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SATELLITE REARING OF *AEDES* MOSQUITO EGGS: SYNCHRONIZED EMPIRICAL TEST OF A NOVEL MASS REARING MODEL

KAREN L. DOBSON,[1] KAI BLORE,[2] JENNIFER A. HENKE,[3] KIM Y. HUNG,[3] TIM MORGAN,[4] TANYA POSEY,[5] SOKANARY SUN,[4] OLIVIA SYPES,[2] NICOLAS P. TREMBLAY[5] AND STEPHEN L. DOBSON[1,6,7]

ABSTRACT. Mosquito suppression strategies based on "rear and release" of male mosquitoes are attracting renewed interest from governments, municipalities, and private businesses. These include irradiation-based sterile insect technique, *Wolbachia*-based technologies, and genetic modification. Each of these approaches requires the mass rearing and release of adult male mosquitoes, which typically is accomplished via a rearing facility near the release site. Although some release programs have relied on centralized rearing and shipment of adult males, adult male mosquitoes are relatively fragile, and their fitness can be diminished by temperature fluctuations, humidity, nutritional deficiencies, and other stresses that occur during shipment. Furthermore, expensive, expedited shipment is typically used to maximize the amount of adult lifetime in the field following the release. In contrast, *Aedes aegypti* and *Ae. albopictus* eggs can be desiccated and stored for long periods. They are small, and many millions of eggs can be shipped without specialized environmental conditions and using less expensive means. Here we examine a model in which mosquito eggs are centrally produced and then mailed to satellite rearing facilities. As a control, a replicate set of eggs was reared at the factory of origin. At each of the rearing sites, cloud-based software was used to track and compare rearing at the different locations. The results demonstrate similar rearing outcomes (i.e., egg hatch, immature development, and number of adult males) at each of the different sites for both species. We discuss the outcome in relation to downstream applications and potential future studies.

KEY WORDS *Aedes aegypti*, *Aedes albopictus*, mass rearing, sterile insect technique

INTRODUCTION

Autocidal strategies are not new, but interest in them has been reinvigorated recently by multiple factors including: a dearth of existing mosquito control tools; increased public and regulatory concern surrounding potential health and environmental impacts of chemical control tools; insecticide resistance development among the targeted mosquito populations; the spread of invasive *Aedes* mosquito species and their associated pathogens; and an insufficient ability to impact cryptic breeding sites (Morrison et al. 2008). Autocidal mosquito control approaches are predicated on the mass release of adult males, which mate with indigenous conspecific females to either induce sterility or produce nonviable offspring. Males are released because they do not bite or transmit pathogens. Autocidal approaches include classical irradiation-based sterile insect technique, *Wolbachia*-based incompatible insect technique, and approaches based on genetically modified mosquitoes (Ritchie and Johnson 2017, Ritchie and Staunton 2019, Benedict 2021).

The cost of mass-producing male mosquitoes has been a tenacious impediment to the wide-scale adoption of autocidal technologies, with costs including the construction and maintenance of the mass rearing facility and labor (Anaman et al. 1994, Dyck et al. 2005, Pascacio-Villafan et al. 2017, Vreysen et al. 2021). The facility and labor cost influences include parameters such as the colony size and space required, along with the effort and time needed for each of the different mass rearing steps. Therefore, savings in operating costs can be realized at a specific facility if one or more steps can be eliminated at that facility, i.e., shifted to a separate, centralized facility. For example, if "bloodfeeding" can be obviated for a facility, then cost savings can result from the reduction in the factory space, labor, reagent, and equipment costs associated with bloodfeeding.

Conceptually, the mass rearing of male mosquitoes can be divided into 2 components: the "Egg Production" (EP) facility and "Male Rear and Release" (MRAR) facility (Fig. 1). The EP facility requires the complete mosquito life cycle, including female bloodfeeding and egg collection, often occurring across multiple gonotrophic cycles. Female mosquitoes are integral to the EP facility. In contrast, the MRAR facility operations are not a cycle but a unidirectional flow, starting with eggs as an input and resulting with adult males as the output. Female mosquitoes are an unwanted byproduct of the MRAR

[1] MosquitoMate, Inc., 210 Malabu Drive, Lexington, KY 40502.
[2] Anastasia Mosquito Control District, 120 EOC Drive, St. Augustine, FL 32092.
[3] Coachella Valley Mosquito and Vector Control District, 43420 Trader Place, Indio, CA 92201.
[4] Orange County Mosquito and Vector Control District, 13001 Garden Grove Boulevard, Garden Grove, CA 92843.
[5] Greater Los Angeles County Vector Control District, 12545 Florence Avenue, Santa Fe Springs, CA 90670.
[6] Department of Entomology, University of Kentucky, Lexington, KY 40506.
[7] To whom correspondence should be addressed.

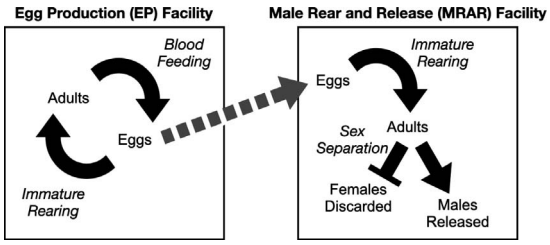

Fig. 1. The rearing model is based on the "Egg Production" (EP) and "Male Rear and Release" (MRAR) facilities. While the EP facility operations are a cycle, the MRAR operations are a unidirectional flow, beginning with eggs (provided from the EP facility) and ending with the release of adult males.

facility, which are separated out during the rearing process and eliminated prior to eclosion.

Dividing the mass rearing process into the EP and MRAR facilities provides additional operational flexibility. Specifically, an EP facility does not necessarily need all of the equipment for sex separation, irradiation, or other processes used in the preparation of males for release. Similarly, a MRAR facility does not need space, labor, and equipment for bloodfeeding or egg collection. Egg collection is space intensive, because adult cages are typically held for multiple gonotrophic cycles, which can tie up valuable floor space and cages for weeks. The MRAR facilities also have simplified arthropod containment needs, because few female adults should occur at a MRAR facility. Most females are removed and discarded prior to reaching adulthood at the MRAR facility, which lowers the risk of female escape, relative to an EP facility that propagates millions of adult females.

A centralized EP facility can oversee ongoing quality control steps such as periodic outcrossing, molecular tests for *Wolbachia* infection, and repeated fitness testing. Thus, the MRAR facilities do not necessitate trained staff or expensive equipment required for the quality control steps. Furthermore, the "MRAR only" rearing process benefits from simpler quality control requirements relative to that of an EP facility. The EP facility must maintain careful control to avoid inbreeding, which would affect mosquito fitness. In contrast, a MRAR facility can disregard this concern and optionally combine multiple generations into a single rearing pan if wanted. Inbreeding is not a concern with an output that is all males, and the autocidal efficacy will not be diminished if multigenerational cohorts are released simultaneously.

For the bifurcated EP/MRAR model to work, a method for egg storage and shipment is required. Egg storage and shipment methodology exists for many important mosquito species, including *Culex*, *Anopheles*, and other mosquitoes. The eggs of *Aedes aegypti* (L.) (yellow fever mosquito) and *Ae. albopictus* (Skuse) (Asian tiger mosquito) can be desiccated and stored for long periods (Kuno 2010, Mayilsamy 2019, Martinez-Garcia et al. 2021). The EP/MRAR model also requires consistent methodology at the satellite MRAR facilities, to provide reliable quality of the resulting males. Furthermore, an effective EP/MRAR model will require good communication and data sharing between the EP and MRAR facilities, to detect and identify problems and maintain optimal quality control.

Here we report a test of the bifurcated EP/MRAR model. Specifically, a commercially operated mass rearing facility in Kentucky acts as the EP facility and simultaneously provides *Ae. aegypti* or *Ae. albopictus* eggs to 4 satellite MRAR facilities in California and Florida. Cloud-based software was used to track and compare rearing that occurred concurrently in Kentucky, California, and Florida. Monitored parameters included environmental conditions, egg hatch rates, development time, survival, and the resulting sex ratio.

MATERIALS AND METHODS

The mosquito strains used in the study were wild-type *Ae. albopictus* and *Ae. aegypti*. The *Ae. albopictus* colony (ALB) was established at the MosquitoMate mass rearing facility (MM), using eggs that were field collected at Lexington, KY, in 2021. The *Ae. aegypti* colony (GYP) was established from eggs that were field collected in Monroe County, FL, in 2020 and generously provided by the Florida Keys Mosquito Control District. The ALB eggs were reared at MM and the Anastasia Mosquito Control District (AM). The GYP eggs were reared at MM, Greater Los Angeles County Vector Control District (GL), Orange County Mosquito and Vector Control District (OC), and Coachella Valley Mosquito and Vector Control District (CV).

A total of 6 replicate ALB and 12 replicate GYP groups were made. The eggs and larval diet were supplied by MM and shipped to the satellite rearing locations. Eggs were shipped overnight from MM to each remote location in Styrofoam insulated boxes, and in each case, the eggs arrived within 24 h of shipment. The environmental conditions experienced by the eggs were monitored during shipment using HOBO units (HOBO MX100; Onset, Bourne, MA), and in all cases, the temperature remained between 15°C and 30°C during shipment.

"Food capsules" were made at MM and provided to all facilities for daily feeding of mosquito larvae. Food capsules consisted of 500 mg liver powder (MB Biomedicals, San Diego, CA) in a cellulose capsule (Size 00 Vegetarian Capsules; Herb Affair, Chicago, IL). The schedule and number of capsules was predetermined as a set schedule. Target rearing conditions for all trials were: room conditions of 27 ± 1°C and 65 ± 5% RH; and larval water temperature of 24 ± 1°C.

As a nonshipped control, 3 ALB and 3 GYP pans were reared at MM, identically to the satellite rearing

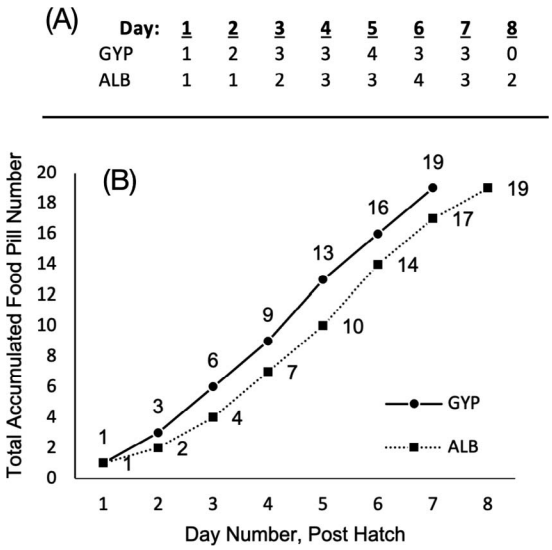

Fig. 2. Feeding schedule for the *Aedes aegypti* (GYP) and *Ae. albopictus* (ALB) pans. Food capsules were added daily, based on species and larval development. (A) Number of food capsules added daily. (B) Cumulative number of food capsules added.

sites. For consistency among the different sites, the shared protocol was based on a predefined schedule and 24-h interval timing. Specifically, all work would be conducted beginning at 24-h intervals from the initial hatch time. While facilities in different time zones could operate at times that differed from each other, within a facility, all work occurred at multiples of 24 h from the hatch time, e.g., a lab that hatched eggs at 11 a.m. local time would subsequently feed pans and do additional work beginning at 11 a.m. on subsequent days.

For the experiment, approximately 7,500 ALB eggs or 6,500 GYP eggs were hatched by submersion in polycarbonate larval pans (Cambro#DB18263CW148 18 × 26 × 3 inch; Webstaurant, Lititz, PA) filled with 4 liters of tap water (approximately 2.5 cm deep) and a food capsule. Egg number was estimated based on weight, i.e., 56 mg ALB eggs and 57 mg GYP eggs. Three pans were established at each satellite site and 6 pans (3 ALB and 3 GYP) at the MM site.

For ALB eggs, eggs were hatched at MM, GL, OC, and CV on the same day. Each site varied slightly with vertical pan placement on racks or shelving, tools used for daily pan temperature readings, and light/dark cycle settings for immature rearing. Due to evaporation, low water levels were observed at two of the insectary sites (OC, CV), and tap water was added to those pans.

Food capsules were added to the GYP and ALB pans using a predetermined sequence shown in Fig. 2. Water and room temperatures were monitored daily using a combination of thermometers (e.g., Fisher#13-201-556; Waltham, MA) and HOBO probes (HOBO MX2303; Onset), and larvae were examined daily for growth and mortality.

On day 9 and day 8 for ALB and GYP, respectively, the immature mosquitoes were poured through a mesh screen sieve (#32 Lumite, 7250Q; Bioquip, Compton, CA), rinsed, and then transferred into a plastic container (#1286064; Webstaurant) holding approximately 750 ml water. The container was placed inside a cage to contain the eclosing adults. On day 11 and day 10 for ALB and GYP, respectively, the predefined protocol was to remove containers from the cages and then freeze the cages to kill the mosquitoes. Subsequently, the dead mosquitoes were sorted and counted by sex. Throughout the experiment, data at each of the facilities were collected using the proprietary TRACKER software (MosquitoMate, Lexington, KY). Statistical analyses consisted of Student's *t*-tests and ANOVAs were performed using JMP 16.2 software (SAS Institute, Cary, NC).

RESULTS

Prior to egg shipment, a subsample of eggs was hatched at the MM site, resulting in 78.7% and 90.6% hatch for ALB and GYP eggs, respectively. Following shipment, egg hatch was measured again at both the MM and satellite facilities. For the ALB line, no significant difference ($t = 2.1$, df = 5, $P > 0.06$) was detected between the two sites, with an average egg hatch of 73.2% and 79.3% at the MM and AM locations, respectively. For the GYP line, similar hatch rates were observed at three of the sites, with GL reporting a lower hatch, $F = 6.1$; df = 3, 8; $P < 0.02$. Specifically: MM, 91.6%; OC, 92.8%; GL, 86.1%; and CV, 93.0% hatch rates were recorded.

As shown in Fig. 2, the ALB and GYP larvae received a predetermined, consistent feeding pattern at each of the rearing locations. The GYP larvae developed faster than ALB and were fed at an accelerated pace relative to ALB larvae (Fig. 2). Room conditions targeted a water temperature of 24°C and were generally consistent, with less than 2°C variation at all the rearing sites (Fig. 3). At each site, there was some temperature variation between pans. Pans positioned lower on the rack tended to be slightly cooler than the higher pans, resulting in slightly slower pupation rates.

Immature developmental rate was estimated by observing rearing pans, with key observations including the onset of pupation. For the ALB pans, pupation was observed at MM and AM on day 7 and day 8, respectively. For the GYP pans, the GL site reported accelerated development, with observations of pupation on day 6. The remaining 3 GYP sites observed pupation on day 7.

Approximately 2,000 males reached the adult stage from each ALB pan, with one exceptional AM pan producing 1,368 males (Fig. 4). Because female mosquitoes develop slower and because the cages were frozen before all pupae had eclosed, all

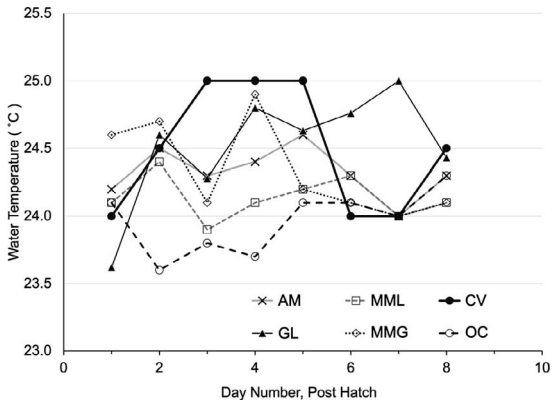

Fig. 3. Rearing pan water temperature was recorded daily at the 6 rearing sites: Anastasia Mosquito Control District (AM), Greater Los Angeles County Vector Control District (GL), Orange County Mosquito and Vector Control District (OC), Coachella Valley Mosquito and Vector Control District (CV), MosquitoMate *Aedes aegypti* (MMG), and MosquitoMate *Ae. albopictus* (MML).

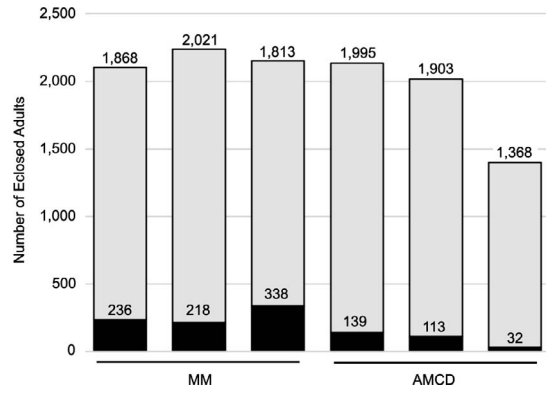

Fig. 4. The number of female (black) and male (gray) *Aedes albopictus* adults counted after freezing the cages on day 11.

cages were male biased. The percent ALB males differed between the AM (95.2% male) and MM (87.8% male) locations, $t = 3.4$, $df = 5$, $P < 0.02$.

A majority of the GYP pans produced 2,000 males or more (Fig. 5). Only 2 pans produced fewer than 1,950 males, with one each at MM and CV. As with the ALB cages, all GYP cages were male biased due to the protocol of freezing prior to full eclosion. The percentage of GYP males was not observed to differ significantly ($F = 1.4$; $df = 3, 8$; $P > 0.3$) among the 4 sites, with 65.9%, 60.3%, 66.7%, and 62.4% male at the CV, GL, MM, and OC locations, respectively. Because females develop slower than males, those pans with the most adults also had a greater proportion of females (Fig. 6).

DISCUSSION

For both *Ae. albopictus* and *Ae. aegypti*, the comparison of the local and satellite rearing sites demonstrate that the male quantities resulting at the satellite sites are no lower than those reared locally, and that this was consistently observed despite different people operating with different equipment at different rearing sites. Specifically, despite the shipment of eggs to satellite rearing facilities, there was no observed reduction in the number of males produced remotely. Observations of developmental timing were generally consistent between facilities.

The experimental design of a strict 24-h interval timing was chosen to maximize the comparability among the satellite sites that were operating in different time zones. Had the goal been to maximize production, then each site would have been allowed to readjust timing and allow for additional male eclosion. For example, the highest GYP adult numbers were observed at the GL site, which had the highest temperatures during the later immature developmental period (Fig. 3).

While this study demonstrates comparable male quantity between the satellite MRAR facilities, future studies examining this model should also focus on male quality. Several assays have been developed to examine male quality, including male flight ability, size, longevity, and mating competitiveness (Vreysen et al. 2021). Presumably, immature mosquitoes that are reared identically should result in similar quality male adults, and no detrimental effects would be expected, even if there is egg stress during shipment. However, egg mortality that occurs during shipment (O'Neill et al. 2018, Denton et al. 2022) can result in different densities of larvae in pans, which can affect the quality of male adults. While no egg mortality attributable to shipping was observed in this study, additional downstream work can examine extended shipping durations and additional conditions, e.g., temperature extremes.

Importantly, import and export regulations must be considered in relation to the shipment of live mosquitoes and the bifurcated EP/MRAR model. In the USA, there are no current restrictions or permits required for the interstate shipment of lab-reared mosquitoes, provided they have not been exposed to pathogens. However, international shipment or local shipment in some countries do require permitting, which could affect the EP/MRAR model and should be considered in selecting an appropriate mass production model (Denton et al. 2022).

A model based on satellite rearing sites requires good communication and tools for data sharing. In this experiment, we relied on the proprietary, cloud-based TRACKER software to record feeding, monitor rearing pans, and record adult male and female numbers. Not only is this software useful for monitoring quality control parameters, it can also be used to capture critical data required for regulated technologies. For example, *Wolbachia* technology in the USA is regulated by the Environmental Protection Agency, and accurate record keeping is required

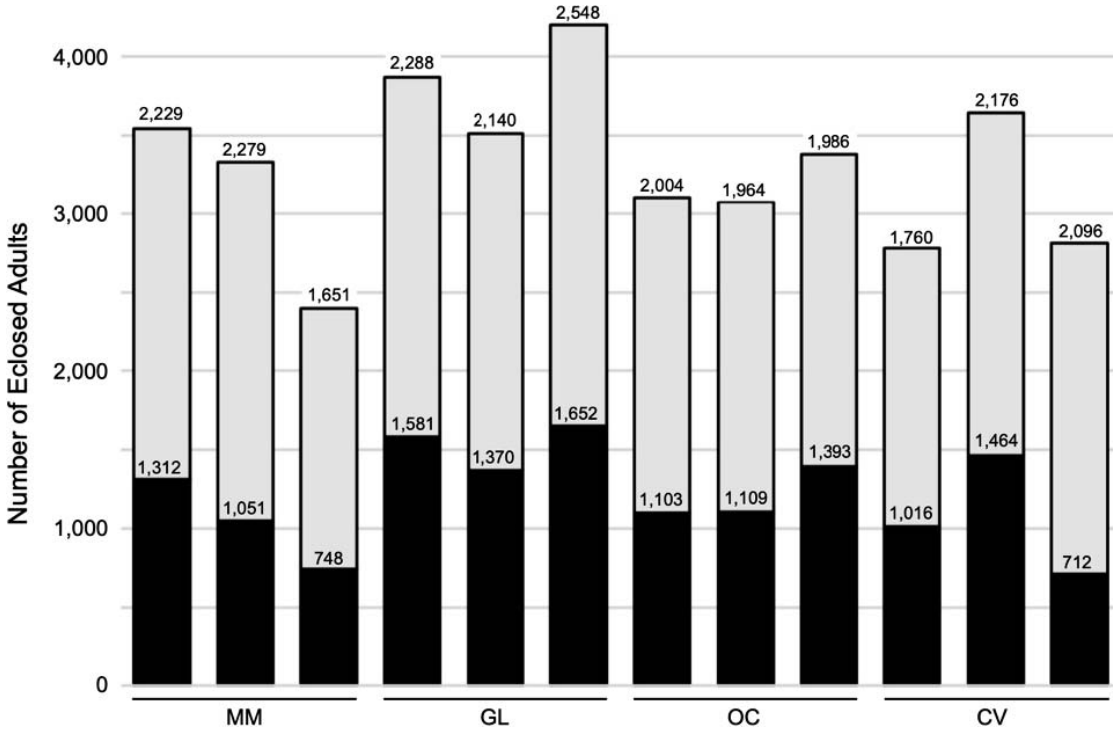

Fig. 5. The number of female (black) and male (gray) *Aedes aegypti* adults counted after freezing the cages on day 10.

similar to other regulated pesticides. Production "batches" of males are recorded, and reports to federal and state authorities are required. Therefore, an effective tool for tracking production, quality control, communication, and reporting is critical, especially when operations include multiple parties, as required by the EP/MRAR model.

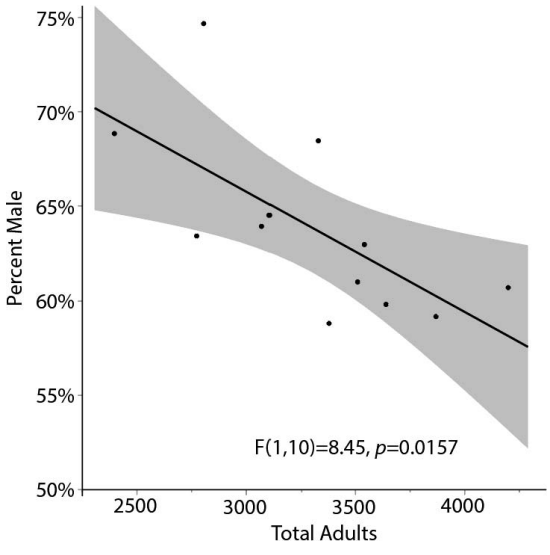

Fig. 6. An example of the correlation between percent male and total number of eclosed adults. Those pans with the most eclosion (i.e., due to greater time and/or higher temperatures) resulted in more female adults prior to experiment termination by freezing.

In conclusion, the initial trials support the potential utilization of the EP/MRAR model by demonstrating consistent quantities for both *Ae. aegypti* and *Ae. albopictus* in multiple, remotely located laboratories. Additional studies would be useful to further examine the model through the incorporation of additional fitness comparisons (e.g., size, mating competitiveness), and the deployment of satellite reared males in field trials to suppress mosquito populations.

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