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Original Article

A Standardized Rearing Procedure for *Aedes aegypti* (Diptera: Culicidae): Implications for Laboratory-Based Vector Competence and Control Studies

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Abstract

Background: *Aedes aegypti* is a major vector of numerous arboviruses, including dengue, Zika, chikungunya and yellow fever. This study aimed to establish a standardized and practical procedure for maintaining and propagating an *Ae. aegypti* colony under controlled insectary conditions in Bandar Abbas, Iran.

Methods: Environmental parameters were routinely monitored. Larvae were reared with an appropriate diet under a regular feeding schedule, while adult mosquitoes were provided with a sucrose solution and blood meals. The correlation between the duration of egg paper drying and the hatching percentage was evaluated. Hatching rates were compared across three media: cooled boiled water, yeast and larval food.

Results: A strong negative correlation was observed between drying duration and hatching percentage, with a Pearson correlation of −0.818 ($p< 0.001$). Gradual drying of egg papers over a period of two days resulted in a hatching rate of over 90% without any hatching stimulant. No significant differences in hatching rates were found among the three media, as indicated by $F= 0.601$ ($p= 0.578$). In a novel observation, female mosquitoes were found to deposit approximately 20−30% of their eggs directly on the water surface inside ovitraps under insectary conditions in Iran.

Conclusion: This study provides a reliable, low-cost model for rearing *Ae. aegypti* in research insectaries and facilitates future studies on vector control.

Keywords: *Aedes aegypti*; Dengue fever; Insectary; Rearing; Iran

Introduction

Aedes aegypti is one of the most important vectors of human viral diseases, including dengue, Zika, chikungunya and yellow fever. The expansion of this species in recent decades, along with climate change, urbanization and human displacement, has significantly increased the need to manage and study its biology (1). The success of vector control programs, vaccine development, genetic studies and the evaluation of novel strategies such as the sterile insect technique (SIT) and the release of *Wolbachia*-infected strains depends on the availability of stable colonies and laboratory standards. The mass rearing of mosquitoes for control purposes may become as common as the mass production of insecticides in the near future (2). Understanding the population biology of mosquitoes is a prerequisite for their control, and effective control strategies require detailed knowledge of mosquito biology (3).

Recent research has demonstrated that optimizing environmental parameters-such as tem-

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perature, relative humidity, larval diet and photoperiod-can considerably improve breeding efficiency and the sustainability of laboratory colonies. Because insectary conditions and available resources vary across regions, the development and evaluation of locally adapted, cost-effective rearing techniques are crucial for successful mosquito rearing and subsequent experimental applications (2).

Although various protocols have been proposed for rearing *Ae. aegypti* (4–6), their practical application depends on the facilities, equipment and environmental conditions of each country; therefore, the quality and results of implementing the methods may not be the same in different countries. In the present study, we describe a practical and reproducible approach for rearing *Ae. aegypti* mosquitoes under controlled insectary conditions. Environmental and nutritional conditions were tailored to utilize locally available materials and infrastructure. We also determined the optimal dry period for maximum egg hatching and evaluated the egg hatching rate using three inexpensive and readily available media in the insectary. This method aims to enhance larval survival, boost fecundity and ensure colony stability for further research on vector control.

Materials and Methods

Insectary facility and building conditions

The insectary used in this study was established in a separate, access-controlled area to minimize environmental fluctuations, prevent the entry of contaminants, and reduce the risk of accidental mosquito escape. The facility was designed to regulate temperature, humidity and light conditions, creating a stable environment for maintaining *Ae. aegypti* colonies. The insectary was located separately from the staff building and was windowless.

The insectary consisted of designated areas for different developmental stages of the mosquito, including eggs, larvae and adults. This spatial separation was established to minimize cross-contamination, enhance colony management and improve environmental control during the rearing process. To prevent mosquito escape, the entrance to the insectary was equipped with two doors. Additionally, the ceiling was kept low to make it easier to capture any mosquitoes that might escape from the cages and fly freely within the insectary.

To ensure optimal conditions for mosquito development, the insectary was equipped with ventilation and environmental control systems. The room temperature was kept at approximately 27–28 °C, and the relative humidity was maintained between 65% and 75%. A photoperiod of 12 hours of light followed by 12 hours of darkness was implemented to simulate favorable biological conditions. These parameters were monitored and recorded daily to guarantee environmental stability throughout the rearing period.

The rearing room was equipped with a sink that provided hot and cold running water. Floor drains featured appropriate filters with a mesh size of 100 μm to prevent the accidental release of mosquitoes, particularly eggs, larvae and pupae. Shelves designed for holding larval trays and adult cages were constructed from stainless steel and aluminum. The bases of these shelves were placed in containers filled with water and positioned away from the walls. The interior walls of the insectary were painted in bright colors to help detect, recapture, or eliminate any mosquitoes that managed to escape from the cages.

Routine sanitation procedures were implemented to minimize microbial contamination and maintain the health of the colony. This included environmental cleaning, washing of equipment and proper waste management. All trays, containers and adult cages were thoroughly cleaned before use and disinfected when necessary. Access to the insectary was restricted to authorized personnel only, and all activities were conducted in accordance with biosafety precautions and measures to prevent any escapes.

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No visitors were permitted entry to the insectary. This policy aimed to minimize the risk of mosquitoes contracting pathogens from infected individuals. Staff members in the insectary were also screened to confirm they were free of mosquito-borne diseases.

The insectary was established in accordance with the guidelines for biosafety and biosecurity in mosquito rearing facilities, as published by the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA) (7). Overall, the physical and operational setup provided a stable and reproducible environment for establishing and maintaining *Ae. aegypti* colonies, while also supporting the evaluation of biological parameters under controlled conditions within the insectary.

Equipment required for the insectary

To rear adult *Ae. aegypti* mosquitoes, we constructed cages measuring 50 × 50 × 50 cm. The frames of these cages were made from 5-mm white plastic tubes, which were lined with a fine white mesh. Additionally, stainless steel and aluminum cages were utilized. Each cage featured a front panel with a wide sleeve opening for easy access. The bottom of the cage frame consists of a plastic or aluminum plate, allowing for the easy placement of ovitraps and pupal bowls at the bottom of the cage (Fig. 1A).

An ovitrap (a black plastic container with a 1.5 L volume, 12 cm in height, and 16 cm in diameter) and specialized paper were used to collect eggs laid by adult female mosquitoes inside the cage (Fig. 1B).

Larvae were reared in white plastic trays measuring 28× 37× 8 cm. The white color of the trays enhanced the visibility of both larvae and pupae (Fig. 1C).

Other equipment needed for the insectary: refrigerator, thermometer, hygrometer, timer for regulating light–dark cycles, 50 mL glass bottle for sugar solution, sugar, paper towels, electric kettle for boiling water, plastic pipettes, lidded plastic containers, 50–100 L bucket for storing dechlorinated water (filled with water and left uncovered for 24 hours to allow chlorine dissipation), aspirator, commercial fish food for larval feeding, special ovitrap paper, wash bottle, pupal bowls and net for larval trays.

Providing initial samples to start rearing in the insectary

To begin rearing in the insectary, male and female *Ae. aegypti* mosquitoes were collected using a human-baited bed net trap and an aspirator from different areas of Bandar Abbas city and transferred to an adult cage. A 10% sugar water solution was placed inside the cage, which was then carefully transferred to the insectary. Additionally, eggs were collected using ovitraps placed in the field and transferred to the insectary. After a two-day drying period, the eggs were submerged in water. Third-instar larvae were then identified using a valid morphological key (8). Once the pupae emerged, they were transferred to an adult cage. Adult mosquitoes were given a blood meal. The first generation was also identified to confirm that the species was *Ae. aegypti*, using a valid key (8).

To confirm the absence of dengue virus infection in the mosquitoes, we tested the first generation using the NS1 detection kit as a preliminary screening test. The SD Bioline NS1 Antigen Kit (Standards Diagnostic, Gyeonggi-do, Republic of Korea) was used for this purpose. We separated the thorax and abdomen of blood-fed adult mosquitoes. Then, we added 50 microliters of PBS to the abdomen, homogenized the mixture, and centrifuged it. The resulting supernatant was placed in the well of the test kit. After 15 minutes, we observed a single band in the control strip. This indicated that the colony tested negative for dengue virus infection (9). Also, some samples were sent to the Arbovirus Reference Laboratory, and RNA extraction and multiplex RT-PCR were done. Routine screening was maintained for subsequent generations. Once we confirmed the absence of infection in the mosquitoes, we began rearing *Ae. aegypti* in the insectary.

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Rearing of adult *Aedes aegypti* mosquitoes in an insectary

A 10% sugar solution was provided for the adult male and female mosquitoes to serve as a source of energy. The prepared sugar solution was poured into a sterile 50 mL glass bottle. A piece of paper towel was inserted into the bottle to serve as a wick. The bottle was then placed inside the adult cage. The sugar solution and paper towel were replaced every other day, and the bottle was thoroughly washed with hot water before being refilled with fresh sugar solution.

Female mosquitoes were ready for blood feeding 24–48 hours after emerging from the pupal stage. In the insectary, artificial blood feeding was performed twice a week. To increase blood feeding, the sugar solution was removed from the adult cage six hours before feeding and returned after feeding was completed.

For the artificial blood feeding experiment, fresh human blood obtained from the Iranian Blood Transfusion Organization (collected in CPDA-1 [citrate phosphate dextrose adenine] anticoagulant solution) was used. The blood was stored at 4 °C. The elapsed time between blood collection and use was 2–4 hours. A 6–8 cm glass funnel was sterilized in an oven. The funnel's opening was sealed with Parafilm, and the blood was poured through the funnel tube to form a thin layer on the Parafilm surface. To warm the blood and attract the mosquitoes, the funnel containing the blood was placed in hot water at a temperature of 40–42 °C for 10 minutes. Afterward, the funnel was positioned on top of the adult mosquito cage, and two warm water bags were placed around it to help maintain the temperature. The mosquitoes fed on the blood for a duration of two hours. The water in the bags was replaced with warm water every 30 minutes to ensure the blood temperature remained consistent. The blood feeding took place during the daytime (Fig. 2A).

Egg collection

Eggs were collected using ovitraps. Two days after blood feeding, an ovitrap containing 500 mL of water was placed inside the adult mosquito cage (Fig. 2B). The use of 450 mL of dechlorinated water combined with 50 mL of water from the larval rearing tray in the ovitrap significantly increased mosquito oviposition. Oviposition began on the third day after blood feeding and continued for three days, typically during the night or dark periods in the insectary. The peak of egg-laying occurred on the second day of oviposition. Eggs were deposited on paper above the water surface. Each day, the egg-laden paper was removed from the ovitrap and replaced with a new sheet. On average, each sheet contained between 1,500 and 3,000 eggs (Fig. 2C).

Drying egg papers

Eggs required an additional maturation period before hatching (10). To aid in this embryonic maturation, the egg papers were gently dried after being removed from the ovitrap. First, dead mosquitoes adhering to each paper were removed using a wash bottle with dechlorinated water, taking care not to dislodge the eggs. Each paper was placed on a paper towel at the bottom of a lidded plastic container, ensuring that the eggs did not come into contact with the paper towel and that the papers did not overlap. The container was closed and kept under insectary conditions for 24 hours. After this period, the lid was opened, and the papers were transferred to a clean, uncovered plastic container, where they remained under insectary conditions for an additional 24 hours (Fig. 2D). Following two days of gradual drying, embryonic development was completed.

To assess the correlation between the duration of egg-paper drying and the percentage of egg hatching, we dried papers containing 250 eggs each for periods ranging from 0 to 30 days. Each time point included three replicates. The eggs were manually counted on the paper using a handheld magnifying lens. Once the target count was reached, the corresponding section of the paper was marked and cut. This manual counting procedure was performed

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consistently for each replicate to maintain a standardized egg density. The percentage of hatching was calculated 24 hours after the papers were immersed in water.

Egg hatching and larval rearing

The hatching percentage of eggs was compared using three different media: cooled boiled water, yeast and larval food (fish food). In the first medium, the larval tray was filled with 1 L of cooled boiled water, and a paper containing 250 eggs was immersed in the water (The eggs on the paper were counted, and the paper was cut into pieces containing 250 eggs). In the second medium, yeast was used. The larval tray was filled with 1 L of dechlorinated water, and 0.2 g of yeast was added to the water. The following day, a paper containing 250 eggs was immersed in the tray. In the third medium, fish food was used. This food (branded TetraMin Tropical) was in the form of small flakes, and its mix included a combination of protein 46%, fats 11%, fibre 3%, vitamins and minerals; one gram of ground fish food was mixed with 10 mL of dechlorinated water and added to a larval tray, which also contained dechlorinated water. After 24 hours, the paper containing 250 eggs was immersed in the tray. Each method was carried out in triplicate on the same day, and the egg hatching percentage was calculated 24 hours after immersion.

After two days of drying, the egg papers were immersed in larval trays containing 1 L of cooled boiled water, with each paper holding approximately 1,500 eggs. The trays were covered to minimize oxygen exposure and improve hatching success for 24 hours (4). After this period, the first instar larvae hatched from the eggs. At that point, 1 L of dechlorinated water was added to the tray, and the larvae were fed fish food (Fig. 2E).

The fish food was ground, and 5 grams of it were mixed with 50 mL of dechlorinated water. 10 mL of the resulting mixture was added to each larval tray to feed the newly hatched larvae during the first two days after hatching. The paper was left in the water for an additional 24 hours, then removed and disposed of by immersing it in boiling water. The papers were later sealed in plastic bags and taken out of the insectary. As the larvae developed, the volume of feeding solution was gradually increased. On the third and fourth days after hatching, 20 mL was added, and from the fifth day until the fourth larval instar, 40 mL of the larval food mixture was added to each tray.

The water in the larval trays was changed daily to remove larval exuviae from the surface. To do this, a portion of the surface water was removed each day using a pipette, and clean dechlorinated water was added to the trays.

Sorting larvae and pupae

In an insectary maintained at a temperature of 27–28 °C and 65–75% relative humidity under a photoperiod of 12 h light: 12 h dark, the larval stage lasted between 8–10 days. A plastic pipette or dropper was used to separate pupae from larvae. Male and female pupae can be distinguished by size, as female pupae are larger than male pupae, and female pupae are darker in color. *Aedes aegypti* pupae can be reliably sexed by the differences in the genital lobe shape (at the end of the pupal abdominal segments just below the paddles). In males, the genital lobe is distinctly smaller and exhibits a more rounded or blunt appearance at the apex (11). Approximately 40% of the pupae were female.

The pupae were rinsed with dechlorinated water using a tea strainer and then transferred to a wide bowl filled with clean dechlorinated water. This bowl was placed inside an adult mosquito cage. After emerging, adult mosquitoes temporarily rested on the water surface or along the edges of the pupal container before taking flight. To prevent overcrowding in the pupal container, care was taken not to fill it, and the water level was kept below the rim (Fig. 1D, 1E). A total of 3,000 to 4,000 pupae were introduced into each cage measuring 50×

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50× 50 cm. A female-to-male ratio of 1:1 is generally suitable for colony maintenance, but a ratio of 3:1 is used to enhance egg production in the insectary (4, 11). Consequently, each cage contained up to 3,000 females and 1,000 males. This ratio was applied only for selected generations and achieved by manual size-based pupal separation.

Results

Under laboratory conditions, female mosquitoes displayed a three-day gonotrophic cycle, laying eggs three days after blood feeding. The results revealed a strong negative correlation between drying duration and hatching percentage (Pearson correlation= −0.818, p< 0.001). Specifically, as the drying duration increased, the hatching percentage decreased (Fig. 3).

Gradual drying of egg papers for two to three days resulted in a hatching rate of over 90% without the need for a hatching stimulant solution. In contrast, eggs on papers that were not dried and were immersed in water immediately after removal from the ovitrap hatched at only 18% after 24 hours. Extending the immersion time to 72 hours increased the hatching rate to 50%; however, the larvae from these undried eggs exhibited asynchronous development, with all larval instars present in a single tray. Rapid drying of the egg papers within one day led to over 70% of eggs shriveling and failing to hatch (Fig. 3).

Observations showed that male and female mosquitoes emerging from eggs dried for two days were larger than those emerging from eggs dried for more than 20 days. All other rearing conditions were identical among groups (Fig. 2F).

After evaluating the three types of egg hatching media and finding no significant difference among them, cooled boiled water was chosen for egg hatching in this study due to its low cost. A one-way ANOVA was performed to evaluate the impact of different media on the hatching success of *Ae. aegypti* eggs. The results showed that the mean hatching rates did not differ significantly among the three media tested (cooled boiled water, yeast and larval food) (F= 0.601, p= 0.578).

It was observed that female mosquitoes laid about 20% to 30% of their eggs on the surface of the water in the ovitraps. This was especially evident in cages with a pupal bowl alongside the ovitrap, likely due to the bowl's white inner surface (Fig. 2G). These eggs were transferred to larval trays and reared to adulthood without a drying period. It was observed that female mosquitoes, when ready to lay eggs, deposited them on any available moist surface, even in the absence of free water. Figure 2H shows eggs laid on a paper towel soaked with a 10% sugar solution.

Observations in the insectary revealed that *Ae. aegypti* larvae primarily feed on food particles that settle at the bottom of larval trays. Both larvae and pupae of *Ae. aegypti* tended to aggregate in the corners and along the bottom of the rearing tray (Fig. 1F).

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Fig. 1. Rearing of *Aedes aegypti* mosquito in the insectary, Bandar Abbas, Iran. A: Plastic and aluminum cages for rearing adult mosquitoes. B: Ovitrap and egg paper. C: Larva white plastic trays. D, E: Containers containing pupae inside the cages. F: The tendency of larvae and pupae to aggregate in the corners and along the bottom of the rearing tray

Fig. 2. Rearing of *Aedes aegypti* mosquito in the insectary, Bandar Abbas, Iran. A: Artificial blood feeding. B: Ovitrap placed inside the cage. C: Eggs are on paper above the waterline. D: Placing *Aedes aegypti* egg paper on a paper towel to dry in a plastic container. E: Commercial fish food used to feed larvae. F: Differences in adult female *Aedes aegypti* size emerging from eggs subjected to different drying durations. Left: mosquito after 2 days of drying. Middle: mosquito after 20 days of drying. Right: mosquito after 30 days of drying. G: *Aedes aegypti* eggs deposited above the waterline and on the surface of the water in the insectary. H: Egg-laying behavior of *Aedes aegypti* mosquitoes on paper towels soaked in a 10% sugar solution in the insectary

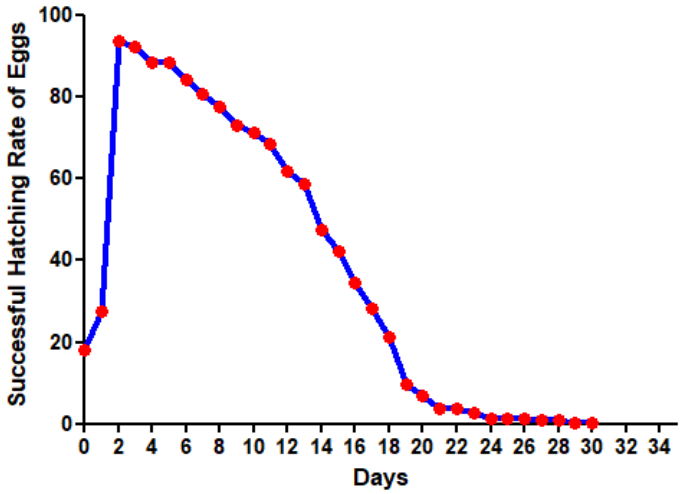

Fig. 3. The impact of egg paper drying duration on hatching percentage of *Aedes aegypti* eggs in the insectary, Bandar Abbas, Iran

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Discussion

The *Aedes aegypti* mosquito is typically known to lay its eggs on the moist surfaces above the waterline (12). However, in the present study, we observed that these mosquitoes laid 20–30% of their eggs directly on the water surface during each oviposition cycle. To our knowledge, this is the first report of *Ae. aegypti* laying eggs on the water surface in Iran. Similar findings have been noted in other studies conducted in natural habitats and insectaries (13–16).

Field and laboratory studies have documented that eggs are sometimes deposited directly on the water surface in ovitraps, buckets and artificial larval habitats, accounting for 28–62% of all eggs. Factors such as relative humidity, lack of suitable rough substrates, and possible kairomones in the water have been suggested as contributors to increased egg-laying observed on the water surface in these studies (14, 15, 17). Eggs laid on the surface hatch more quickly but are also more vulnerable to desiccation (14).

In the current study, we observed that egg-laying on the water surface occurred in ovitraps filled with larval tray water, as well as in bowls containing pupae and pupal exuviae. This preference may be influenced by chemical cues present in the water where *Ae. aegypti* larvae had previously developed, suggesting that this environment is suitable for oviposition and larval growth. This behavior of *Ae. aegypti* is significant from both entomological and epidemiological viewpoints. Eggs laid on the water's surface hatch immediately without a drying period, which can lead to quicker population establishment at new breeding sites. This behavior is, in fact, a cornerstone of their evolutionary success; a dual-path strategy that maximizes fitness under unpredictable environmental conditions. The water surface oviposition strategy acts as the population expansion mechanism during periods of consistent humidity, whereas the waterline oviposition strategy functions as the resilience mechanism against environmental conditions (14, 15). Therefore, when using ovitraps for monitoring and surveillance, it is crucial to account for eggs laid on the water's surface in addition to those deposited above the waterline. Neglecting to consider this may result in an underestimation of infestation levels and reproductive activity in a specific area. Understanding the behavior of *Ae. aegypti* is crucial for enhancing the accuracy of vector surveillance and can aid in developing more effective vector control strategies.

This study evaluated how the duration of egg paper drying affects the hatching percentage of *Ae. aegypti* eggs, to determine whether extended drying and storage periods impact egg survival and hatching in controlled environments. The resistance of *Ae. aegypti* eggs to drying out is a significant advantage for their long-term storage. Under suitable conditions, the eggs can remain viable for up to one year (3). However, the duration of desiccation affects the hatchability of the eggs. The ability of *Ae. aegypti* eggs to resist desiccation is closely linked to the formation of the serosal cuticle and the biosynthesis of chitin during embryonic development (18, 19). This ability to tolerate desiccation may help explain the species' successful invasion of temperate regions around the world, enabling it to survive harsh conditions and establish new populations (20).

Prasad et al. (21) found that *Ae. aegypti* eggs survive desiccation by acquiring desiccation tolerance during the late stages of development. A shift in metabolic pathways toward lipid degradation and polyamine accumulation confers desiccation resistance in the eggs. Furthermore, rapid lipid degradation is required to provide energy upon re-entry into water, enabling the eggs to survive and hatch after reabsorbing water.

The present study showed a strong negative linear relationship between drying duration of egg papers and egg hatching percent-

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age. Gradual drying of egg papers for two to three days resulted in hatching of more than 90% of eggs without the use of a hatching stimulant solution. Short-term gradual drying allows the egg membrane to remain permeable to water. This method is suitable for maintaining successive generations in an insectary (22). Under appropriate rearing conditions, 70–80% or more of the eggs are expected to develop into adult mosquitoes, indicating a satisfactory survival rate for routine laboratory rearing (4). In the present study, egg hatchability exceeded 90% following a 2-day gradual drying period and subsequent immersion in cooled boiled water, demonstrating that the rearing conditions were optimal for egg hatching.

Desiccation for a period of 4 to 15 days can lead to partial water loss from the eggs and reduce embryonic metabolism, which may result in lower hatchability. If desiccation continues for more than 15 days, the eggs enter a state of desiccation resistance. During this phase, the eggshell becomes more rigid and less permeable. Under these conditions, when these eggs were reimmersed in water, many embryos may struggle to exchange water and gases, leading to a significant reduction in hatchability (22, 23).

After investigating the hatching rates of *Ae. aegypti* eggs in three different media (cooled boiled water, deionized water and bacterial broth), Zheng et al. (24) concluded that water alone is not enough to trigger hatching in eggs that have been stored for more than two weeks. They suggested that, over time, the embryos of *Ae. aegypti* enter a dormant state at the end of embryogenesis.

Other factors affecting egg hatchability include storage temperature (27±1 °C) and relative humidity. The relative humidity should be sufficiently high to prevent excessive desiccation but low enough to avoid premature hatching. Additionally, the quality of oviposition paper also affects egg viability. The environmental microbial load is another contributing factor; for instance, fungal contamination during dry storage can damage the eggshell (24). Kumari et al. (25) investigated how temperature affects the timing of hatching and the survival of *Ae. aegypti* eggs. They found that egg viability remained above 80% when temperatures were between 16 and 31 °C. However, exposing *Ae. aegypti* eggs to temperatures outside their typical range can cause physiological stress and hinder their development (26). Therefore, it is essential to maintain temperatures within a proper range to ensure successful hatching, development and reproduction (27).

Zheng et al. (24) evaluated two long-term storage methods for *Ae. aegypti* egg papers. The first method, conventional storage, involved placing the egg papers in zip-lock bags inside a sealed black plastic box. The second method, water storage, consisted of keeping the egg papers in sealed plastic cups containing oxygenated deionized water. The authors reported that water storage is an effective alternative because it maintains egg viability for a longer period compared to conventional storage. They found that bacterial broth is the most effective medium for hatching, and after five months of storage, an impressive hatching rate of 85% was recorded.

The present study suggests that the duration of egg drying may affect the size of *Ae. aegypti* larvae and adults, although its effects are minimal under optimal rearing conditions. Under environmental stress, however, prolonged egg drying can reduce larval energy reserves, leading to delayed development and potentially smaller larvae (21, 28). Given that, in this study, the insectary and larval feeding conditions were the same, and the water in the rearing trays was changed daily to prevent oxygen depletion, the primary factor affecting the reduced size of larvae and adults was the duration of egg desiccation. This finding is significant and warrants further quantitative investigation and analysis of morphometric data (such as wing length, body weight) from different drying duration groups.

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In this study, egg hatching obtained using three different media-cooled boiled water, yeast and larval food-did not differ significantly. Lower dissolved oxygen levels accelerate hatching. Several studies have used cooled boiled water (11), yeast and larval food (29, 30), and bacterial broth (24, 31) in hatching media to reduce dissolved oxygen levels.

During the hatching of eggs, the processes of water and gas exchange are essential. When eggs are immersed in water, they initially absorb a small amount of water, which helps reactivate their metabolic activity. The eggshell is equipped with microscopic pores that facilitate this water uptake. A significant factor that influences egg hatching is the level of dissolved oxygen. Unlike many other organisms that require high levels of oxygen for development, hatching in *Ae. aegypti* occurs in response to reduced oxygen levels. Reduced dissolved oxygen levels may signal to the embryo that the water contains organic matter and potential food sources, indicating that the environment is suitable for larval development (22, 23).

Hatching media are generally more expensive than cooled boiled water. The FAO/IAEA recommends hatching eggs in cooled boiled water, regardless of whether larval food is present, or in pre-prepared distilled water that includes larval food (4). However, cooled boiled water may not be ideal for mass rearing because the boiling process removes dissolved oxygen from the water. But in bacterial broth, the metabolism of bacteria gradually decreases the levels of dissolved oxygen over time (24). Additionally, for eggs in deeper dormancy, cooled boiled water may be unable to disrupt this barrier. To overcome this barrier, eggs require specific chemical compounds in the water, usually produced by microorganisms or organic matter; however, cooled boiled water lacks such chemical stimuli (18).

Our observations in the insectary showed that *Ae. aegypti* larvae primarily feed on food particles located at the bottom of larval trays. Souza et al. (32) also noted that *Ae. aegypti* larvae primarily feed on organic matter either suspended in the water column or deposited at the bottom of rearing containers. Finely ground food enhances larval feeding efficiency and promotes nutrient uptake, larval growth and pupation by making food particles more accessible to *Ae. aegypti* larvae (33, 34). Over the past 50 years, researchers have explored and recommended a variety of materials as larval diets for maintaining mosquito colonies. Ideally, these diets should be readily available, easy to process and store, and cost-effective (35).

Aedes aegypti larvae aggregate in both natural habitats and insectaries as a behavioral adaptation to enhance survival and optimize resource use. Aggregation is influenced by factors such as predator avoidance, light, temperature, food availability, overcrowding, shelter and chemical signaling and may become more pronounced under environmental stress (36, 37).

At each stage of *Ae. aegypti* rearing, problems may arise during colony establishment and maintenance. Completing the life cycle in the insectary requires special care at each of the four developmental stages: egg, larva, pupa and adult. Common rearing problems linked to each stage are outlined below, concerning their most critical requirements.

Egg papers should be transported in sealed containers. Transporting egg papers with the eggs exposed to air can result in complete egg mortality. If egg papers are not fully dried before being stored in zip-lock bags, this can promote mold growth. Additionally, asynchronous hatching may result from insufficient oxygen depletion in water. Older eggs or those that have not completed drying and embryonic development may also contribute to asynchronous hatching (3, 35).

During the larval stage, inadequate nutrition, overfeeding, overcrowding and microbial contamination can negatively affect development and survival. Insufficient food slows growth, disrupts molting, increases mortality and produces smaller adults, while excessive feeding

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can cause foam formation that impairs respiration and may kill larvae and pupae. Foam formation is influenced by temperature, diet, feeding rate and larval density. Dietary restriction may alter adult lifespan and generally reduces fecundity and mating performance. Proper feeding, stocking density, and hygienic rearing conditions are therefore essential for successful larval development (3, 35, 38).

Inadequate larval nutrition can increase pupal mortality and reduce pupal size, resulting in undersized adults. To reduce pupal mortality, pupae should be removed from larval trays, transferred to clean water, and subsequently placed in adult mosquito cages (3, 35).

Maintaining appropriate insectary conditions, including temperature, humidity and photoperiod, is critical for adult mosquito survival, mating, blood feeding and oviposition. High temperatures, low humidity, overcrowding, unsuitable cage size, improper timing of blood feeding, prior access to sugar, host preference and inadequate oviposition substrates can reduce reproductive success. It is important to adjust the size of the oviposition container based on the mosquito density in the cage (3, 35, 38, 39). Carvalho et al. (11) reported that a maximum of 1,000 males and 3,000 females could be maintained in cages measuring 30× 30× 30 cm. However, in our study, we used larger cages measuring 50× 50× 50 cm, which were equipped with larger ovitraps. This approach was intended to prevent overcrowding of mosquitoes within the cages and to provide sufficient egg-laying sites for all blood-fed mosquitoes.

After hatching, jars, containers and egg papers should be sterilized with boiling water or frozen for at least three days before being washed or disposed of. It is important to wear gloves and change them when handling different strains or species to prevent cross-contamination (4).

Conclusion

Insectary-reared mosquitoes are essential for research on mosquito-borne diseases and the development of innovative vector control strategies. Environmentally sustainable methods, such as the sterile insect technique (SIT), the release of insects carrying a dominant lethal gene (RIDL), and *Wolbachia*-based interventions, rely heavily on the large-scale production of high-quality mosquitoes. In this study, we established a standardized protocol for rearing *Ae. aegypti* under local insectary conditions. By precisely controlling environmental and nutritional parameters, we achieved stable colonies with high survival rates and reduced operational costs. Although egg hatching is the most critical stage of colony establishment, we successfully ensured larval development and adult emergence once hatching took place. Overall, the described rearing procedures provide a reliable and reproducible framework for maintaining *Ae. aegypti* colonies and will support future research on mosquito biology, vector competence and the development of novel vector control interventions.

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Ethical considerations

The authors declare the ethical approval code as No.: IR.TUMS.MEDICINE.REC. 1402.029.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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