

Aquatain® Causes Anti-Oviposition, Egg Retention, Oocyte Melanization and Triggers Female Death in *Ae. Aegypti*

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Abstract

Background: In arboviral disease systems where the virus can be transmitted from male to female and from one generation to the next, targeting the female (especially when she is pregnant) can help alter the persistence of the virus in nature and its transmission. This is typical of *Ae. aegypti*, which has been unmanageable due to the development of insecticide resistance. Despite evidence that monomolecular surface films prevent the selection of genetic resistance, their potential in *Aedes* vector control remains largely unexplored.

Methods: We examined the oviposition, egg retention, oocyte melanization, and female mortality of the Cayman Islands strain of *Ae. aegypti*, using choice and no-choice bioassays involving Aquatain® Mosquito Formulation (AMF).

Results: When presented with similar opportunities to oviposit in two sites treated with AMF and two others with water (control), egg deposition rates were significantly higher in water than in oil presence. We also observed a matching pattern of egg deposition preference in arenas with more options in AMF-treated sites. Females laid appreciably more eggs when water was the only available medium than when all sites were treated with AMF. Also, considerably more mature eggs were withheld in the AMF no-choice arena than in the no-choice test involving only water. Internal oocyte melanization was not observed in females from the oviposition arenas with the lowest oil presence (equal-choice and water-based no-choice); in contrast, this physiological response intensified as the number of AMF-treated sites increased. Female death occurred at high rates in AMF-treated environments, and this response increased with the increasing presence of such egg deposition sites.

Conclusions: This study demonstrated that AMF acted as a deterrent signal to ovipositing *Ae. aegypti* and an indirect adulticide. Referring to its previously reported direct toxicity on the aquatic stages of this mosquito and its biodegradability, AMF should be incorporated as a critical component in integrated control strategies for dengue and related diseases.

Background

Despite several decades of control efforts, mosquito vectors and the diseases they transmit are still significant threats to human health [1, 2]. Beyond disease transmission, mosquitoes pose serious menaces to human quality of life [3] and economic development [4]. This is the case for most Caribbean islands [5]. This region has recently experienced an unprecedented epidemic of chikungunya, Zika viruses, and febrile illness due to dengue [6]. These public health threats, in particular, the link of the Zika virus to reproductive health, pregnancy, and congenital disabilities [7, 8, 9] have stimulated increased interest in *Aedes aegypti*, the primary vector of all viruses previously mentioned [10].

Efforts to prevent outbreaks of these diseases have mostly counted on the use of insecticides, but success has been limited due to the development of insecticide resistance [11, 12]. Apart from increased resistance to the four existing families of adulticides—pyrethroids, organophosphates, carbamates, and

organochlorines [13, 14], the control of *Ae. aegypti* with larvicides is hindered by a reduced susceptibility [15], international scrutiny and deregulation for the toxicity against biodiversity [16] and the existence of inconspicuous larval habitats [17, 18].

This mosquito disperses eggs across multiple natural and artificial containers associated with human activity [19, 20, 21]. Despite the multitude of potential breeding sites, *Ae. aegypti* has been reported to successfully locate those that can guarantee the success of the offspring and to ignore others that are unsuitable for larval development completion [22, 23, 24]. This ability to identify high-quality oviposition sites and select those that provide increased larval performance is mediated by many parameters, but most studies focused on the role of olfactory cues [25] and habitat physics [20]. Although there has been a focus on how some components of the site's physical structure affect egg deposition choice [26], there has been little research work regarding the physical features of water bodies, known to present several visual signals [20]. These include texture and reflectance from the water surface, which have been reported to influence the oviposition of container-breeding mosquitoes including *Ae. aegypti* [27, 28, 29, 30]. Surface tension is a property of water that plays an integral part in the development and survival of aquatic insects, including mosquitoes [31, 32]. Surface tension can be generated when a water surface is covered with an oil film [33, 34]—putting oil over water results in an elastic behavior of the water, causing it to acquire a minor surface area possible [35]. Such surface tension reduction produces a film at the air-water interface [20]. *Ae. aegypti* adults are incapable of resting on the water's surface when its tension is reduced [36]. Because this mosquito prefers a high level of surface tension, it exhibits high mortality responses by drowning when exposed to water with reduced surface tension [36].

In addressing dengue vector control, the World Health Organization [37] and many other mosquito scientists [38, 23] have called to take oviposition into account, not only because of transovarial transmission of viruses [39] but because preventing oviposition can to disrupt the life cycle and reduce the population growth [23]. Due to these potentials positive control outcomes, some studies have been done addressing the use of oil to prevent mosquito oviposition, but most of these investigations have used either essential oils [40, 41, 42], lecithin monolayers [43, 44, 45] or agnique MMF monomolecular surface film [46], all of which known to be easily breakable by wind and rainfall [47, 48]. Although a recent study [49] has tested an oil film with high resilience to breakages by wind and rain—Aquatain® Mosquito Formulation [50], the authors did not address oviposition and *Ae. aegypti*. Here, we assessed the oviposition, egg retention, oocyte melanization, and female mortality of *Ae. aegypti* in response to different levels of presence of Aquatain® Mosquito Formulation (AMF) in potential breeding sites.

Methods

Colony maintenance

The *Ae. aegypti* mosquitoes used here came from a colony maintained at the insectarium of the Mosquito Research and Control Unit (MRCU) where environmental conditions were as were $26.5 \pm 1^\circ\text{C}$ temperature, $65 \pm 4\%$ relative humidity, and photoperiod of 13:10 (light:dark) with 1 hour of dusk. The

colony originated from larvae collected from different containers in George Town in November 2020. Routinely, larvae were reared at a ratio of 100 to 150 larvae in plastic trays filled with 800 ml tap water. Larval food (ground Tetramin, Tetra, Germany) was supplied once daily, and the rearing medium was replaced with fresh medium once before the commencement of pupation. Pupae were placed in polystyrene cups (50-mL capacity) and transferred to BugDorm cages (30 × 30 × 30 cm, MegaView Science Co., Ltd., Taichung, Taiwan). Emerging adults had continuous access to 10% sucrose solution. Females were artificially offered blood meals once every two weeks. Three days after blood feeding, eggs were collected, air-dried under insectary conditions, and stored for colony maintenance or experiments.

The making of experimental subjects

To obtain gravid females, egg samples from the colony stock were submerged in tap water, and four larval population replicates, each with 200 newly hatched larvae was reared as outlined above. They were fed every two days with Tetramin. As performed for the colony, the rearing water was replaced with fresh water before the third food supply, following others [24]. Pupae were held in plastic cups and transferred into cages holding a 10% sugar solution. Three to four days old females were offered blood meals for 10 min. Fully engorged females that digested blood meals for three days were considered gravid and used as experimental subjects.

Experimental features

All oviposition tests were performed following a published experimental design [24, 51] with slight adjustments. Fig. 1 shows the oviposition bioassay design. The egg deposition arena was composed of four acrylic containers (depth = 7.3 cm, diameter = 3.3 cm), each with a segment of white paper (length = 8 cm, width = 8 cm) that layered the entire surface of the container and served as an egg deposition substrate. These containers were each at a given corner of the cage, and to obviate any likely location bias, the positioning and setting of the oviposition sites was modified for bioassay replicate, adopting the previously described clockwise replication blueprint [24, 51]. In this scheme, an oviposition test replicate accorded the layout of the four oviposition containers within the cage. All bioassay types were repeated four times, and for each replication, a batch of twelve fully blood-fed females that had digested their meals for three days, new egg deposition substrates, and new oviposition media were used. All oviposition arenas were equipped with a sugar source supplied by a cotton wick soaked with a 10% sucrose solution. The sugar supply apparatus was replaced with fresh solution and cotton wick once during the oviposition period, which lasted ten days. All oviposition bioassays were performed where temperature and relative humidity were $28 \pm 2^\circ\text{C}$ and $70 \pm 3\%$, respectively.

Chemical and test concentration

Aquatain® Mosquito Formulation—AMF from (Aquatain Products Pty Ltd., Australia) containing 78% of the active ingredient polydimethylsiloxane (also called silicone), was used in this study at 1 ml/m², the application rate recommended by the manufacturer [50]. The test oviposition medium was generated referring to the approved application rate and considering the dimensions of experimental containers

used in this study. Each container used as an oviposition site has a diameter of 3.3 cm and a surface area of 33.16 cm². Based on these dimensions, a volume of 3.316 µl of AMF was added to 150 ml of water, and the resulting solution was used as an oviposition medium. The exact final volume in water (150 ml + 3.316 µl) served as a water medium. For convenience, containers with the test medium or water were designated as “AQ” and “W”, respectively.

Bioassays

To ascertain whether or not the presence of AMF influences the oviposition responses of *Ae. aegypti*, four bioassay types were run following others ([51] Dieng et al. 2017). In the first test, a total of twelve gravid females were placed in a BugDorm cage (30 × 30 × 30 cm) into a cage with an oviposition arena comprising four W containers (W1, W2, W3, and W4) and a sugar supply source. A second bioassay was performed as illustrated above, but the twelve females were given options to lay eggs in four AQ containers (AQ1, AQ2, AQ3, and AQ4). In the third test, the twelve females were given equal chances to lay eggs in two AMF-treated containers (AQ1 and AQ2) and two others with water (W1 and W2). In the fourth bioassay, the twelve females were presented with more options to oviposit in AMF-treated containers (AQ1, AQ2, and AQ4) than in water (W1).

Data collection and statistical analyses

At the end of the oviposition period (10 days), dead females were counted in each cage replica of each bioassay type and immediately frozen alongside survivors. Egg depositions were checked in all bioassays by procedures from Dieng et al. [51] with the help of a stereomicroscope (Motic SMZ-171-TLED; Motic Instruments Inc.; Schertz, USA). The total numbers of eggs laid in each container replica were quantified by counting the eggs present on the surface of the water media, paper substrates, and the lower surfaces of the bottom of the containers. The resulting numbers were used to determine the percentages of eggs laid in a given oviposition container replicate using the following formula: total number of eggs deposited in a given media divided by the total number of eggs deposited in all media of a given bioassay replicate multiplied by 100. The mean values of numbers egg deposited in a given bioassay replica by summing up all numbers of eggs in a given bioassay divided by the total number of experimental females. Percentages and mean values were utilized to score oviposition responses. Non-dried females were dissected, and the two ovaries were examined for egg presence using the stereomicroscope. The numbers of eggs withheld were counted for each dissected female, and the number of eggs produced by a female was defined as the sum of eggs laid and retained, as to follow Farjana and Tuno [52]. As did Dieng et al. [51], the egg retention rate for each dissected female was calculated as the total number of eggs withheld divided by the total of eggs produced × 100. The percentage of eggs retained by a given female was defined as the total number of eggs retained divided by the total number of eggs produced. At each dissection occasion, the numbers of eggs that were white/light grey, dark grey with black spots, and black were recorded. We considered white/light grey, dark grey, and black colored eggs as non-melanized, melanizing and melanized, respectively. The numbers of eggs in such states were used to determine the percentages of non-melanized, melanizing, and melanized eggs. The differences in oviposition, egg retention, and eggshell melanization responses were analyzed

by nonparametric test (*Kruskal-Wallis*), and whereas female mortality responses were examined by Analysis of variance (ANOVA) from Systat version 13 [53]. Dwass-Steel-Christchlow-Fligner test was used to assess the significance of the differences between mean values for oviposition, egg retention, eggshell melanization, and Tukey's post hoc test for mean mortality values. In all analyses, $p < 0.05$ scored statistical significance.

Results

Oviposition responses to AMF-treated water in various competition levels with water

When water was the only medium, eggs were found in each of the four containers. Of the 2084 eggs laid, 30.13% (628/2084), 20.49% (427/2084), 22.69% (473/2084) and 26.68% (556/2084) were deposited in W1, W2, W3 and W4, correspondingly. The mean number of eggs deposited was 130.25 ± 21.31 per container (W1: 157.00 ± 37.68 ; W2: 106.75 ± 45.87 ; W3: 118.25 ± 43.59 ; W4: 139.00 ± 56.25). Egg deposition did not differ between these containers ($p = 0.862$) (Fig. 2A). When given equal chances to oviposit in two containers holding water supplemented with AMF and two others with water, *Ae. aegypti* females laid eggs in all containers, but oviposition responses varied appreciably with container medium. Of the 1185 eggs oviposited by the 12 females, 95.44% (1131/1185) were deposited in containers with water, and 4.56% (54/1185) in AMF-treated water. The mean egg deposition in containers with water (141.37 ± 10.84 eggs, range 93 – 184; W1: 152.25 ± 15.19 ; W2: 130.50 ± 15.47) was substantially higher than that found in containers with AMF supplement (6.75 ± 4.97 eggs, range 0 – 41; AQ1: 2.50 ± 0.95 ; AQ2: 12.00 ± 9805) ($p = 0.001$) (Fig. 2B). When there were more choices to lay eggs in containers with water treated with AMF, the 12 females laid 783 eggs in total, including 643 in the container with water and 140 in the three cups containing AMF, corresponding to 82.12% and 17.88% of the total, accordingly. Egg deposition was significantly lower in the presence of the oil (11.66 ± 5.13 eggs, range: 0 – 63; AQ1: 9.25 ± 4.38 ; AQ2: 6.25 ± 4.62 ; AQ3: 19.50 ± 14.72) than when water was the only oviposition medium (160.75 ± 50.77 eggs, range: 78 – 287) ($p = 0.003$) (Fig. 2C). When AMF-treated containers were the only oviposition sites, *Ae. aegypti* deposited eggs in all four containers. A total of 540 eggs were deposited by the forty-eight females, of which 44.07% (238/540), 22.6% (122/540), 14.44% (78/540), and 18.88% (102/540) were oviposited in AQ1, AQ2, AQ3, and AQ4, respectively. Egg depositions ranged from 0 to 112 and averaged 33.75 ± 8.39 eggs per container (AQ1: 59.50 ± 25.97 ; AQ2: 30.50 ± 8.13 ; AQ3: 19.50 ± 15.65 ; AQ4: 25.50 ± 10.44). There were no appreciable discrepancies in oviposition response between the four cups ($p = 0.375$) (Fig. 2D).

Comparative oviposition responses relative to option availability and egg retention

In the water-based no-choice test, the forty-eight females (twelve females per cage replicate) produced a total of 2734 eggs, and the mean number of eggs they laid individually was 43.43 (2084/48). This latter measurement was 24.68 (1185/48), 16.31 (783/48), and 11.25 (540/48), when *Ae. aegypti* females were

provided with (i) matching oviposition options, (ii) more oviposition opportunities in containers supplemented with AMF, and (iii) in the AMF-based no-choice setting, respectively. The total egg deposition when water was the only oviposition medium was 1.75, 2.66, and 3.86 times higher than when half, three-fourths, and all oviposition sites were treated with AMF, respectively (Table 1). Eggs were retained in all experimental oviposition designs (Table 2), but egg retention responses varied significantly with oviposition arena layout ($p < 0.0001$). In the no-choice test involving only water, a total of 649 eggs were recovered from the ovaries of the forty-eight females ten days after blood meal uptake; this means that they retained 23.73% (649/2734) of their total egg production. In the equal-choice test, 462 eggs were collected following the dissections of the twenty-eight surviving females a week after blood digestion. When more containers were treated with the oil, 1669 eggs were retained by the forty-one surviving females. This value was 968 eggs for 38 females when containers with the oil were the only egg deposition sites. The mean number of eggs retained per female in the water-based no-choice trial (13.52 ± 3.49 eggs, range: 0 – 76 eggs) was appreciably lower than that in the equal-choice experiment (17.11 ± 3.99 eggs; range: 0 – 63 eggs) ($p < 0.0001$), which, in turn, was substantially lower than that obtained when there were more containers treated with the oil (40.70 ± 4.88 eggs, range: 0 – 107 eggs; $p < 0.0001$). Individual females also retained arithmetically fewer eggs in the water-based no-choice compared to the AMF-based no-choice trial (25.94 ± 3.98 eggs, range: 0 – 71 eggs), which in turn recorded significantly more eggs retained per female than the equal-choice ($p < 0.0001$).

Oocyte melanization patterns in responses to AMF exposure

Figure 3 shows the egg melanization responses and variations after a 10-day oviposition period. When water was the only medium present in the oviposition arena, and when there was an equal chance for oviposition in two containers with water and two others with AMF-treated water, none of the females dissected had melanized eggs. Of the 1669 eggs retained by the forty-one females that were provided with more oviposition opportunities in AMF-treated containers, 76.45% (1276/1669) and 15.27% (255/1669) were partially and fully melanized, respectively. When containers treated with the oil were the only oviposition sites, 85.13% (824/968) and 14.87% (162/968) of retained eggs were moderately and fully melanized, accordingly. Numerically, the mean number of partially melanized eggs in the no-choice test involving AMF (21.68 ± 3.63 eggs) was lower than that when egg deposition options were biased towards containers supplemented with AMF (31.12 ± 4.86 eggs), but there was no significant discrepancy between the two mean values ($p = 0.087$). Females tended to have less fully melanized eggs when all sites were treated with the oil (4.26 ± 1.44 eggs) than their counterparts maintained in the arena with the sole egg-laying option in water (6.22 ± 1.40 eggs), but the difference was insignificant ($p = 0.092$).

Mortality responses of *Ae. aegypti* females during oviposition in AMF presence

There were significant differences in the survival of *Ae. aegypti* females between the different oviposition opportunities ($p < 0.0001$). When containers with water were the only egg deposition sites, the mean female mortality was $4.16 \pm 4.16\%$, and oscillated between 0 and 16.66%). Arithmetically, this latter mean

was far lower than that recorded when females were given equal choices to oviposit in containers with AMF-treated water and two others with water ($16.66 \pm 4.16\%$); however, the difference was insignificant ($p = 0.539$). The mean mortality rate of females in the “more AMF options” experiment ($42.833 \pm 7.21\%$, range: 25 – 58.33%) was significantly greater than that of the “equal-choice experiment ($p = 0.034$), which in turn, was numerically lower than that from the AMF-based no-choice test. There was no significant difference in female death when there were more options in containers holding AMF-treated water and when only containers with AMF-treated water were present ($p = 0.901$) (Fig. 5).

Discussion

The current study revealed significant adverse impacts of the label dose of the silicone-based monomolecular film on *Ae. aegypti*. Oviposition responses were deficient as the presence of containers with the oil increased. Egg retention and internal oocyte melanization rates were high in oviposition arenas dominated by the presence of containers with the oil; such effects decreased in water presence, especially under water-based no-choice conditions. Death occurred at greater rates among females maintained in arenas with the increased presence of oviposition sites treated with the oil compared to arenas with elevated water presence.

The appeal or repulsiveness of competitive egg deposition sites to gravid mosquito females is dictated by a diversity of factors, among which the most important is physico-chemistry of the water media [54] and the prospect of unfavorable conditions for larval development completion [55, 20]. Various physical elements have been evidenced to prevent egg deposition in mosquitoes [20, 56, 57]. In particular, water surface tension has been shown as a critical factor influencing oviposition [22]. Water tension typically occurs when oil is added to water; following addition, the layer of water molecules and that of oil molecules balance force between the two interfaces, causing the reduction of the surface tension at the water surface [35] and the apparition of a film [32]. *Ae. aegypti* has been reported to select oviposition sites concerning the possibility of larval development completion [55, 22, 23, 24]. In mosquitoes, the presence of an oil film on the surface of the water of potential breeding habitats has often been associated with reduced survival. For instance, Mbare et al. [50], dealing with *Anopheles* vectors, noted a 90% larval mortality and over 80% adult emergence inhibition with an AMF film. Dawood et al. [32], working with *Culex pipiens*, investigated the effects of AMF on different immature stages. They found significantly far greater mortality rates in first-, second- and fourth-instar larvae and pupae in treated containers than untreated ones; they attributed these impacts to the ability of AMF to alter the water surface tension. In a recent study, Kavran et al. [49] examined the lethality of AMF on the immature forms of two mosquito species and reported higher mortality rates of the juveniles of the dengue vector, *Ae. albopictus*. In an earlier study, Ngrenngarmert et al. [58] assessed the lethal potential of a silicone-based monomolecular film against *Ae. aegypti* and reported significant larval and pupal mortality rates. Besides the immature stages, the presence of oil can affect the behaviors of adults [48], including ovipositing females [43]. The mechanism by which this phenomenon occurs has been well documented. According to Likura et al. [59], mosquito legs are highly hydrophobic, and this trait causes a weight-supporting force on water surfaces. For a successful egg-laying to occur, a gravid female must generate a repulsive force

from to legs to be able to use the surface of the water as a foothold [58, 59] and evaluate its quality [20]. However, when silicone oil is added over the water surface, it generates an attractive capillary force that drags legs towards the water [58], inducing a short contact time and an escape response [59]. A shortened contact time and startle will result in an incomplete exploration of the quality of the oviposition site and thus a decreased probability of egg deposition. The current study was carried with two colorless media oviposition, i.e., AMF-treated water (150 ml of water + 3.316 µl of AMF) and water (150 ml + 3.316 µl). All the containers used in oviposition bioassays had a similar configuration, and no food was added to any container; as such, differences in oviposition responses due to difference in configuration is unlikely. Based on the reports mentioned earlier and our methodology, the observed low oviposition responses in AMF-treated containers could be explained by an attempt of the gravid females to maximize survival for both offspring and themselves. It is possible that the ovipositing *Ae. aegypti* females associated AMF presence with poor nutritional quality and reduced chances of larval development completion. It is also likely that AMF has reduced the water surface tension, which in turn has prevented gravid females from either landing or staying long enough on the surfaces of near container edges to release their eggs.

The egg retention rates in *Ae. aegypti* females placed in environments dominated by the presence of containers treated with AMF were far greater than that of females kept in arenas with only water as oviposition medium. Several studies have reported egg retention in mosquitoes relative to oviposition site features. Chadee et al. [60], working with the species studied here, increased egg retention behavior seven day-post blood meal when females were not provided with egg-laying substrates.

Dealing with a dengue vector, Satho et al. [24] assessed changes in its oviposition responses in no-choice and choice bioassay involving different coffee extract concentrations. They observed that *Ae. albopictus* females withheld increased numbers of mature eggs when cups with highly concentrated extract were the only available sites. Seenivasagan et al. [23] reported that the topical repellent diethyl phenylacetamide deterred egg depositions by the *Ae. aegypti* females, which retained around half of their egg productions. Xue and colleagues [61] investigated oviposition behaviors in *Ae. albopictus* in response to DEET and noticed that the females retained high numbers of mature eggs. Bibbs et al. [62] observed that when egg deposition sites were contaminated with a fast-acting pyrethroid insecticide (Transfluthrin), gravid females of dengue vectors withheld almost half of their eggs. The studies from Seenivasagan et al. [23] and Bibbs et al. [62] tested *Ae. aegypti* and reported 49 and 50% oviposition deterrence. Using AMF for this same mosquito species, we obtained egg retention rates ranging between 64.61 and 68.06%, showing deterrence outcomes that are above those mentioned above. Egg retention comes with many physiological and behavioral effects [63]. These include egg resorption [64], initiation of subsequent vitellogenic cycle [65], follicle morphological changes [66], visual, olfactory, and tactile responses of mosquitoes [67], and egg dissemination pattern [60].

Although we did not assess the effects mentioned above, our study revealed some information on melanization, known to play various crucial roles in insects [68]. There was a clear link between oviposition medium type and eggshell melanization level. No oocyte melanization occurred when gravid

females were exposed to water or AMF-treated water in balanced competition with water contained. In contrast, egg groups dissected from the ovaries of females exposed to an increased presence of AMF-treated water showed a high prevalence of melanized eggs, those from arenas holding exclusively AMF-treated water, where all retained eggs exhibited different levels of melanization (whitish/Light grey—non-melanized, dark grey with black spots—partially melanized and black—fully melanized). In dengue vectors, this biochemical process [69] generally starts after oviposition [70, 71]. Newly laid eggs that are whitish and smooth [72] undergo a melanization/sclerotization process [73] to become gradually black and hard after two to four hours [72, 74, 75]. During the process, a cascade of enzymatic synthesis and activities involving the phenoloxidase [75] and DOPA decarboxylase [76, 70] lead to the production of melanin. Recently, Isoe et al. [71] assessed the genetic aspects of melanogenesis in *Ae. aegypti*, and found that the eggshell organizing factor 1 (EOF1) plays an essential role in melanization. They suggested that partial melanization prior to oviposition is caused by the loss of EOF1 activity, which alters the chemical balance within the oocytes, which in turn activates other eggshell components to initiate the melanization process prematurely. They also noted that EOF1-deficient mosquitoes have eggs with different melanization levels, varying from non-melanized to completely melanized eggshells. In the current study, gravid *Ae. aegypti* females were presented with AMF-treated water as oviposition medium in different competition levels with water for the same period (10 days). Although we did not investigate these phenomena, contingent on the elucidations from the studies quoted above, it is presumable that the synthesis and levels of the phenoloxidase and decarboxylase were high in the eggs of females exposed solely to AMF-treated water and when three-fourths of the oviposition containers were treated with AMF. It is plausible that the observed disparities in melanization levels between AMF-treated water-exposed females and non-exposed or less exposed females ensued due to the high activity levels of these enzymes. Such effect of AMF treatment on the activities of phenoloxidase and decarboxylase was possibly less marked in females with no (water treatment) or reduced exposure (equal-choice and three-fourths treatments) to AMF. It is also conceivable that the females exposed solely to AMF-treated water had more EOF1 expression levels and activities. During the 10-day oviposition period, the mortality rates of gravid females were high in arenas with the increased presence of AMF-treated oviposition containers, especially the “AMF-based no-choice treatment”, compared to water-based no-choice treatment”, thus denoting the presence of adulticidal factors of the AMF medium. AMF is a polydimethylsiloxane (PDMS, 80%)–based liquid that is not toxic [32] as indicated by its certification for use in drinking water [77, 78]; so, death due to toxic chemicals is unexpected. The observed differences in female mortality responses may have been due to at least two causes. First, reducing the water surface tension in the AMF-treated containers, particularly in the AMF-based no-choice and three-fourths treatments, may have produced strong, attractive capillary forces that dragged the legs of females towards the water, consequently increasing the likelihood of sinking and death. In support of this assertion, on the water surface, but not on the water surface with a silicone oil film, female mosquitoes can use the surface as a foothold to lay their eggs, using a maximal repulsive force from their legs [59]. Second, the untimely internal oocyte melanogenesis during egg retention may have occasioned the production and accumulation of toxic compounds among females with increased exposure to AMF-treated water, which elevated the possibility of intoxication. In favor of this contention, it has been shown that phenoloxidase activity in the insect

hemolymph can produce quinonoid toxic compounds [79] and that melanin and its quinone intermediates are toxic to hosts [70].

Conclusions

We observed that treating all, three-fourths or half of potential breeding sites of *Ae. aegypti* in a given environment with AMF altered its potential contribution to the next generation. AMF treatment prevented females from laying sizeable numbers of eggs, caused increased egg retention and internal egg melanogenesis, which are conducive to reproductive unsuccess. AMF inoculation in potential developmental sites also triggered the death of gravid females, thus denoting adulticidal potentials. Evidently, AMF performed as an oviposition deterrent and as an indirect killer of ovipositing *A. aegypti* females; this latter observation would be a supplemental benefit to vector control programs. These attributes, coupled with its previously recognized direct lethality on the immature stages of dengue vectors [58, 49], advocate for the incorporation of AMF into integrated approaches for dengue vector control. As AMF is safe for humans [77, 78, 50], its use in domestic areas where *Ae. aegypti* thrives naturally, may be widely accepted. In addition, as AMF's mode of action is physical, its use and rotation with other larvicides can help mitigate insecticide resistance issues.

Declarations

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Authors' contributions

HD and AW conceived of and designed the study; ST, HS, CM, WME, ZE and HD performed the bioassays; HD and AW analyzed the data; HD drafted the manuscript. All authors brought essential insights and all of them read and approved the final manuscript.

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Availability of data and materials

The data supporting the results of this paper are available upon request.

Ethics approval and consent

The study protocol was approved by the ethics committee of MRCU.

Consent for publication

Not applicable

Competing Interest

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, tables 1 and 2 are only available as a download in the Supplemental Files section.

Figures

Fig. 1.

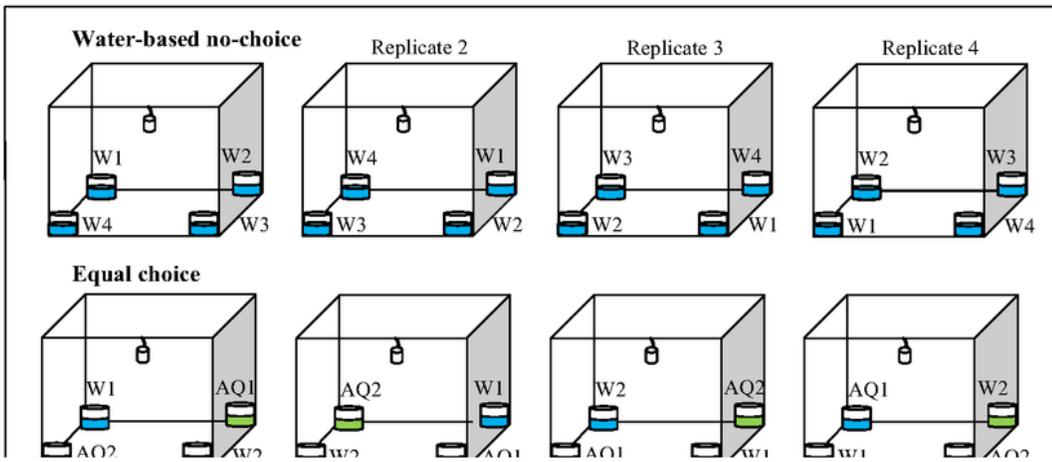


Figure 1

Oviposition bioassay design. The containers were placed on the dish, which was positioned at the bottom center of the cage. Containers were placed such that each was at an identical distance from the adjacent container. A bioassay replicate coincided with one arrangement of the four containers.

Figure 2

Responses of gravid *Ae. aegypti* females when given a choice to oviposit in four cups: **A:** Water-based no-choice [150 ml + 3.316 µl of water 1 (W1), 150 ml + 3.316 µl of water 2 (W2), 150 ml + 3.316 µl of water 3 (W3) and 150 ml + 3.316 µl of water 4 (W4)]; **B:** Equal choice [3.316 µl Aquatain® in 150 ml of water (AQ1), 3.316 µl Aquatain® in 150 ml of water (AQ2), 150 ml + 3.316 µl of water 1 (W1), and 150 ml + 3.316 µl of water 2 (W2)]; **C:** More oviposition opportunities in containers with water [3.316 µl Aquatain® in 150 ml of water 1 (AQ1), 3.316 µl Aquatain® in 150 ml of water 2 (AQ2), 3.316 µl Aquatain® in 150 ml of water 3 (AQ3), and 150 ml + 3.316 µl of water 1 (W1)]; **D:** Aquatain®-based no-choice [3.316 µl Aquatain® in 150 ml of water 1 (AQ1), 3.316 µl Aquatain® in 150 ml of water 2 (AQ2), 3.316 µl Aquatain® in 150 ml of water 3 (AQ3), and 3.316 µl Aquatain® in 150 ml of water 4 (AQ4)].

Fig. 3.

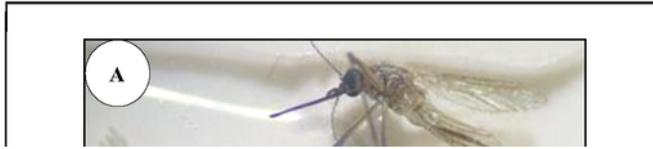


Figure 3

Melanization response patterns of the oocytes of gravid *Ae. aegypti* females after a 10-day exposure to Aquatain® Mosquito Formulation—(AMF)-treated water in various competition levels with water. **A:** Whitish/light grey eggs (non-melanized) when water was the only medium present in the oviposition arena (water-based no-choice) and when there was an equal chance to oviposit in two containers with water and two others with AMF-treated water (Equal choice); **B:** Dark grey with black spots (melanizing or

partially melanized) when AMF-treated water was the only medium present in the oviposition arena (water-based no-choice); and **C**: Black eggs (fully melanized) in AMF-based no-choice bioassay.

Fig. 4.

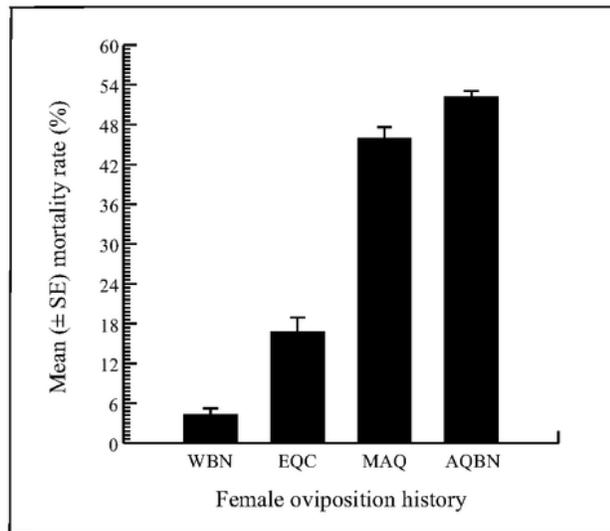


Figure 4

Mortality responses of gravid *Ae. aegypti* females during a 10-day oviposition period in the presence of containers in Aquatain® Mosquito Formulation—(AMF)-treated water under different competition levels with containers holding a water medium. In the graph, WBN, EQC, MAQ, and AQBN stand for “Water-based no-choice”, “Equal-choice”, “More AMF choices” and “AMF-based no-choice”, respectively.

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