trembley 1944).

Despite the optimal water temperature variation obtained and the minor evaporation achieved, the top trays in the fully stacked rack setting had a higher temperature and an unacceptable evaporation rate of the initial water. A technical solution based on the creation of a cover for the rack structure is under evaluation. Another possible solution for this problem could be based on the exclusion of larvae from the top two trays leaving these trays in place as a thermal insulator.

The tests performed for the evaluation of the stress induced on larvae and pupae by tilting and collection procedures showed no relevant alteration of mortality and metamorphosis rate in comparison with unprocessed insects. The small percentages of recollection of the mixtures processed from the top tray cannot be directly converted to predictions for the entire unit but surely suggest that a certain portion of pupae and larvae will be lost during this procedure. We observed that the insects remaining were mainly stuck in the upper part of the plastic curtain and could be recollected after extra rinsing of the rack. This experiment suggests that the 2 min used for the rack rinsing at the end of collection should be prolonged to 5 min to complete the harvesting.

The rack has been conceived to be an independent rearing unit and for this reason is equipped with a basket collector. When tilting and rack rinsing has been completed the basket can be removed from one side of the rack for larval and pupal collection. We expect that many racks will be aligned side-by-side, and for the collection procedure the tilted racks will be rolled out for basket removal. If desired, several racks without collector baskets could also be aligned over a common collection trough, in which case the racks would not need to be moved after tilting for larva-pupa collection.

During the mass-production process, it is envisioned that racks would be filled with trays, possibly in the tray cleaning area. These would be rolled to the rearing floor where they would be filled with water, food, and either eggs or larvae. When pupae formed, the contents would be drained into the basket that would then be taken to the larva/pupa separator. Floor drains would collect the waste rearing water. The rack would either be cleaned or refilled with remaining larvae which would complete development. Finally, the rack would be taken to the kitchen for cleaning where the trays would be loaded into a commercial washer.

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