Video Article

Maintaining *Aedes aegypti* Mosquitoes Infected with *Wolbachia*

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Abstract

Aedes aegypti mosquitoes experimentally infected with *Wolbachia* are being utilized in programs to control the spread of arboviruses such as dengue, chikungunya and Zika. *Wolbachia*-infected mosquitoes can be released into the field to either reduce population sizes through incompatible matings or to transform populations with mosquitoes that are refractory to virus transmission. For these strategies to succeed, the mosquitoes released into the field from the laboratory must be competitive with native mosquitoes. However, maintaining mosquitoes in the laboratory can result in inbreeding, genetic drift and laboratory adaptation which can reduce their fitness in the field and may confound the results of experiments. To test the suitability of different *Wolbachia* infections for deployment in the field, it is necessary to maintain mosquitoes in a controlled laboratory environment across multiple generations. We describe a simple protocol for maintaining *Ae. aegypti* mosquitoes in the laboratory, which is suitable for both *Wolbachia*-infected and wild-type mosquitoes. The methods minimize laboratory adaptation and implement outcrossing to increase the relevance of experiments to field mosquitoes. Additionally, colonies are maintained under optimal conditions to maximize their fitness for open field releases.

Video Link

The video component of this article can be found at https://www.jove.com/video/56124/

Introduction

Aedes aegypti mosquitoes are responsible for transmitting some of the most important arboviruses in the world, including dengue, Zika and chikungunya[1]. These viruses are becoming an increasing threat to global health as the widespread distribution of *Ae. aegypti* in the tropics continues to expand[2,3,4]. Female *Ae. aegypti* preferentially feed on human blood[5] and thus tend to live in close proximity to humans, particularly in urban areas where populations are most dense. Through this close association with humans they have also adapted to breed in artificial habitats, including tires, pots, gutters and water tanks[6,7]. *Ae. aegypti* also readily adapt to laboratory environments where they can be maintained without any special requirements after being collected directly from the field, unlike some other species in the *Aedes* genus[8,9,10]. Their ease of maintenance has seen them studied widely in the laboratory in a broad range of fields, ultimately aiming to control the diseases mosquitoes may transmit.

Traditionally, arboviral control relies heavily on the use of insecticides to reduce mosquito populations. However, there is increasing interest in approaches where modified mosquitoes are reared in the laboratory and then released into natural populations. Released mosquitoes may be modified genetically[11,12,13], biologically[14,15], through irradiation[16], chemical treatment[17,18], or with combined techniques[19] to either suppress populations of mosquitoes or replace them with mosquitoes that are refractory to arboviral transmission[20].

Wolbachia are bacteria that are currently being used as a biological control agent for arboviruses. Several strains of *Wolbachia* were recently introduced into *Ae. aegypti* experimentally using embryonic microinjection[21,22,23,24]. These strains reduce the capacity of arboviruses to disseminate and replicate in the mosquito, diminishing their transmission potential[23,25,26,27,28]. *Wolbachia* infections are transmitted from mother to offspring, however certain strains induce sterility when infected males mate with uninfected females[22]. *Wolbachia*-infected males can therefore be released in large quantities to suppress natural mosquito populations, as recently demonstrated in other *Aedes* species[15,29]. However, since *Wolbachia* also inhibit arboviral transmission in *Ae. aegypti*, mosquitoes can also be released to replace native populations with poorer vectors. *Ae. aegypti* infected experimentally with *Wolbachia* are now being released into the field in several countries using this latter approach[14,30,31].

Wolbachia-based approaches for arboviral control rely on a sound understanding of the interactions between *Wolbachia*, the mosquito and the environment. *Wolbachia* occur naturally in a broad range of insects, and the strains introduced into mosquitoes are diverse in their effects[32]. As new *Wolbachia* infection types are introduced into *Ae. aegypti*[24], it is necessary to characterize each strain for their effects on mosquito fitness, reproduction and arboviral interference under a range of conditions. Rigorous experimentation in the laboratory is therefore required to evaluate the potential for *Wolbachia* strains to succeed in the field.

Open field releases of *Ae. aegypti* with *Wolbachia* infections can often require thousands to tens of thousands of mosquitoes per release zone to be reared each week[14,30,31]. The success of initial releases can be improved by releasing mosquitoes of a large size to maximize their fecundity[33]

and mating success[34,35]. Mosquitoes should also be adapted to the conditions they will experience in the field, however long-term laboratory rearing may cause changes in behavior and physiology which could impact field performance[36,37,38].

We describe a simple protocol for rearing *Ae. aegypti* in the laboratory using basic equipment. This protocol is suitable for both wild-type and *Wolbachia*-infected mosquitoes, the latter of which can require special attention as some *Wolbachia* strains have substantial effects on mosquito life-history traits[39,40]. The rearing conditions avoid overcrowding and competition for food to produce mosquitoes of a consistent size, which is critical for vector competence and fitness experiments, and ensures that the mosquitoes are healthy for field release[41]. We also take precautions to minimize laboratory adaptation and inbreeding by reducing selective pressures and ensuring that the next generation is sampled from a large, random pool. However, laboratory environments are distinctly different from field conditions, and long-term maintenance under relaxed conditions could reduce the fitness of mosquitoes upon release into the field[37,42,43]. We therefore cross females from laboratory lines to field-collected males periodically, resulting in colonies that are genetically similar for experimental comparisons and that are adapted to the target field population[39]. The methods do not require any specialized equipment and can be scaled up to rear tens of thousands of individuals per week for field releases. The protocol also prioritizes the fitness of mosquitoes within and across generations, an important consideration for insects destined for establishment in natural populations. The protocol is suitable for most laboratories that require maintenance of *Ae. aegypti*, particularly for experimental comparisons where a consistent quality of mosquitoes and relatability to the field are important.

Protocol

Blood feeding of mosquitoes on human subjects was approved by the University of Melbourne Human Ethics Committee (approval #: 0723847). All volunteers provided informed written consent.

1. Larval Rearing

NOTE: Mosquitoes are held at 26 ± 0.5 °C and 50-70% relative humidity, with a 12:12 h (light:dark) photoperiod for this colony maintenance protocol. These conditions are similar to the average climatic conditions in Cairns, Australia and within the optimal thermal range for *Ae. aegypti* survival and development[44,45,46]. High temperatures can result in the loss of *Wolbachia* infections from mosquito colonies and should be avoided[47]. We maintain at least 500 individuals per population to minimize inbreeding; maintaining colonies of a smaller size can have fitness consequences [Ross *et al.* unpublished]. Under these conditions and assuming adequate nutrition, the average generation time is 28 days (see **Table 1**).

1. Submerge the eggs on substrate in trays (**Figure 1A**) containing 3 L of water (reverse osmosis water or aged tap water, generated by leaving tap water in trays for 24 h prior to use), ~300 mg of fish food (one crushed tablet, see **Table of Materials**) and a few grains of active dry yeast to induce hatching[48].
2. One day after hatching, use a glass pipette to transfer approximately 500 larvae to trays containing 4 L of water (**Figure 1B**), count using a clicker counter. Add two crushed fish food tablets to each tray. If needed, use containers of different sizes for rearing larvae (**Figure 1A**), but keep larval densities below 0.5 larvae/mL to avoid overcrowding.
3. Check the trays daily to ensure that the larvae have sufficient food; add approximately two food tablets to the trays every two days. Provide food *ad libitum* but ensure that 0.5 mg/larva/day is available during this period to ensure development is synchronous and body size is consistent, otherwise the results of experiments may be confounded (see Representative Results).
4. Take care to avoid the overfeeding of larvae, particularly in smaller rearing containers with less water surface area and volume. If the water looks cloudy or if there is significant larval mortality, replace it with fresh water; mortality should be negligible if larvae are fed optimally.

2. Adult Emergence

NOTE: Larvae will begin to pupate from five days after hatching if well fed and the majority should pupate by seven days after hatching. Adults will begin emerging approximately two days after pupation if maintained optimally at 26 °C (see **Representative Results**). Larval development is typically unaffected by *Wolbachia* infections when ample food is provided[23,39,49].

1. Seven days after hatching pour the entire contents of the tray through a fine mesh (pore size 0.4 mm). Keep the filtered larval water for later use in ovicups (see "Blood Feeding and Oviposition" section). Invert the mesh and dip it into a plastic container with 200 mL of water to transfer the pupae. Provide additional food if any larvae remain.
2. Prepare the adult emergence cages (**Figure 1C**) by providing two cups of 10% sucrose solution (**Figure 1F**) and two cups of moist cotton wool to prevent desiccation (**Figure 1E**).
3. If the pupae do not need to be sorted by sex, place the lidded containers of pupae into the cage and leave the lid slightly ajar to allow the adults to emerge into the cage. Alternatively, place an inverted funnel over the container to minimize drowning. Ensure all adults have emerged before removing the container from the cage to prevent selecting against slow developers.

3. Pupal Sexing for Outcrossing

1. If the pupae do need to be sorted by sex (*e.g.*, for outcrossing), pipette the pupae from larval trays and separate the sexes (**Figure 2**) into plastic containers (**Figure 1A**) with 200 mL of water every 24 h until the desired number of each sex has been reached. Place lids on the containers and leave them closed.
2. Adults will emerge into the containers; confirm their sex before releasing into cages (**Figure 2C**). Remove any adults sexed incorrectly with an aspirator within 24 h of emergence before they reach sexual maturity. Once the sexes have been confirmed, release the adults into cages every 24 h.
3. **To obtain *Wolbachia*-infected colonies of a similar genetic background to a natural population, outcross by adding *Wolbachia*-infected females from laboratory colonies to cages of uninfected males derived from eggs collected by ovitraps in the field[39], maintaining the prescribed density of 500 individuals per population.**

1. Repeat the outcrossing for at least three consecutive generations to produce colonies that are at least 87.5% similar genetically to the field population[39]. Critical: Ensure that the sexes are correct at this stage (see step 3.1).

4. Female *Ae. aegypti* are usually refractory to further insemination within hours of mating[50]. When outcrossing colonies, allow the females and males to mature in separate cages for two days and then aspirate the females into the male cage to provide an equal opportunity to all males.

4. Blood Feeding and Oviposition

1. **Wait for at least three days after the last female has emerged before blood feeding to allow ample time to mature. Blood feed the females within two weeks of emergence to prevent excessive mortality, particularly for mosquitoes with *Wolbachia* infections that adversely affect longevity[22,24,49]. Remove the sugar cups the day prior to feeding to improve feeding rates.**
    1. Ask a volunteer to insert their forearm into the cage to allow the female mosquitoes to feed. Most females should feed to repletion within 5 min, but to reduce selection against slow feeders, leave the forearm in the cage for 15 min, or until all females are visibly engorged; a latex glove to protect the hand from bites is optional but recommended.
    2. Two days after blood feeding, place two plastic cups containing larval rearing water and lined with a strip of sandpaper (**Figure 1G**) (or filter paper (**Figure 1H**)) into the cage for females to lay eggs. Partially submerge the sandpaper strip in the water to keep it moist. Remove other sources of water to prevent the females from laying their eggs outside the oviposition cup.
    NOTE: Tap water may be used in the cups, but larval-rearing water encourages oviposition[51,52] and females will lay their eggs more synchronously.

5. Egg Collection and Conditioning

1. The females will lay eggs on the sandpaper just above the water line; collect and replace the strips of sandpaper daily until no more eggs are laid. Note that oviposition may continue for up to one week.
2. Partially dry the sandpaper strips by gently blotting them on a paper towel for 30 s, taking care not to dislodge the eggs. Then, wrap the strips in a sheet of dry paper towel and place it into a sealable plastic bag (**Figure 1I**).
3. Check the condition of the eggs under a dissecting microscope (**Figure 3**). If the sandpaper strips are too wet, eggs may hatch before being submerged in water (**Figure 3B**), but if dried too harshly, eggs may collapse (**Figure 3C**).
4. The eggs can be hatched at any time beyond three days post-collection; hatch all eggs from each colony, collected across all days, in the same container of water to ensure that the next generation is sampled from a large, random pool of individuals.
5. For long-term storage, keep the eggs in a sealed container at a high (> 80%) humidity at around 20 °C. Under these conditions, eggs without *Wolbachia* can be stored for several months while maintaining high hatch rates[53,54].
6. As some *Wolbachia* infections greatly reduce the viability of eggs with age[49,55], hatch the eggs from *Wolbachia*-infected lines within one week of collection to prevent excessive mortality for the relevant strains. Blood feed the females again after one week if more eggs are needed.

Day	Step
0	Hatch eggs
1	Count larvae into trays
7	Transfer larvae and pupae to colony cages
17	Blood feed female adults
21	Begin collecting eggs
25	Finish collecting eggs
28	Hatch eggs

Table 1: Overview of the *Ae. aegypti* colony maintenance schedule at 26 °C. The timing of the female blood-feeding and the hatching of eggs is flexible, but long durations at these stages should be avoided, particularly for mosquitoes infected with *Wolbachia*, in order to minimize mortality. Following this schedule minimizes selection against mosquitoes that are fast or slow to develop or mature at all life stages, provided that larvae are fed optimally.

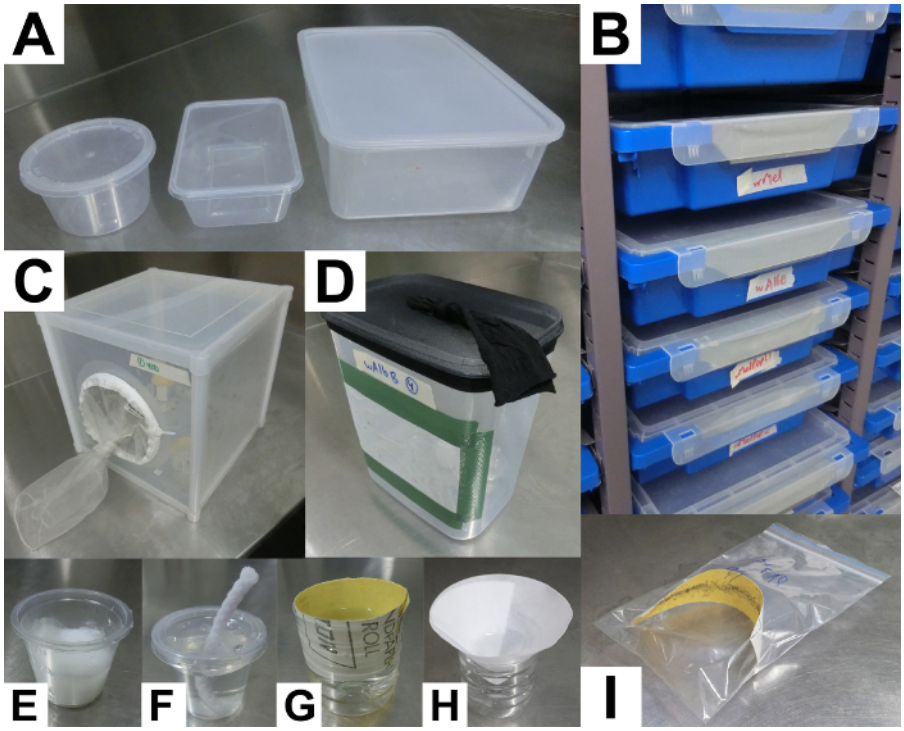

Figure 1: Equipment used for rearing *Ae. aegypti* in the laboratory. (**A**) Plastic containers used for hatching eggs or rearing larvae with volumes of 500, 750 and 5,000 mL (from left to right). (**B**) Trays used for rearing larvae at a controlled density, usually 500 larvae in 4 L of water. (**C**) 19.7 L and (**D**) 3 L cages used for housing adults. A density of 25 adults or less per liter should be maintained to provide sufficient space. (**E**) 35 mL cup with moist cotton wool provided as a source of water to adults. (**F**) 35 mL cup with sucrose solution provided through a cord or dental wick as a source of sugar. (**G-H**) Cups filled with larval rearing water and lined with an oviposition substrate of sandpaper or filter paper (**G** and **H**, respectively). (**I**) Zip-lock bag used for storage of sandpaper strips or filter paper. Black spots on sandpaper are mosquito eggs. Please click here to view a larger version of this figure.

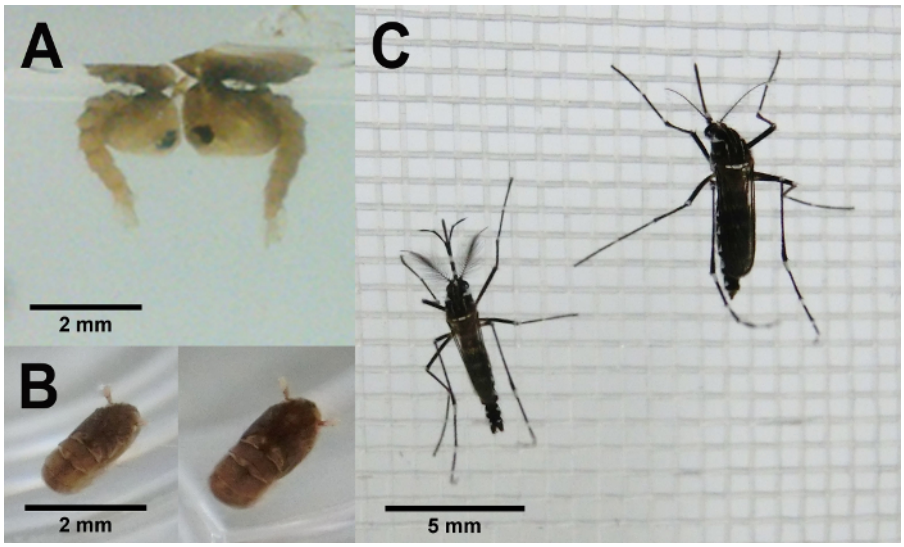

Figure 2: Lateral (A) and dorsal (B) views of pupae and adult *Ae. aegypti* (C) demonstrating their sexual dimorphism. Males are positioned on the left and females on the right in each panel. When optimally fed, male and female pupae are distinguished by size; females are larger than males (**A**) and have a relatively bulbous cephalothorax compared to males which have flatter sides (**B**). Male adults are easily distinguished from females under all rearing conditions, primarily by their plumose antennae and long palps. Please click here to view a larger version of this figure.

Figure 3. Four day old *Ae. aegypti* eggs under different conditions. (**A**) Intact eggs on sandpaper strips maintained at a high (> 80%) humidity but without any visible moisture. Hatch rates should be above 90% for wild-type *Ae. aegypti* if maintained correctly. (**B**) Eggs that hatch before being submerged in water (precocious hatching) are distinguished by a detached egg cap and visible larva. This indicates that the sandpaper strip was kept too moist. (**C**) Eggs that are dried too harshly may collapse, and are clearly visible by their concave appearance. If the sandpaper becomes stiff this also indicates that the eggs may be too dry. Please click here to view a larger version of this figure.

Representative Results

Figure 4 demonstrates the effects of suboptimal nutrition on the development of *Ae. aegypti* larvae. When containers are provided with 0.25 mg of food per larva per day or less, the development time increases for both males and females, and is less synchronous than in containers provided with 0.5 mg of food. If adequate food is not provided throughout the duration of larval development, this could have an adverse impact on the maintenance schedule. Slow-developing individuals are at risk of being selected against, blood feeding may be delayed, and there is a higher risk of adult mortality before reproduction occurs.

Figure 5 shows the wing length (an estimate of body size) of *Ae. aegypti* adults reared under a variety of nutrition regimes. Wing lengths of both males and females decrease substantially and become more variable when nutrition is suboptimal. Uniform body sizes are important for experimental comparisons as body size is positively associated with fecundity, and large mosquitoes are expected to exhibit greater fitness under field conditions[33,34,35].

The effects of *Wolbachia* infections on the above traits are described in other studies but generally there is little to no effect[23,39,49].

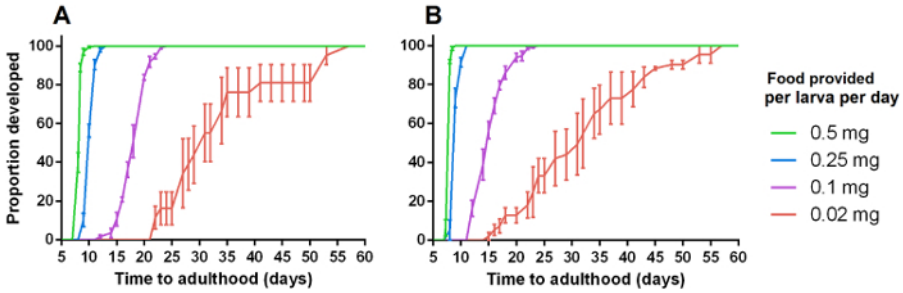

Figure 4: Cumulative proportion of *Ae.aegypti* (A) females and (B) males developing to adulthood under different food regimes at 26 °C. 100 larvae were reared in containers of 500 mL water (a larval density of 0.2 larvae per mL), provided with different levels of food (see **Table of Materials)** and scored for their development time. Only larvae that survived to adulthood were included. Error bars are standard errors, with n = 4 replicates per treatment. Please click here to view a larger version of this figure.

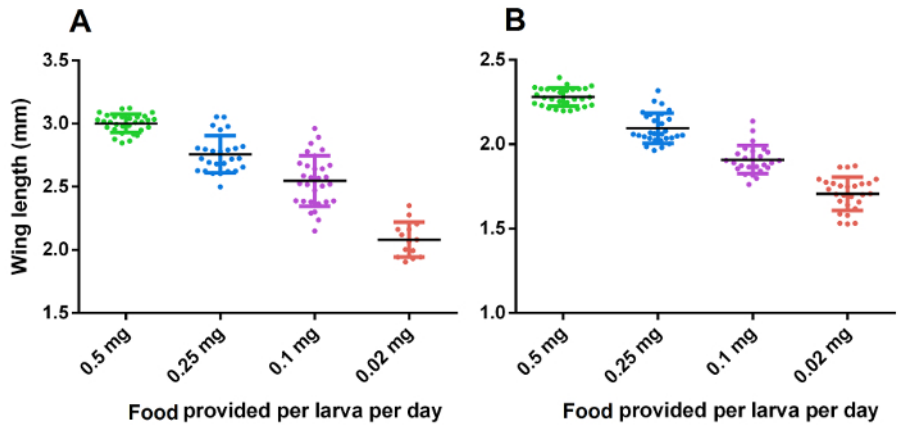

Figure 5: Wing length of *Ae.aegypti* (A) females and (B) males developing under different food regimes at 26 °C. 100 larvae were reared in containers of 500 mL water (a larval density of 0.2 larvae per mL) and provided with different levels of food. A subset of adults was then measured for their wing length by using previously described methods[56]. Error bars are standard deviations. Please click here to view a larger version of this figure.

Discussion

Following the protocol presented here for the maintenance of *Wolbachia*-infected *Ae. aegypti* should ensure that healthy mosquitoes of a consistent quality are produced for experiments and open field releases. In contrast to other protocols that prioritize the production of mass quantities of mosquitoes (see reference[57]), the methods are focused on maximizing their fitness, both within generations by implementing relaxed rearing conditions, and across generations by minimizing inbreeding, selection and laboratory adaptation. This protocol is also designed specifically for *Ae. aegypti* with *Wolbachia* infections but should be suitable for any type of *Ae. aegypti*. However, it is not suitable for rearing mass quantities (in the order of millions per week), which can be necessary for sterile or incompatible insect releases that require high numbers to achieve population suppression[57,58].

There are some critical steps that should be followed carefully. It is important to feed the larvae optimally and avoid overcrowding for the duration of their development. This will ensure that the mosquitoes develop synchronously and are of a consistent size. Special care should also be taken when conditioning the eggs; larvae can very easily hatch too soon or eggs can desiccate if the oviposition substrates are too wet or dry, respectively. At all steps in the protocol, we recommend allowing enough time for as many individuals in the colony as possible to complete each stage. Selection against individuals that are slow to develop, mature, blood feed, oviposit or hatch will likely lead to the loss of genetic variation.

We note some further considerations for the maintenance of *Wolbachia*-infected mosquitoes that are not described in the above protocol. It is possible for *Wolbachia* infections to be lost from laboratory colonies, and we therefore recommend that colonies be routinely monitored for their *Wolbachia* infection status. We use quantitative polymerase chain reaction (qPCR)[49,59] to test at least 30 individuals from each *Wolbachia*-infected line every generation. If any individuals test negative for the appropriate *Wolbachia* infection, colonies can be purified by isolating as many females as possible from the affected colony and then using progeny from infected mothers only, to found the next generation. The cause of the loss of some *Wolbachia* strains from laboratory colonies is largely unknown, but could be explained by the failure of temperature controls, as high temperatures can cause the loss of *Wolbachia* infections[47,60].

Colonies and experiments may also become contaminated with individuals from different lines if care is not taken when rearing. Contamination can result from careless pipetting of larvae, mixing up batches of eggs, mislabeling cages or incorrectly sexing pupae in crosses. Special care should therefore be taken when handling colonies with different *Wolbachia* infection types. Thoroughly clean any oviposition cups, rearing trays and cages before reusing them, clean out pipettes before handling each new tray of larvae and clean the mesh each time when transferring larvae and pupae to other containers. Furthermore, inspect fingers for eggs when handling sandpaper strips and use fresh paper towels when drying each strip, ensure that adults are the correct sex before releasing them into cages, and deal with any escapees quickly. Taking these precautions should avoid most contamination, but colonies should still be routinely monitored using diagnostic assays[59].

Female *Ae. aegypti* require a blood meal in order to lay eggs, and laboratories around the world provide them in a variety of ways, from membrane feeding systems[57], restrained animals[61] and, to a lesser extent, artificial blood[62]. However, mosquitoes with experimental *Wolbachia* infections often perform poorly on non-human blood, and may exhibit reduced fecundity and hatch rates, and incomplete transmission of *Wolbachia* to their offspring[63,64,65]. Maintaining the receptiveness of females to human odors is also important for mosquitoes to be released into the field, and feeding them through membranes or other animals may compromise this ability[37]. We therefore opt to use the forearm of human volunteers for this colony maintenance protocol, though other methods are required under some circumstances. Blood should be supplied through other means when working directly with arboviruses, and care should be taken when colonies originate from field populations with high viral loads as transovarial transmission of arboviruses may occur[66]. Human volunteers should also not blood feed if they have recently visited arboviral endemic countries as there may be a risk of transmission.

Our maintenance protocol aims to minimize laboratory adaptation and selective pressures, but there is room for improvement. Leftwich *et al.*[42] provide further recommendations for maintaining the fitness of mosquitoes for open field releases, including the use of more diverse and natural larval diets, reducing the density of adults in cages, and providing a complex environment. These considerations may enhance the fitness of *Ae. aegypti* to a greater level, though currently there is no evidence of fitness changes due to laboratory adaptation under the maintenance protocol described here [Ross *et al.* unpublished]. Some additional measures may not be feasible for laboratories with limited space and resources, but are nevertheless worth investigating. Maintaining large population sizes, avoiding major selective pressures through rearing protocols and implementing periodic outcrossing to field mosquitoes should help in ensuring high fitness of *Ae. aegypti* for open field releases. The principles involved in the approach can be applied to rearing other disease vector species for release to manipulate or suppress natural populations.

Disclosures

The authors declare that they have no competing financial interests.

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