**SPECIAL ISSUE: STERILE INSECT TECHNIQUE**

**Enhancements to the mass-rearing cage for the malaria vector, *Anopheles arabiensis* for improved adult longevity and egg production**

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**Abstract**

Innovations in mosquito mass-rearing techniques are essential in the quest to develop sterile insect technique methods to fight mosquito vectors of disease. This study reports modifications to the Food and Agriculture Organisation/International Atomic Energy Agency (FAO/IAEA) mass-rearing cage (MRC) for mosquitoes to support the behaviour of adult *Anopheles arabiensis* Patton (Diptera: Culicidae) and to maximise egg production. The effects of an improved sugar-feeding device, and the addition of resting sites and a black cloth shroud to create an artificial horizon (visual contrast of light vs. dark at the edge) were assessed for their effect on adult longevity and egg production. Egg production of adults resulting from larvae reared in individual free-standing trays vs. those reared in the same trays in the IAEA/FAO larval rearing rack was also compared. Finally, the effect of blood feeding and frequency of egg collection from the MRC on average egg production per batch was investigated. Overall, the modifications to the MRC enhanced adult longevity, and the improved cage prototype allowed the collection of more eggs overall from a cohort of adults than was possible using the original and previous cage prototypes. These stepwise improvements are important for the development of economical and logistically efficient mass-rearing systems for the malaria vector *An. arabiensis*.

**Introduction**

Malaria remains a serious threat to world health even though tremendous progress has been made over the last decade in reducing its morbidity and mortality; 214 million malaria cases and 438 000 deaths were estimated in 2015, most of them in children under five and pregnant women in sub-Saharan Africa (WHO, 2013). To achieve elimination of the various vector-borne diseases, additional complementary control methods are needed. Amongst those being advocated is the sterile insect technique (SIT), a species-specific and environmentally friendly vector control method that relies on the mass release of sterilised insects (Knipling, 1959). The potential of SIT for mosquito suppression has been demonstrated in a promising feasibility study in Italy (Bellini et al., 2013), but successful application will rely on maintaining a continuously high release ratio of sterile-to-fertile males within the target area and on the competitiveness of the sterile males after release.

To control *Anopheles arabiensis* Patton (Diptera: Culicidae) in a 20-km2 pilot site in Merowe, Northern State, Sudan, a facility with the capacity to produce around 10 million sterile males per week is planned (Robinson et al., 2009). In order to sustainably and affordably produce such large numbers of adults on a daily basis, novel methods and materials for the mass rearing of mosquitoes are needed (Benedict et al., 2009).

The Insect Pest Control Laboratory (IPCL) of the joint Food and Agriculture Organisation/International Atomic Energy Agency (FAO/IAEA) Division of Nuclear Techniques in Food and Agriculture has been supporting studies to assess the feasibility of implementing area-wide control programs with a SIT component to control the important malaria vector *An. arabiensis* and *Aedes aegypti* (L.) and *Aedes albopictus* Skuse (vectors of dengue, chikungunya, and zika). The IPCL has been leading the development of mass-rearing equipment methods for *Anopheles* and *Aedes* mosquitoes for deployment in field projects in member states such as Sudan (Robinson et al., 2009), South Africa (Munhenga et al., 2011), and others (Lees et al., 2015).

Optimizing the mass-rearing environment is a balance between matching the natural habitat and the biological needs of the species, and economic efficiency and high production rates (Balestrino et al., 2014). The quality of food sources (Gilles et al., 2011; Damiens et al., 2012, 2013), conducive oviposition sites (Balestrino et al., 2010), and adequate space for pre-copulative flight and resting sites should all be addressed in the development of a mass-rearing system (Benedict et al., 2009). Constant access to a sugar source is also known to enhance survival and mating success (Gary & Foster, 2001) and therefore increase the number of inseminated females and hence egg production, a benefit when production of large numbers of eggs in a single batch is beneficial when producing mosquitoes for release.

Although an adult mass-rearing cage (MRC) (Balestrino et al., 2014) and a larval mass-rearing unit (Balestrino et al., 2012) have been developed for *An. arabiensis*, they have not been fully validated under conditions of routine mass rearing. Preliminary observations of swarms forming at the lower part of the MRC confirm that the environment is conducive to the mating behaviour of *An. arabiensis* but adult longevity and egg productivity were impaired relative to small-scale rearing (JRL Gilles, pers. obs.). Incremental improvements to the cage and rearing regime were therefore planned and their impact was assessed in turn. The FAO/IAEA larval rearing unit consists of a mechanized stainless steel rack that can hold 50 large mosquito mass-rearing trays (Balestrino et al., 2014), validated for *Ae. albopictus* mass rearing but not yet for *Anopheles* species, apart from some preliminary testing (Maïga et al., 2016).

This study therefore aimed to validate and optimize the use of the *An. arabiensis* MRC in combination with the IAEA/FAO larval rearing rack, and to improve adult survival and egg production through modification of a prototype assessing the effects of different sugar feeders and the addition of resting sites. The effects of blood feeding frequency (daily vs. four blood-feeds per week) and egg collection frequency in MRC on productivity were also investigated.

**Materials and methods**

**Mosquito strain rearing and egg collection**

The Dongola strain of *An. arabiensis*, originating from Dongola, Sudan, was maintained at the IPCL (Seibersdorf, Austria) following the Anopheline rearing procedure described by Damiens et al. (2012),in laboratory rearing cages (30 × 30 × 30 cm) (Model 1; Burgdorm, Taipei, Taiwan) with ad libitum access to 5% sugar and meals of defrosted, defribrinated bovine blood provided to females. Eggs were collected twice a week in plastic cups surrounded by black paper and containing a wet sponge covered by a filter paper.

For experiment 1, pupae were reared on a small scale in trays (30 × 40 × 8 cm) with 1.5 l of de-ionized water until pupation (Damiens et al., 2012). For experiments 2 and 3, pupae were reared on a large scale using either the mass-rearing rack system or individual trays kept on the bench top (Balestrino et al., 2012), both filled with 4 l of de-ionized water and seeded with 4 000 eggs per tray. Larvae were reared in a climate-controlled room maintained at 30 ± 1 °C and 70 ± 10% r.h., and fed with a 1% FAO/IAEA larval diet (5 g tuna meal, 5 g bovine liver powder, and 4.6 g vitamin mix per litre) according to IAEA *Anopheles* mass-rearing protocols (Maïga et al., 2016).

**Modification of original mass-rearing cage for experiments**

The original MRC prototype (Balestrino et al., 2014; C1 in Figure 1) was modified in several ways to improve the efficiency of the mass rearing cage for *An. arabiensis*. First, two methods of delivering sugar were trialled. The original sugar feeder (Figure 2, FC1) was made of a rectangular aluminium tube (6 cm diameter) sealed at each end; an inlet pipe with a watertight stopper was connected 5 cm from one end for addition of sugar solution from outside of the cage. Thirty holes (2.5 cm diameter) along the tube on the same side as the inlet pipe and covered with 20-μm stainless steel mesh allowed mosquitoes to feed. The adaptation tested was to simply add six 100-ml cups each containing 80 ml of 5% sugar solution and a coffee filter paper (Melitta 1×4 Original FSC C095206) (Figure 2, FC2) to the top of the original rectangular tube (Figure 1, C2). Secondly, the rectangular sugar tube was replaced altogether with a new device (Figure 2, FC4-6): a tube (220 cm long, 5 cm diameter), mounted through holes positioned halfway up the stainless steel cage frame, sealed with a rubber plug on both ends and with an upwards facing elbow plumbing fixture on the front end to fill the device. To discard the sugar solution for cleaning, the front rubber plug from the elbow plumbing fixture was removed and turned down. Two litres of 5% sugar solution poured into the sugar-feeding device before loading the cage with mosquitoes lasted for the entire duration of a cage cycle (20 days). The tube contained three slots into which filter papers were inserted [Whatman paper, 2589 A Bogen sheets, cut into three 25 × 20 cm sheets, folded along the vertical axis (25 × 10 cm), each end sealed with tape] (Figure 1, C4-6). The aim of this arrangement was to provide an increased surface area on which the adults could feed.

To test the effect of additional resting sites, a black box (15 × 120 × 35 cm; IKEA) was hung inside the cage as an internal, dark resting site (Figure 1, C3, C5); mosquitoes could enter and exit through openings from the bottom part. For experiments 2 and 3, instead of a black box hung inside the cage, a black cloth shroud was added to both provide a dark resting site along the upper part of the cage and to create an artificial horizon: a visual contrast of light and dark at the edge of the cloth that aimed to stimulate natural swarming behaviour and thus increase mating (Figure 1, C6).

**Experiment 1: Effect of sugar feeding method and resting site provision on adult longevity and egg production**

Two sets of trials were performed in order to assess the effect of sugar feeder type and internal resting sites on adult longevity and egg production. In the first set, three MRCs (Figure 1, C1-3,) were loaded with 7 000 pupae over three consecutive days; pupae were added over this period of time rather than by adding all 7 000 from a single day’s pupation so that the male-to-female ratio was close to 1:1. Pupae numbers were estimated by volume and they were divided into two or three plastic cups (10 × 10 × 6 cm) filled with water for introduction into the MRCs. A sample of 50-100 pupae was sex separated daily under a stereomicroscope to estimate the subsequent sex ratio in the cages (data not shown). A blood meal was offered daily in the form of defrosted, defibrinated bovine blood in the modified Hemotek membrane feeding system (Discovery Workshops, Accrington, UK; Damiens et al., 2013), for 1 h in the morning from the 4th day after the last pupae were added to the cage. The number of females taking a blood meal was estimated by observation. Each day dead pupae and adults were removed from the cage by draining the trough using the outlet valve through two nested, progressively fine sieves (300 and 50 µm; Retsch, Haan, Germany). Male and female mortality was recorded daily for 12 days after adult emergence. The first egg batch was collected 3 days after the first blood feeding. Eggs were collected through sieves and air dried on filter paper for quantification according to the method of Maïga et al. (2016). Repeated rinsing of the trough using the hose attachment and spraying of the trough walls through the netting using a squeeze bottle was needed to remove dead adults and eggs clinging to the metal surface. The trough was refilled with 2 l distilled water daily to facilitate oviposition. Four egg batches were collected from each cage and average egg production was compared between single cages of each type.

In the second set of trials, cages C1, C4, and C5 (Figure 1) were used to test the effect of the new sugar-feeding device (that replaced the aluminium sugar feeder of the original cage, C1) and the presence of resting sites. Females were blood fed daily for 2 h. Based on observations of the low numbers of females feeding in the previous set of cages, for these trials the Hemotek feeding devices were covered with a black cloth (50 × 50 cm) laid on top of each cage (during blood feeding only) to stimulate blood feeding. Daily mortality was recorded and eggs were collected and quantified as described above. Nine egg batches were collected from each cage type and the average egg production was compared between single cages of each type.

**Experiment 2: Validation of larval rearing method for egg production**

As the rearing method of immature (aquatic) stages affects the condition of adult mosquitoes, egg production in the improved MRC prototype (C6, Figure 1) was compared between five replicate cages filled with pupae reared in individual free-standing mass-rearing trays vs. five replicate cages filled with pupae reared in the rack system. In each case 4 000 eggs were rinsed into each tray and reared according to the method described by Maïga et al. (2016). Pupae were picked by hand from the individual free-standing trays or by tilting the rack, and separated from larvae by swirling in an Erlenmeyer flask with tap water. Each cage was loaded with 10 000 pupae and blood meals were offered (for 2 h) as in experiment 1. Eggs were collected daily over nine consecutive days.

**Experiment 3: Effect of blood feeding and egg collection frequency on egg production**

The effects of two blood-feeding and egg-collection regimes on the average number of eggs produced per batch per cage were compared using the improved MRC prototype (C6, Figure 1), with three replicates of each schedule: daily blood feeding and daily egg collection (DBF/DEC) vs. four blood feeds and two egg collections per week (4BF/2EC). Each cage was loaded with a single cohort of 15 000 pupae (male and female in approximately equal numbers) collected on the same day from the mass-rearing rack system. After being left in the cage overnight to emerge, cups were removed and dead pupae were counted. A blood meal was offered as described above, either on a daily basis starting on day 3 (DBF), or 4× per week on days 3, 4, 6, and 7 (4BF). Egg batches were collected from each of three replicates, 8× in the DBF/DEC treatment and 3× in the 4BF/2EC treatment, starting on day 7 after adult emergence in both cases.

**Data analysis**

All statistical analyses were performed using GraphPad (San Diego, CA, USA) and RStudio v.0.99.903 (RStudio, Boston, MA, USA). Comparisons of survival curves were performed with a log-rank (Mantel-Cox) test. A comparison of multiple survival curves between cage types C1-C2-C3 vs. C1-C4-C5 was performed using the Bonferroni method (α = 0.05). Egg numbers were estimated using an equation (Weight (mg) = (0.00399 × Number of counted eggs) + 0.536) described by Maïga et al. (2016) and compared between MRC types (experiment 1). One-way ANOVA was used to compare egg production between MRC models in experiment 1. For analysis of the effect of rearing method on egg production in experiment 2, a linear mixed effect analysis (*lme*4 package) with square root link was performed with egg number defined as dependant variable and intercepts for replicate and egg batch assigned as random effects. P-values were obtained by likelihood ratio test of the full model with rearing method effect against the model without the rearing method effect. In experiment 3, data were √x transformed to normalize distribution and then analysed with a paired t-test to compare the mean egg production per week between DBF/DEC vs. 4BF/2EC treatments. Egg numbers were checked for normality of error distribution (Kolmogorov-Simirov normality tests) before analysis for all experiments.

**Results**

**Effect of sugar provision and resting sites on adult longevity and egg production**

The addition of cup-style sugar feeders to the original MRC improved both male and female adult longevity (log-rank (Mantel-Cox) test, male: χ2=15.93, df=2, P=0.003, female: χ2 = 8.446, d.f. = 2, P = 0.015). Males lived longer in cages with additional sugar feeders [median survival of 8 days (C2) compared to 5 days in cages without sugar feeders (C1)]. However, the resting site did not improve adult longevity (male: χ2=0.006, d.f. =1, P = 0.94, female: χ2 = 1.267, d.f. = 1, P = 0.26). In this setting, females lived longer in cages with additional sugar feeders than in cages without sugar feeders (C1) (χ2 = 7.395, d.f. = 1, P = 0.006). Few eggs were collected from any of these three MRCs [C1: mean (of four egg batches per cage type) ± SE = 244 ± 124, C2: 42 ± 20, C3: 131 ± 83; ANOVA: F5,18 = 2.572, P = 0.063).

When adult longevity was compared in MRC models C1, C4, and C5, the new MRC prototype with the internal resting site (C5) gave improved adult longevity compared to C1[median survival of 11 days for C5 compared to 7 days for cages C1 and C4] ( log-rank (Mantel-Cox) test, male: χ2 = 81.44, female: χ2 = 24.26, both d.f. = 2, P<0.0001). When a resting site was available, males lived longer than females (median survival 11 vs. 8 days) though the resting site seems to be beneficial to both males and females. Similar numbers of eggs were collected from the three MRC models [C1: mean (of nine egg batches per cage type) ± SE = 2 621 ± 901, C4: 2 355 ± 1 032, C5: 2 261 ± 540; ANOVA: F2,24 = 0.3273, P = 0.72].

**Validation of larval rearing method for egg production**

No difference in egg production was observed as a result of larval rearing in individual free-standing trays compared to trays loaded into a mass rearing rack (χ2 = 1.1877, d.f. = 4, P = 0.88), with daily mean egg production of 23 256 ± 2 082 and 20 255 ± 2 283, respectively. Neither replicate effect nor egg batch was significant in either rearing method.

**Effect of blood feeding and egg collection frequency on egg production**

More eggs per batch were obtained in cages treated with four blood feedings and two egg collections per week (4BF/2EC) compared to cages given a daily blood feeding and daily egg collection (DBF/DEC) (paired t-test: t = 2.596, d.f. = 8, P = 0.032). When females were fed daily and eggs were collected on a daily basis (DBF/DEC) the mean (± SE) egg number per collection was 26 810 ± 4 797 (ca. 5 eggs per female per collection), with females laying most of their eggs during the first 3 days. The mean number of eggs per batch ranged from 6 860 ± 2 084 to 56 170 ± 22 236 with this feeding regime (Figure 3). When females were fed 4× per week and eggs were collected twice per week, the mean number of eggs per collection was 46 662 ± 10 621 (ca. 8 eggs per female per collection), and mean number of eggs per batch ranged from 14 627 ± 9 389 to 94 115 ± 21 860 (Figure 3). The total number of eggs collected from the cage was 214 478 ± 18 010 (DBF/DEC) and 139 986 ± 31 863 (4BF/2EC).

**Discussion**

The adult and larval mass-rearing units have been tested here for the first time for *An. arabiensis* mass rearing and performance in terms of mean egg production. Larval rearing conditions dictate adult fitness (Ng'habi et al., 2005; Yahouédo et al., 2014) and so it was important to validate the production of pupae in the FAO/IAEA larval rearing rack (Balestrino et al., 2012). In this experiment, neither handling of pupae (picking by hand from individual trays or collection by tilting the rack) nor rearing conditions seemed to affect fecundity. This study is the first demonstration of a complete *An. arabiensis* rearing cycle using the rack system (eggs-larvae-pupae) and the mass-rearing cage (pupae-adults-eggs). Some modifications of the original prototype cage design appeared to enhance male and female *An. arabiensis* longevity and the improved cage prototype led to collection of more eggs from a cohort of adults than was possible using previous cage prototypes. Daily blood feeding with daily egg collection may not be the most efficient rearing regime in *Anopheles* mass-rearing facilities, as a reduced frequency of blood feeding and egg collection yielded a higher number of eggs per batch.

Increasing the availability of sugar solution to adults in the cage increased adult longevity, with adults feeding more readily from filter paper wicks than from plastic cups (H Maïga, pers. obs.), likely due to the increased surface area. Neither sucrose nor glucose has any fragrance and so the sugar sources are probably located incidentally using the water vapour plume (MQ Benedict, pers. comm.). The aluminium of the original sugar tube may have been responsible for the high mortality observed in the original mass-rearing cage directly, not just because it provided a limiting surface area for adults to feed. Aluminium is reactive and when in contact with water forms aluminium oxide which can cause cuticle abrasion and induce gut damage. Huang et al. (2013) have also shown that nano-alumina might have adverse effects on the central nervous system in *Drosophila*. When sugar is available, the presence of a resting site seems to be beneficial to both males and females, with the combination of better access to sugar and a place to rest increasing longevity.

The improvements to the MRC prototype developed here took adult *Anopheles* biology into consideration in providing optimal conditions. Colonization is an essential process but may induce the artificial selection of genetically homogeneous individuals very different from wild populations. Multiple generations kept in confined conditions under negative selective pressures may induce the loss of characteristics that affect the fitness of released insects (Dame, 1985, Benedict et al., 2009). Marchand (1985) showed that the introduction of a twilight phase and an artificial horizon contrasting with a lit background can stimulate swarming behaviour and lead to higher insemination rates. Facchinelli et al. (2015) recently developed a large cage which induced swarming behaviour in *Anopheles gambiae* Giles in the laboratory and resulted in increased female insemination rates. Though this study was not focused on swarming, the visual contrast of light vs. dark at the edge of the dark external resting site allowing for increased mating may explain increase in egg production.

Though the new MRC prototype increased adult longevity compared to the original cage, no difference in egg yield was observed suggesting that in this setting more eggs were laid early enough, so that differences in longevity did not affect egg production for the 9-day duration of egg collection. However, as the rearing cycle could be extended up to 20 days, more eggs might eventually be expected from the new MRC prototype due to better adult survival. The black box hung inside the cage as a resting site in the first experiment seemed to lead to females preferentially resting rather than blood feeding during the day, possibly explaining the lack of impact on the mean number of eggs collected. In contrast, a black cloth provided both a daytime resting site and an artificial horizon seemed to encourage blood feeding by mimicking the conditions under which *Anopheles* females feed in nature.

An SIT release programme, even for a small geographical area, will require several million adults to be produced weekly. It was therefore important to have effective and economical methods to produce the largest numbers of eggs possible from each MRC by optimising the design and rearing schedule. Damiens et al. (2013) have shown that egg production is higher in small-scale laboratory rearing when females are blood-fed daily compared to every 2 or 4 days. A similar observation was made here: when blood feeding and egg collection was done daily, overall egg production was higher from each cage, though fewer eggs were collected per batch per day than from the cages fed and egged less often. We showed that reducing blood-feeding frequency and collecting eggs twice per week instead of daily, as previously done, yielded a higher number of eggs per batch compared to daily egg collections, around 100 000 eggs from the first batch, to a total of 140 000 eggs from three batches. To fill the 50 trays in one rack with 4 000 eggs each, 200 000 eggs will be needed per cohort, and the more eggs obtained at once from each MRC the better to efficiently use the available resources, given that *Anopheles* eggs are not resistant to desiccation making it difficult to collect and combine eggs collected across more than 1 day. Though Khan et al. (2013) observed that dry storage at 20 °C of eggs in bulk did not affect hatch rate, larval duration, or survival, this approach would mean more work and increased operational costs in a mass-rearing setting than collecting sufficient eggs on a single day.

Although an improved MRC prototype has been developed which enhances adult longevity and egg production, more rigorous testing of combinations of blood feeding and egg collection to optimise the egg yield will still be needed. In addition, removal of access to a sugar solution prior to blood feeding might increase the proportion of females that take a blood meal (Damiens et al., 2013), though the effects of reduced male-to-female ratio as a result of increased male mortality on egg yield should be investigated. A second possible adaptation could be to replace the netting wall panels of the cage with panels consisting of the bottom half in netting and the top half in black cloth to enhance the contrasting colour inside the cage, encourage the mosquitoes to utilize the resting site and hopefully increase longevity, as well as luring them into closer proximity to the blood meal when offered. Other parameters which could be considered for further optimisation include cage size, blood source, adult density, male-to-female ratio, and the effect of daily addition of adults rather than pupae. However, we have successfully demonstrated the potential of the FAO/IAEA mass-rearing system to mass produce *An. arabiensis* for release as part of a vector control programme.

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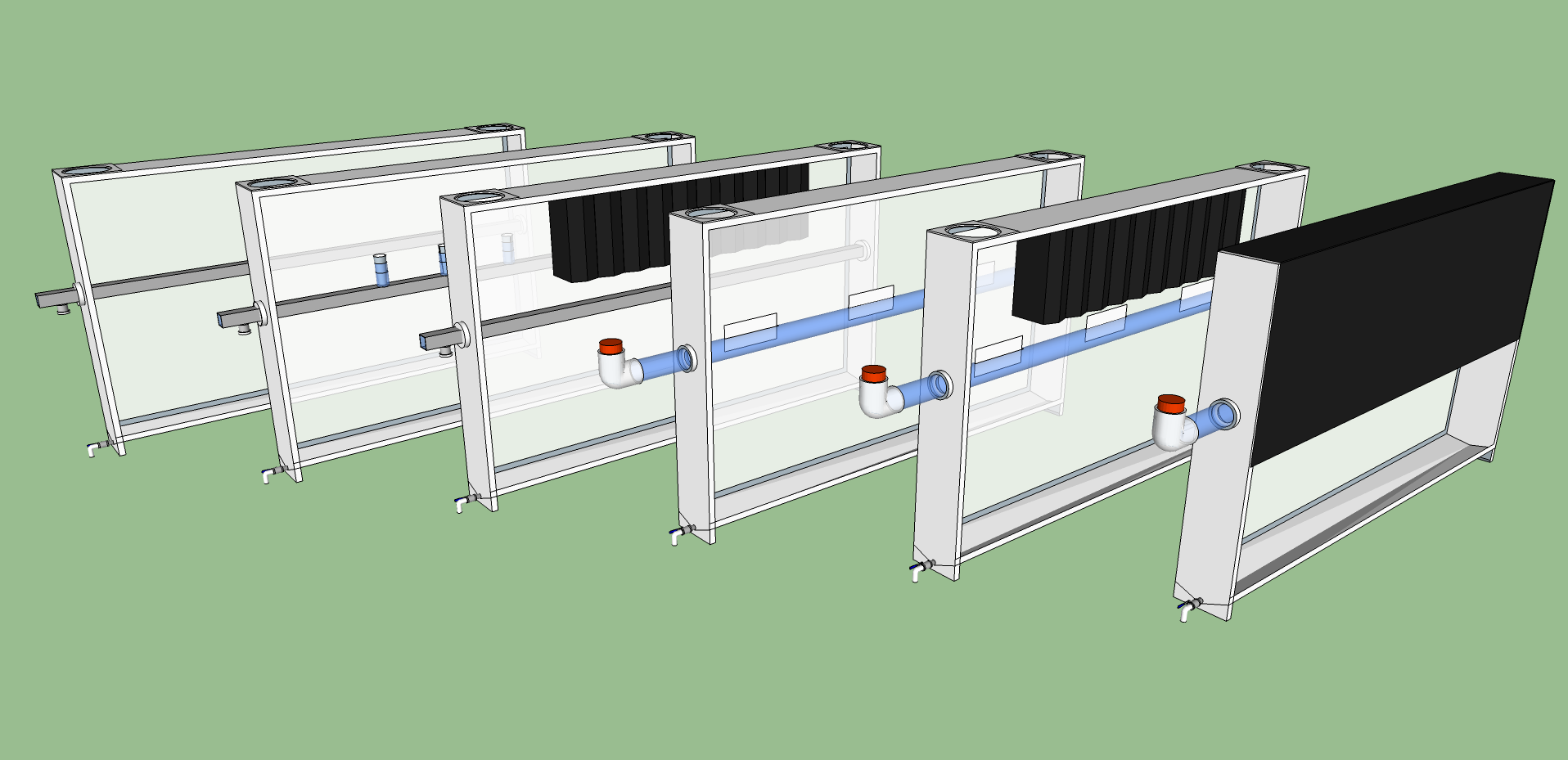
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**Figure captions**

**Figure 1** Six mass-rearing cage (MRC) prototypes were used in the experiments (all 2 × 1 × 0.2 m, hence 400 l volume): original MRC prototype (C1); original MRC prototype with additional cups of 5% sugar solution and coffee filter paper wicks (C2); original MRC prototype with a black box resting site (C3); new MRC prototype with modified sugar tube and filter papers (C4); new prototype with black box internal resting site (C5); and new prototype with external black cloth along the upper part of the cage (C6; ‘improved MRC prototype’).

**Figure 2** Sugar feeder types in cage prototypes. FC1, feeder in cage 1, the original sugar feeder made of aluminium and rectangular tube (6 cm diameter) sealed on each end. On one side of the tube, at 5 cm from the end, an inlet pipe with a watertight stopper was connected for adding sugar solution from outside of the cage. Holes (2.5 cm diameter) were created along the tube on the same side as the inlet pipe and covered with 20-μm stainless steel mesh. FC2, feeder in cage 2, with 100-ml cups each containing 80 ml of 5% sugar solution and a coffee filter paper to the top of the original rectangular tube. FC4-6, feeder in cage 4, 5, and 6: a tube (220 cm long, 5 cm diameter), sealed with a rubber plug on both ends and with an upwards facing elbow plumbing fixture on the front end used to fill the device.

**Figure 3** Mean (± SE; n = 3) number of eggs per batch per cage of daily blood feeding and daily egg collection regime (white bars) compared to cages given four blood feedings and two egg collections per week (grey bars) using the improved mass-rearing cage prototype (C6; see also Figure 1).



C1

C2

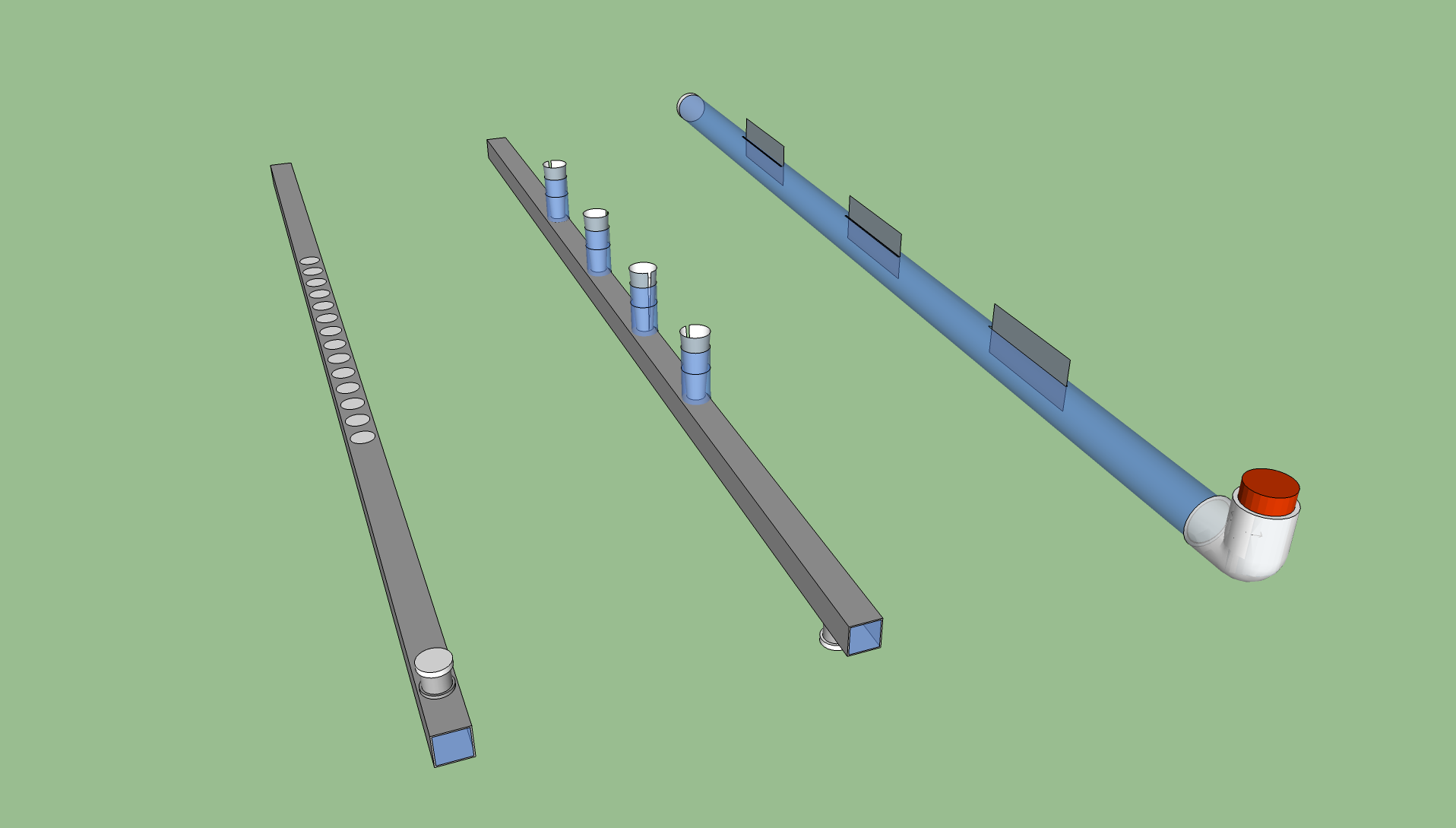
C3

C5

C4

C6

**Figure 1**



FC4-6

FC2

FC1

**Figure 2**

**Figure 3**