

## Review Article

# Lessons from the Mass Production of *Wolbachia*-infected *Aedes aegypti* for Egg Release in the Sleman and Bantul Districts of Yogyakarta

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### ABSTRACT

An efficacy study on *wMel* *Wolbachia*-infected *Aedes aegypti* technology conducted by the World Mosquito Program (WMP) Yogyakarta showed the reducing of dengue incidence in Yogyakarta City. Following this successful result, the intervention was scaled up into two neighbouring districts: Sleman and Bantul. This paper describes our experience in mass production for providing release material for a larger area to reach the deployment target, which includes insectary requirements, mass production protocols, and diagnostic screening. This review may serve as a reference guidance for national mass production for *wMel* *Wolbachia*-infected *Ae. aegypti*.

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### INTRODUCTION

Dengue is still a major public health problem in Indonesia. In the absence of an effective dengue vaccination program, strategies for dengue elimination have relied on vector controls, such as eradication of preadult and adult vectors, chemical control by larvicide and insecticide, and biological control by predators (World Health Organization 2009). The *Wolbachia*-infected *Aedes aegypti* technology introduced by the World Mosquito Program (WMP) is a novel dengue vector control method that complements the existing vector control methods by blocking effects on DENV replication in the vector to decrease the ability to transmit dengue. In addition, *Wolbachia* is maternally inherited through the insect egg cytoplasm and therefore will be sustained once after it is established in the natural habitat of *Ae. aegypti*. The WMP project started with a field site in Townsville, Australia, and to date this project has successfully expanded to more than ten countries (Segoli et al. 2014; O'Neill 2018).

The World Mosquito Program (WMP) Yogyakarta started the

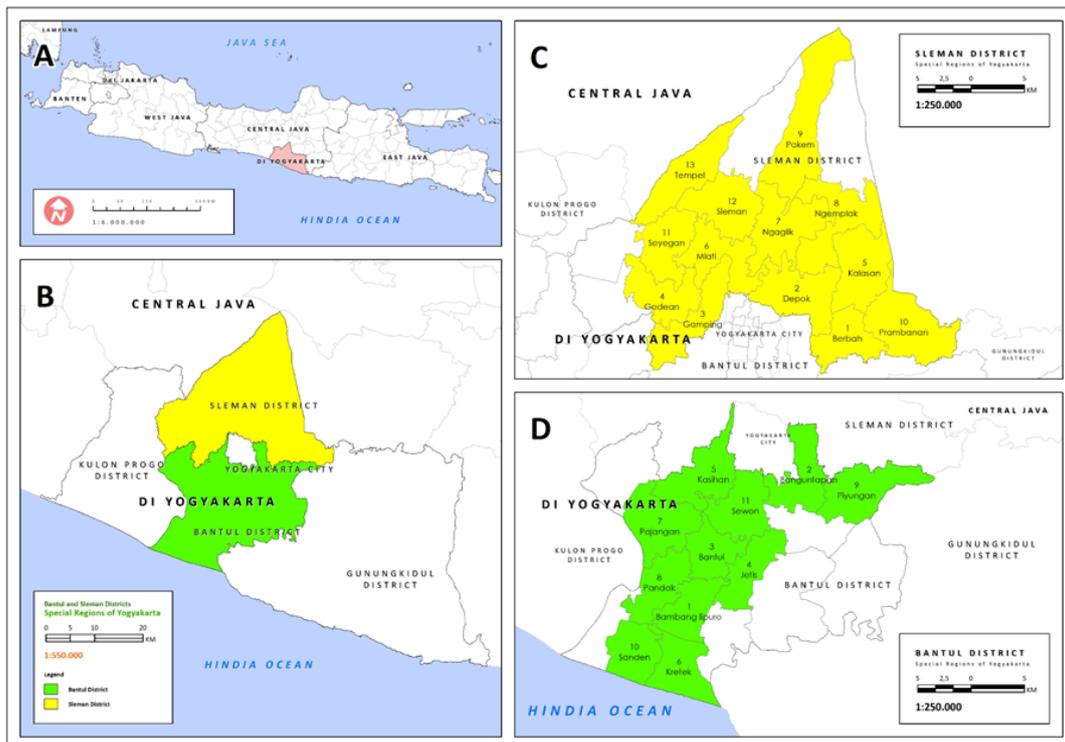
project in 2011. Small-scale releases at the subvillage level, which were conducted in 2014 in Sleman and Bantul Districts, showed the successful establishment of the Wolbachia *wMel* strain in the natural *Ae. aegypti* population (Tantowijoyo et al. 2020). Further efficacy studies in a larger area in Yogyakarta City also showed a decline in dengue incidence by 76-77% and the decline in the rate of hospitalization due to dengue infection by 86% (Indriani et al. 2020; Utarini et al. 2021). Following these promising results, WMP Yogyakarta scaled up the technology to district-wide level in neighbouring districts with the highest reported dengue cases. The strategy of mosquito production shifted from small scale to mass production for increasing the release material supply.

This review describes *wMel* Wolbachia-infected *Aedes aegypti* mass production based on our experience to supply the requirement for egg release materials for wider areas in Sleman and Bantul Districts. This protocol can serve as a reference guide for other institutions involved in nationwide Wolbachia implementation.

## PROTOCOL

### Target production based on release area requirement

The size of the target areas for implementation defines the plan for mosquito production. Sleman and Bantul District are the part of Yogyakarta Special Region (Daerah Istimewa Yogyakarta, DI Yogyakarta; Figure 1A). Sleman District is located at 110° 33' 00" east longitude and 7° 34' 51" and 7° 47' 30" south latitude. It has a total area of 574.8 km<sup>2</sup> and is divided into 17 subdistricts (Figure 1B). There were 13 subdistricts out of the existing 17 subdistricts targeted for release (Figure 1C) due to the high burden of dengue transmission that contributed to 80% of dengue cases in 2017-2019 (official communication with Sleman Health District Office). This area was divided into two release areas: 254.3 km<sup>2</sup> as the administrative area and 122.94 km<sup>2</sup> as the residential area. By releasing

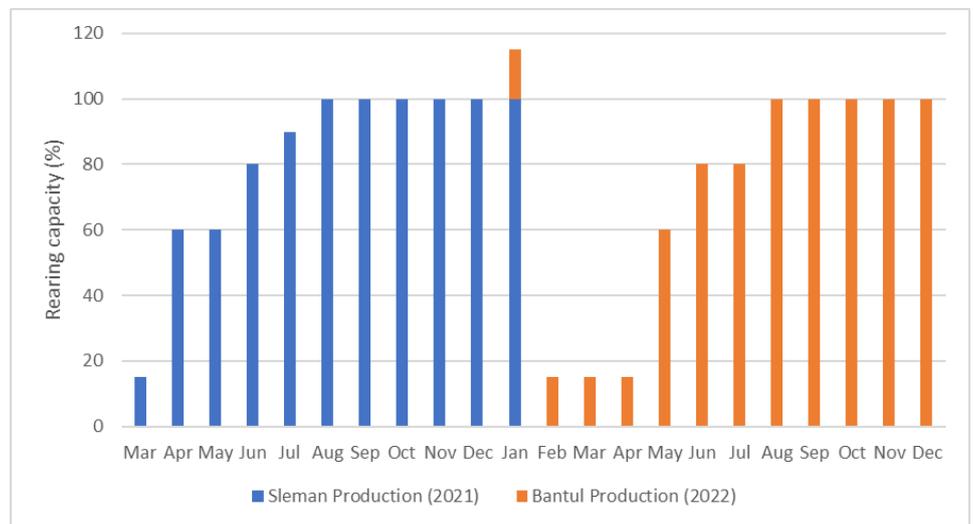


**Figure 1.** Release sites. A. Map of Yogyakarta Special Region (pink), B. Sleman and Bantul District, C and D. The subdistricts of Sleman District and Bantul District those were chosen for release sites (yellow and green, respectively).

Wolbachia-infected *Ae. aegypti* eggs in egg release containers (ERCs) in residential and public spaces, these areas were provided with 22.322 buckets of ERC within 75x75 m grid squares. A total production of 3 million eggs/per week was required for release, and the release was conducted in approximately 12 biweekly rounds over a six-month period with a target of Wolbachia frequency of over 60% by the end of release period.

Bantul District is located between 110° 12' 34" and 110° 31' 08" east longitude and 7° 44' 04" - 8° 00' 27" south latitude. It has a total area of 506.85 km<sup>2</sup> (Figure 1B) and is divided into 17 subdistricts. There were 11 subdistricts out of the existing 17 subdistricts targeted for release (Figure 1D) due to the high burden of dengue transmission that contributed to 75% of dengue cases in 2017-2019 (official communication with Bantul Health District Office). The release area has 254.37 km<sup>2</sup> of administrative area and 75.64 km<sup>2</sup> of residential area with a total human population of 985.770 people. Using the 75x75 m grid area as the basis, a total of 19.117 ERCs were needed, however the number was increased to a total of 24.462 ERCs to address challenges in the field. A total of 4 million eggs/week was produced to accomplish the requirement for the Bantul release area.

The rearing process for Sleman started in January 2021 but due to the COVID-19 situation, the release was delayed until May 2021. In November 2021, we began to prepare the colony for the Bantul area, and the release commenced in May 2022. We gradually set up approximately 130-140 cages to supply the target for the Sleman area and approximately 160-170 cages for Bantul. There was an overlapping time when we maintained two colonies in the last Sleman release and the beginning of Bantul release (Figure 2).



**Figure 2.** Mass production was maintained gradually to reach the maximum capacity of 100%, depending on the release target. There was an overlapping time for maintaining targets in both areas in January 2022.

### Insectary requirement

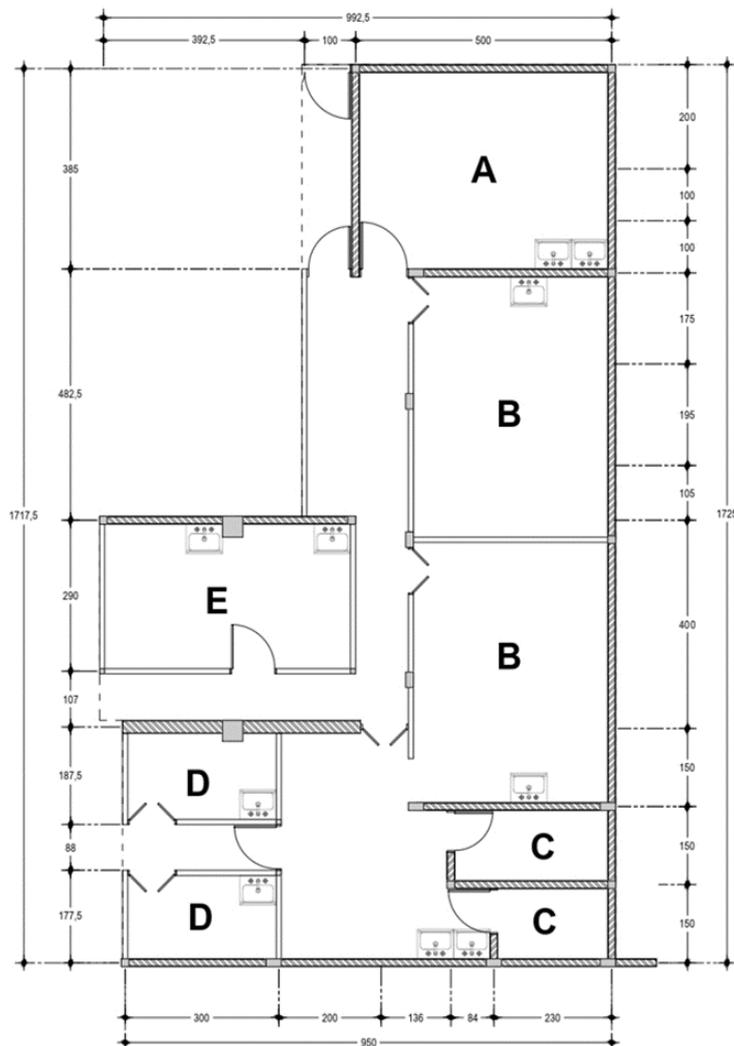
#### Temperature and humidity

The insectary is located in Sleman District, Yogyakarta, which has a suitable temperature range for *Ae. aegypti*. Sleman District recorded average temperature of 27.7 and 27.8 °C, relative humidity of 74.1 and 77.9%, and rainfall of 106.2 and 275.1 mm during the dry season and wet season, respectively. In the insectary, mosquitoes were maintained at 25.5-26.5 °C and 50-70% relative humidity, which are the optimal conditions for *Ae. aegypti* development (Ross et al. 2017). Temperature can influence

the speed of larval development, hatch rate, and even pupal sex ratio (Mohammed & Chadee 2011; Imam et al. 2014). In the temperature range of 28°-34 °C, *Ae. aegypti* can have a better hatching rate rather than above this range. At 40 °C, no eggs will hatch, and there is high larval-pupal mortality (Sukiato et al. 2019). Wolbachia infections from mosquito colonies may be lost due to high temperatures, hence this should be prevented (Ross et al. 2017).

### Room Facility

The insectary consisted of at least five rooms prepared for the preadult rearing room, adult colony room, wild-type colony and extra room, a storage room for equipment and consumables, and a storage room for egg and egg strip release preparation. The preadult rearing room was designed in a humid condition with a relatively natural photoperiod made by a half shade of the roof, including blower air flow for 24 hours. The egg storage room was maintained at an air-conditioned laboratory temperature of 25 °C. The insectary design is shown in Figure 3.



**Figure 3.** The insectary consists of five main rooms (1:100 cm); A. Egg storage room, B. Preadult rearing room, C. Storage room for equipment and rearing consumables, D. Wild-type colony and extra room, and E. Adult colony room.

### Tools and Consumables

The tools for preadult rearing consisted of pipettes, small spoons, buckets of 18,5 cm in diameter and 16 cm in height and cups for pupal emergence containers. During mass production, a total of approximately 450

buckets were used. Tools for adult maintenance were customized cages of 30 x 30 x 30 cm in size muslin cloth and stainless steel frame and that had a, modified sugar cups, and modified oviposition cup (ovicup) for harvesting eggs. The 2 ml tubes and modified cups for screening diagnostic sample collection were used. The list of consumables consisted of Tetramin Tropical Flakes, Tetra Holding (U.S.) Inc., Germany for larvae food, tap water, 10% sugar solution, and 80% ethanol solution for the samples. Detailed pictures of the tools and consumables are shown in Figure 4.



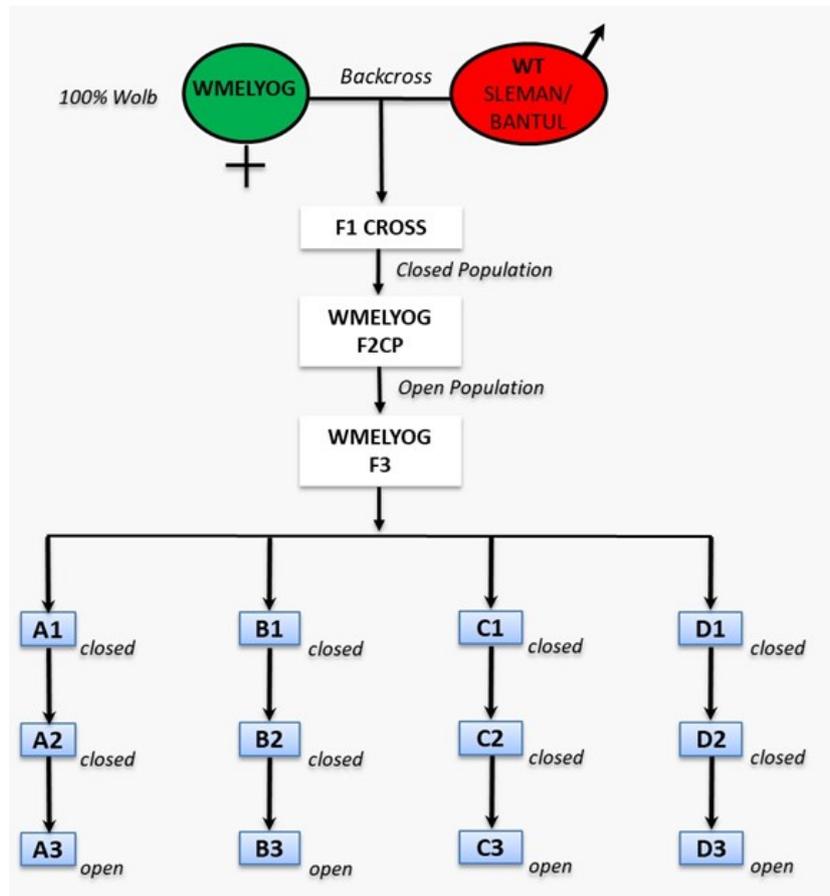
**Figure 4.** A. Tools and consumables for rearing preadult stage, B. Tray to slow-dry eggs with a cloth, C. Emergence cup for the pupae, D. Container ovicup with the strips, E. Modified sugar cup, F. Customized cage, and G. Bucket for larval rearing.

## Production Methods

### Colony preparation (backcrossing-outcrossing)

WMELYOG was the colony of *wMel*-infected *Ae. aegypti* previously used during the Applying Wolbachia to Eliminate Dengue (AWED) trial in the Yogyakarta City (Utarini et al. 2021). The *wMel* Wolbachia-infected *Ae. aegypti* colony mass reared in Yogyakarta was maintained as an open (outcrossing) and closed population at different times. We started the production with backcrossing by mating female *wMel* Wolbachia-infected *Ae. aegypti* with local wild-male *Ae. aegypti* and then continued it with outcrossing. Backcrossing was conducted once with the wild-type males from the Sleman and Bantul populations, and outcrossing was continued once every two cohorts generation colony maintenance to refresh the ge-

netic pool. We performed outcrossing (open colony) by adding 10-20% wild-type males to each successive generation (O'Neill et al. 2019; Garcia et al. 2019). Colony preparation activity was started by placing ovitraps to obtain wild-type *Ae. aegypti* eggs. Ovitraping was performed periodically for one month in Bantul and Sleman in each of the 10 locations. After one week, eggs were collected from the flannel strips in ovitraps, then air-dried slowly for one or two days and pooled until sufficient eggs were acquired. Eggs were hatched and then reared up to the stage of 3<sup>rd</sup> or 4<sup>th</sup> stage of instar larvae to identify *Ae. aegypti* individuals, and these were separated from *Aedes albopictus* individuals, which were sometimes trapped in the same ovitraps. The eggs were further reared to obtain wild-type colonies, which were pooled into three cages for each target area (Dieng et al. 2012; Tantowijoyo et al. 2016) Adult *Ae. aegypti* was set up as a wild-type colony from the release target area. The harvested eggs (F1) were pooled for backcross and outcross material until eight gonotrophic cycles. Eggs were then transferred to a sealed container with a salt solution (2:1) and stored at air-conditioned laboratory temperature (25 °C±2 °C). Wild-type colonies were maintained up to the F2 generation. A simplified version of the flow of the rearing plan is shown in Figure 5.



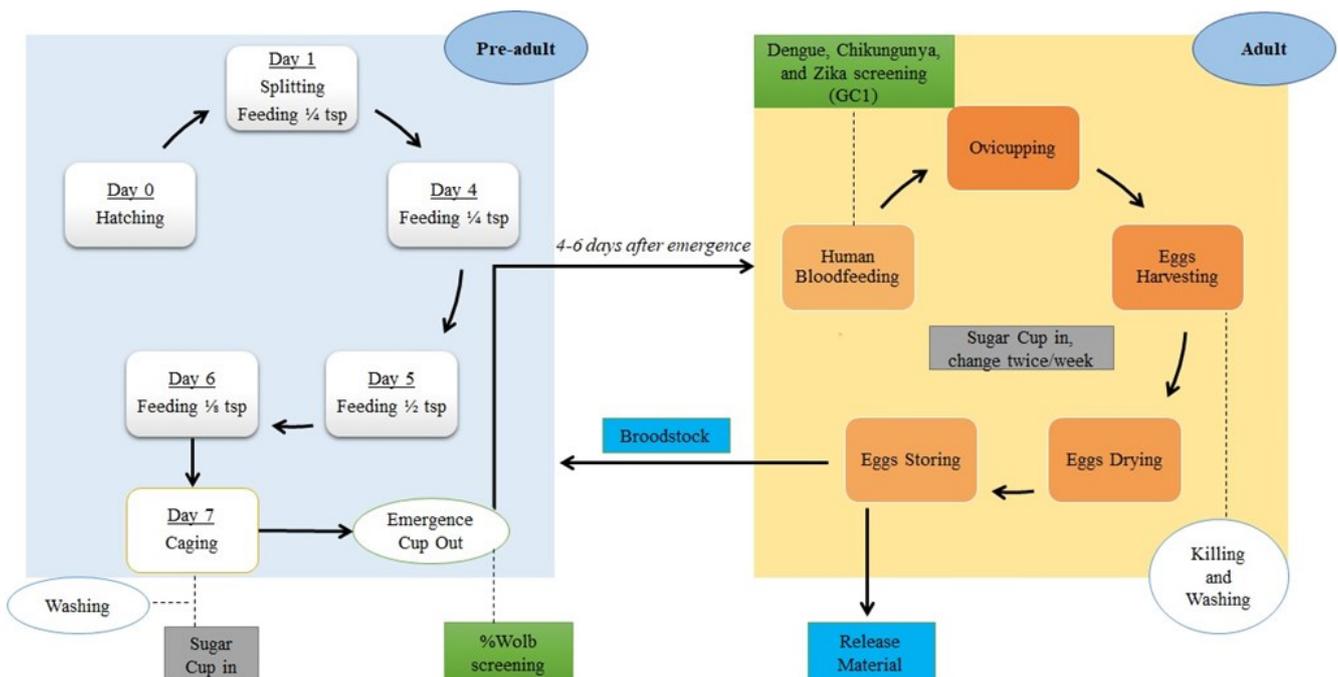
**Figure 5.** Backcrossing and outcrossing of WMELYOG. Female *wMel* Wolbachia-infected *Ae. aegypti* (WMELYOG) were backcrossed to male wild-type mosquitoes derived from the field (Sleman or Bantul). After obtaining the F1 generation (F1 cross), a closed population was generated (WMELYOG F2CP). To obtain a similar genetic background to that of a natural population, outcrosses were performed by adding uninfected males from the field. The colonies (WMELYOG F3) were then divided into four cohorts of ‘anakan’ (brood stock) colonies (A, B, C, and D), and outcrossing was repeated for three consecutive generations. The screening for Wolbachia frequency was conducted in every outcrossing colony, with a threshold of 100% positivity. CP = closed population; WT = wild-type.

### Production Methods for Mass Rearing

Production methods for mass rearing were divided into preadult and adult rearing. Every time hatching was performed, the step continued with three gonotrophic cycles (GC). The whole rearing phase can take one month in length of time. This method is shown in Figure 6.

Every broodstock had approximately 100 flannel strips, each containing around 3000 eggs per strip of *wMel* Wolbachia-infected *Ae. aegypti* and continued to be reared in the preadult step. Preadult rearing room was conditioned with humid temperatures although temperature was not measured regularly. The larvae were reared in the rack trolley system for efficiency. Temperature, density, and nutrition are key factors in larval growth (Imam et al. 2014). The eggs were hatched (Day 0) using yeast solution that was made by adding 0.20 g baker's yeast into 1 L water for early nutrition for the larvae, the larvae were separated on Day 1 to a density of approximately 500-600 individuals per rearing bucket. The best practice, which is also convenient, was hatching the eggs on Thursday, separating the larvae on Friday, and continuing with preadult rearing can be continued on Monday. The larvae were fed immediately after being separated with ¼ teaspoon (tsp) of grounded Tetramin Tropical Flakes to ensure that food was always available during the weekend (Day 2-Day 3). Larval development and rearing water conditions were then monitored every day. The larvae were fed with ¼ teaspoon (tsp) of ground larvae food on Day 4 and ½ teaspoon on Day 5. The feeding time was scheduled and stirred with a zig-zag pattern; this allowed the food to sink as *Aedes aegypti* was the bottom feeder (Kinney et al. 2014).

On Day 7, larval density will reach approximately 500-600 larvae in 1.2 liters tap water per bucket (approximately 80% pupation occurred). All pupae and remaining larvae of each 2-2.5 rearing buckets were sieved and placed in the emergence cups that were filled with clean water. A pinch of food was added to the cups to feed the remaining larvae. Each emergence cup was then transferred into a cage that was clearly labelled with colony type, generation, and the date when the pupae were placed in the cage.



**Figure 6.** Activity flows in colony rearing. The steps are the preadult rearing stage, continuing with the adult stage with the human blood feeding method, and egg harvesting until the 3<sup>rd</sup> gonotrophic cycle (GC3). Tsp = teaspoon.

After reaching the pupal stage, the rearing adult stage began. According to the requirement of material release for Sleman and Bantul, for adult rearing need in a total of 130-170 cages were needed with a capacity of  $\pm$  1000 - 1250 adult mosquitoes in each cage. The adult colony was maintained at an average room temperature. Each colony cage was provided with 10% sugar solution *ad libitum* prepared by soaking cotton balls in a small plastic bowl filled with 1 tablespoon of sugar solution that was changed twice a week. On Days 4-6 after pupae emerged into adults, the females had access to a blood source using a human blood feeding method. Blood feeding was conducted once a week for three gonotrophic cycles by allowing the females to feed for 15-20 minutes on the limb or leg of a healthy volunteer. Exclusionary criteria for the volunteers was not having signs and symptoms of arboviral infection (fever, myalgia, headache, etc.) and not being acutely proven to be infected by arboviral infection (as diagnosed by registered clinicians or by laboratory results). Other exclusionary criteria were (1) taking any antibiotic, (2) a history of allergic reactions to insect bites, and (3) not providing consent before giving blood-meal. Approval for human blood-feeding of mosquito colonies, including field release of mosquitoes, was provided by the Medical-Health Research Ethical Committee, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, with the reference number of KEI0611112011, KE/FK/818/EC, and KE/FK/1274/EC/2021.

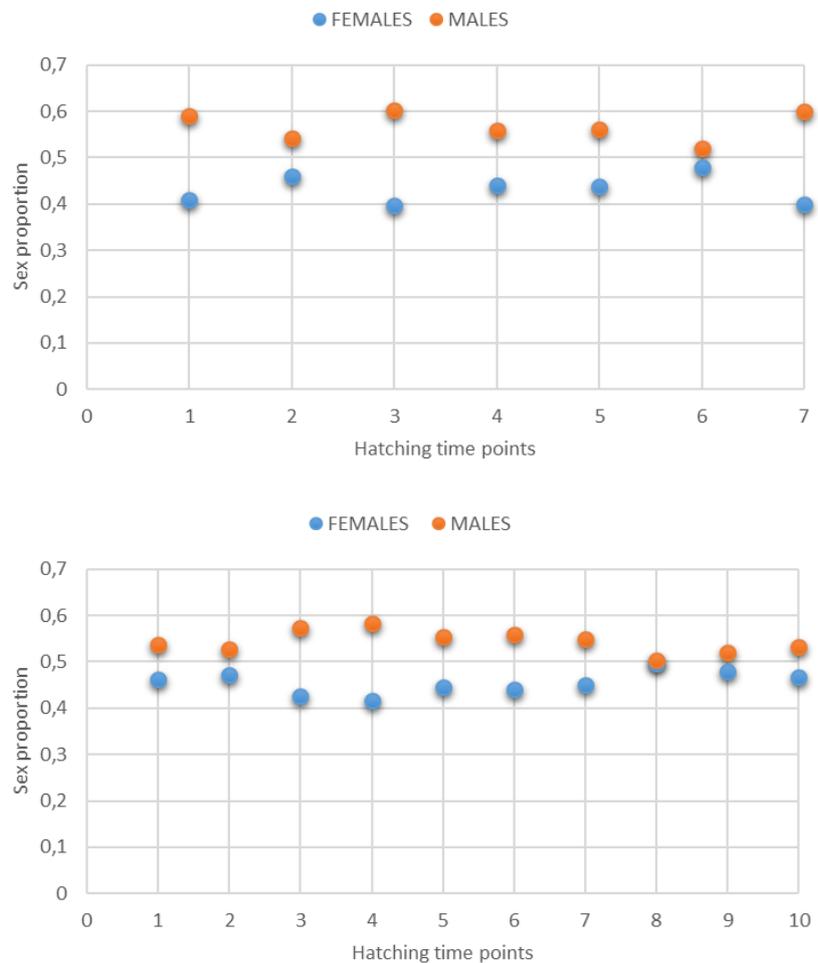
In general, there are many techniques to give blood meals for maintaining female *Ae. aegypti*, including membrane feeding devices (Carvalho et al. 2014) and confined animals (Day & Edman 1984). However, according to several studies, mosquitoes with experimental Wolbachia infections frequently demonstrate poor performance on nonhuman blood, may have lower hatch rates, and may only partially transmit Wolbachia to their progeny (McMeniman et al. 2011; Caragata et al. 2014; Suh et al. 2016).

#### Ratio of Female and Male

The ratio of females and males per cage was taken into account when calculating the insectary necessity and evaluation. We determined that there were always fewer females than males in each rearing bucket. The number of female was substantial due to their fecundity in egg harvesting. In Sleman and Bantul, the female ratio reached 40-50% (Figure 7). The ratio may predict the production expectation. Egg production from each female may vary. In the field, each female can lay between 20-60 eggs, but in the insectary, it can reach up to 100-150 eggs (Clemons et al. 2010; Arévalo-Cortés et al. 2022). Based on our data, each female can produce approximately 70 eggs in the first gonotrophic cycle (GC1), and it will drop in the 3<sup>rd</sup> gonotrophic cycle (GC3) to up to 50 eggs (unpublished result).

#### Egg Harvesting and Production Capacity

*Ae. aegypti* has a strong interest in human blood. Due to this reason and the quality of the eggs, we allowed one-week-old adult colony to be blood-fed by human volunteers once a week (Gunathilaka et al. 2017; Al-Rashidi et al. 2022; Arévalo-Cortés et al. 2022). One cage was only blood fed by one person. After allowing 2-3 days for females to develop their eggs prior to oviposition, the modified ovicup (Figure 4D) was put into the cage. Each modified ovicup had five compartments. By adding wet flannel strips (ovistrips) and pouring water approximately 0.5 cm deep in each compartment, females were allowed to lay the eggs on the flannel. After two days of leaving the ovicup in the cage, harvesting was per-



**Figure 7.** The ratio of female and male *Aedes aegypti* per cage in the insectary to supply release implementation in Sleman (upper) and Bantul (lower).

formed by removing the ovicup from the cage to collect the eggs. The ovistrips were then put into a tray with a dry cloth underneath to remove excess water. Any dead mosquitoes that may be stuck on the ovistrip were removed to minimize fungal growth. The eggs were dried slowly overnight. On the following day, the eggs were transferred to a sealed plastic container with saturated solutions of NaCl (2:1) to maintain humidity at ~75% and stored at an air-conditioned laboratory temperature of approximately 25°C. From all three gonotrophic cycles in each cohort, usually in the 2<sup>nd</sup> and 3<sup>rd</sup> GC, egg production was less than that in the first cycle, so the ovistrip could be reduced from ten pieces of flannels (GC1) to only eight pieces for the 2<sup>nd</sup> GC and six pieces for the 3<sup>rd</sup> GC. This would make the egg clutch easy to observe and cut for the release strips.

We attempted to give a good quality egg to fulfil the release material needed. The newest harvested eggs were prepared to meet the need for release. After selecting the harvested eggs from 10-13 cages for the next parent colony of mass production (broodstock), the remaining eggs were used as egg release material. By using a visual reference that was obtained by counting the egg strips precisely under microscope, the ovistrips were cut into a small piece of between 150-200 eggs/strip for Sleman needs and 250-300 eggs/strip and 300-400 eggs/strip for Bantul. This step needed good practice among the staff. The egg strips were pooled in a large container and transferred to the field staff to be packed for release the following week. By our calculation, the capacity of our

**Table 1.** Production capacity to supply release material in Sleman and Bantul Areas.

Area	Sleman	Bantul
Target release area	126.8 km <sup>2</sup>	76 km <sup>2</sup>
Total cage	130-140	160-170
Density per cage	±1000	±1250
Female ratio	40%-50%	40%-50%
Egg production	GC 1= 3,166,000 – 3,410,000	GC1= 4,677,000 – 4,969,000
	GC2= 2,620,000 – 2,822,000	GC2= 3,870,000 – 4,112,000
	GC3= 2,080,000 – 2,240,000	GC3= 3,072,000 – 3,264,000

production in this implementation can be explained in the table below. It may become important to implement this technology in other areas.

### Diagnostics

During release material preparation, two diagnostic tests were performed, i.e., the Wolbachia frequency screening and arbovirus screening (for dengue, chikungunya, and zika infection). The wolbachia frequency test was performed by screening the samples with a PCR Taqman assay that included the WD0513 gene on a Roche LightCycler 480 while the screening for arboviruses was performed by qRT-PCR as previously described (Yeap et al. 2014; Quyen et al. 2017; Tantowijoyo et al. 2020).

The first Wolbachia rate screening had to be done after backcrossing offspring. This time, the colony had to reach 100% Wolbachia to start mass production. Routine Wolbachia frequency screening was conducted by collecting 100 female and 100 male mosquitoes with a threshold of 96%; if the result was <96%, close colony maintenance had to be performed (just by crossing the offspring against each other) and the screening had to be repeated. Routine arbovirus screening was conducted by sampling ten blood-fed female mosquitoes from each blood feeder of the 1<sup>st</sup> gonotrophic cycle (GC1). If the result corresponded to a positive result in the screening, all the blood-fed cages and their offspring were discontinued or destroyed. Even though one volunteer can feed more than one cage, it is recommended that four cages is the maximum per volunteer due to the drop in egg production when this number is exceeded. In our study, screening for arboviruses never gave positive results.

### CONCLUSION

Wolbachia-infected *Ae. aegypti* mass production plays important role to the success of Wolbachia technology implementation in wider areas. The standardized protocol and sufficiently wide and functional insectary ensure the quality of the entire process. It should be possible to maintain *Ae. aegypti* high fitness for open field releases by maintaining large population sizes, avoiding strong selective pressures through rearing methods, and regularly outcrossing to wild mosquitoes. The techniques do not need any specific tools, and they may be scaled up to produce more mosquito eggs for outdoor releases. For other advancements, particularly for the blood meal feeding system, the approaches are still adaptable. This protocol had successfully been adapted to support the two district-wide Wolbachia implementation programs in Sleman and Bantul, and might serve as a reference for other implementations in Indonesia.

## AUTHORS CONTRIBUTION

All authors reviewed and agreed upon the final manuscript. IF and IN contributed equally to study conceptualization, data collection, writing of the original draft and revision of the final manuscript. BA, DLC, IDU, and NAP contributed to the investigation, data collection, writing the original draft and revision of the final manuscript. US and ES contributed to the investigation and writing of the original draft. AU, RAA, and CI contributed to revision of the final manuscript. EA contributed to study conceptualization, investigation, and revision of the final manuscript. WT designed the research and supervised all the processes, writing of the original draft and revision the final manuscript.

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## CONFLICT OF INTEREST

The authors declare there is no conflicts of interest.

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