Ingested insecticide to control *Aedes aegypti*: Developing a novel dried attractive toxic sugar bait device for intra-domiciliary control

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Abstract (350 words)

Background: Illnesses transmitted by *Aedes aegypti* (dengue, chikungunya and Zika) comprise a considerable global burden; mosquito control is the primary public health tool to reduce disease transmission. Current interventions are inadequate and insecticide resistance threatens the effectiveness of these options. Dried attractive bait stations (DABS) are a novel mechanism to deliver insecticide to *Ae. aegypti*. The DABS are a high-contrast 28 $inch^2$ surface coated with dried sugar-boric acid solution that elicits an ingestion response from *Ae. aegypti* landing on the surface. The study presents the development of the DABS and tests of their impact on *Ae. aegypti* mortality in the laboratory and a series of semi-field trials. *Methods*: We conducted multiple series of laboratory and semi-field trials to assess the survivability of *Ae. aegypti* mosquitoes exposed to the DABS. For laboratory experiments we assessed the lethality, the killing mechanism, and the shelf life of the device through controlled experiments. In the semi-field trials, we released laboratory-reared female *Ae. aegypti* into experimental houses typical of peri-urban tropical communities in South America in three trial series with six replicates each. Laboratory experiments were conducted in Quito, Ecuador, and semi-field experiments were conducted in Machala, Ecuador – an area with abundant wild populations of *Ae. aegypti* and endemic arboviral transmission. *Results*: In the laboratory, complete lethality was observed after 48 hours regardless of physiological status of the mosquito. The killing mechanism was determined to be through ingestion, as the boric acid disrupted the gut of the mosquito. In experimental houses, total mosquito mortality was greater in the treatment house for all series of experiments ($p<0.0001$). *Conclusions*: The DABS devices were effective at killing female *Ae. aegypti* under a variety of laboratory and semi-field conditions. DABS are a promising intervention for interdomiciliary control of *Ae. aegypti* and arboviral disease prevention.

Keywords: *Aedes aegypti*, vector control, toxic sugar bait, attractive bait, semi-field, dengue, arbovirus, ATSB

Background

Arboviral illnesses, including dengue, chikungunya, yellow fever, and Zika, are major contributors to morbidity and mortality in the tropics and subtropics. The burden is particularly apparent in Central and South America: between 2010—2018, the estimated annual number of dengue cases in the region ranged from 500,000 to 2,400,000 [1], and since 2013 it is estimated that there have been a total of >1 million cases of chikungunya [2], and 3-4 million cases of Zika [3]. The viruses causing these diseases are spread mainly by the mosquitoes *Aedes* (*Ae.*) *aegypti* and *Ae. albopictus*, with *Ae. aegypti* serving as the principal vector in many South American countries, including Ecuador [3]. Due to the lack of commercially-available vaccines for most human arboviral diseases, prevention efforts focus on vector surveillance and control methods [4].

Vector control relies heavily on contact-based insecticides, which are available in four main classes: organophosphates, pyrethroids, carbamates, and organochlorines. Indoor residual spraying is a common approach to vector control, for which twelve insecticides are available and approved for human use. This small number of approved insecticides constitutes an impediment for the implementation of effective vector control strategies (such as pesticide rotation cycles) aimed at decreasing the development of resistance to any single insecticide [5]. As a result, pesticide resistance has become a major limitation for current vector control strategies, and is widespread in South American countries [6–8]. Our current reliance on a few chemical molecules to control *Ae. aegypti* is an increasingly flawed strategy, as evidenced by the proliferation of this disease vector across the globe and increasing arbovirus epidemics [9].

In contrast to the contact-based insecticide approach of the public health sector, the agricultural industry has focused on ingested insecticides for pest control. The use of ingested insecticides could be applied in disease control programs and interventions if disease vectors are

successfully led to ingest the insecticide. One solution, attractive toxic sugar baits (ATSB), exploits the nectar-feeding behavior of mosquitoes [10,11] to deliver the insecticide. An ATSB uses a mixture of a lethal agent with sugar water and sometimes an additional attractant [12]. ATSBs have been tested for *Anopheles* spp. [13–17], *Culex* spp. [15,16,18,19], *Ae. albopictus* [20–23], and other vector or nuisance species [16] with a variety of attractants, baits, active ingredients, designs, and placement strategies. Although laboratory bioassays demonstrate that ATSBs are toxic to *Ae. aegypti* [16,24,25], semi-field and field evaluations have had poor results in reducing *Ae. aegypti* populations [26,27], indicating that ATSB devices must be carefully designed and tested for each target species [12].

Compared to other mosquito species, *Ae. aegypti* appear to have a lower propensity for sugar-feeding, preferring human blood meals instead [11]. Despite this, *Ae. aegypti* females will readily feed on sugar in the laboratory, and often feed on plant sugars in the wild [28–30]. However, traditional attractive sugar bait strategies that rely only on sugar as an attractant are likely insufficient to “lure” female *Ae. aegypti* in the natural environment

Herein we present the development of dried attractive bait stations (DABS) (Figure 1), and show results from laboratory and semi-field experiments. In the laboratory we first identified the lethality of DABS (Series 1.1), aimed to identify the killing mechanism of the DABS (Series 1.2), assessed how the physiological status altered the effectiveness of DABS (Series 1.3), and assessed the shelf life of the DABS (Series 1.4). In the semi-field trials, we sought to determine the timing of mosquito mortality (Series 2.1), assess the relationship between DABS exposure time and mosquito mortality (Series 2.2), and to demonstrate these effects in the presence of competing attractants (Series 2.3).

Results

Laboratory experiments

Series 1.1: Effects of DABS exposure on mosquito survival

Mosquitoes exposed to DABS presented 55% survival probability reduction in the first 24 hours post-exposure, and an additional 45% reduction between 24h and 48h post-exposure, resulting in 100% mortality by the end of the experiments (Figure 2). In contrast, mosquitoes exposed to control devices presented only a 1% reduction in their survival probability during the first 24h. This value increased to 2% during the second 24h period, resulting in 98% survival probability by the end of the experiment. Differences between the survival curves of toxic and control treatments were highly significant ($p<0.001$).

Series 1.2: Characterization of the biological mode of action of the device.

After 48 hours, mosquitoes that could still feed (*i.e*. mosquitoes with an intact proboscis), presented 100% mortality when exposed to the toxic device, and 3% mortality when exposed to the non-toxic control device. Mosquitoes that could not feed (those with ablated proboscis) presented 38% mortality regardless of inclusion in the ATSB-device condition or control non-toxic condition. Significant differences were found between the four treatments ($p<0.001$). Post-hoc pairwise comparison determined that (a) the mortality of ablated mosquitoes exposed to toxic devices was not significantly different to the mortality of ablated mosquitoes exposed to control devices, and (b) the mortality of ablated mosquitoes was significantly different from the mortality of whole mosquitoes exposed to toxic devices and whole mosquitoes exposed to control devices (Figure 3).

Mosquitoes that had ingested toxic sugar solution presented histological abnormalities in the posterior midgut. Electron micrographs revealed disruptions in the continuity of the gut epithelium (Figures 4A, 4C). In addition, we found abnormal adipocytes that we hypothesize are undergoing a process of necrosis (Figures 4E, 4F), and an increase in both the size and number of basal infolds in the gut epithelial cells. We hypothesize that boric acid ingestion is the cause of these pathological changes, which contributed to the mortality observed in specimens exposed to

toxic devices. Microscopic images of individuals exposed to control devices presented none of these pathologies on the posterior midgut (Figures 4B, 4D).

Series 1.3: assessment of mosquito biological status on DABS effectiveness

Mosquitoes with both physiological statuses evaluated (blood fed and parous) presented a lower survival probability when exposed to toxic devices than when exposed to non-toxic control devices. Blood fed females' survival probability dropped 13% during the first 24h interval, 22% during the second 24h interval, and 55.6% during the last interval, resulting in 8.89% survival probability by the end of the experiment. Blood fed non-toxic mosquitoes exposed to control conditions resulted in 90% survival probability at the end of 72 hours after having dropped 9%, 1%, and 0% during the three 24-hour intervals, respectively (Figure 5A). Differences between control and toxic treatment survival curves were significant ($p < 0.001$)

Parous female mosquitoes presented a 66% decline in survival probability during the first 24 hours and a 0% survival probability after 48 hours of exposure to the ATSB devices (Figure 5B). These results are significantly different ($p < 0.001$) from the non-toxic control group, that showed a drop in survival probability of 2% during the first 24h, resulting in 98% survival after 48 hours of exposure.

Series 1.4: Assessment of shelf-life of the DABS device

Mosquitoes exposed to toxic devices stored for 38 days showed 0% survival probability after the first 24 hour interval; mosquitoes exposed to control conditions showed 96% survival through 48 hours (Fig. 6A). Differences in survival between conditions were highly significant ($p < 0.001$). Mosquitoes exposed to toxic devices stored for 80 days showed 16% survival probability after the first 24 hour period and 0% survival probability after 48 hours; mosquitoes exposed to control conditions showed 97% survival probability after 48 hours (Fig. 6B). Differences in survival

between conditions were highly significant ($p < 0.001$). Mosquitoes exposed to toxic devices stored for 118 days showed 95% survival probability after the first 24-hour period, 64% survival during the second 24-hour interval, and 35% survival probability after 72 hours; mosquitoes exposed to control conditions showed 96% survival probability after 72 hours (Fig. 6C). Differences in survival between conditions were highly significant ($p = 0.001$).

Semi-field experiments

The results of Series 2.1 (24 hours of exposure to DABS) are shown in Figure 7. Mosquito mortality was 0—6% in the control and 17—57% in the treatment house after 24 hours ($p<0.001$). At 48 hours, mortality was 0—18% in the control and 22—51% in the treatment house ($p=0.003$). At 72 hours, mortality was 0—4% in the control and 0—2% in the treatment house ($p=0.458$). The cumulative mortality of the control was 4—18% and 54—98% in the treatment house ($p<0.001$). Following 24 hours of exposure to DABS in the house, most mosquito mortality was observed within the first 48 hours of the experiment, with no difference in mosquito mortality after this time period.

The results of Series 2.2 (48 hours of exposure to DABS) are shown in Figure 8. Mosquito mortality was 2—23% in the control and 77—100% in the treatment house ($p<0.001$), indicating high mortality from 48 hours of exposure to DABS in the treatment houses.

The results of Series 2.3 (48 hours of exposure to DABS in the presence of competing attractant) are shown in Figure 9. Mosquito mortality was 2—33% in the control and 68—100% in the treatment house ($p=0.006$), indicating that DABS results in high mortality even in the presence of a competing attractant.

When comparing the results of 24 hours (Series 2.1) to 48 hours of exposure (Series 2.2), 48 hours of exposure results in higher mortality at 48 hours ($p<0.001$) in the treatment group (Table 1), with no difference in the control groups ($p=0.151$). When comparing 48 hours of exposure to DABS

only (Series 2,2) and 48 hours of exposure to DABS in the presence of a competing attractant (Series 2.3), there is no effect of a competing attractant on the effect of DABS on mosquito mortality (p=0.784) in the treatment group (Table 1). High mortality from 48 hours of DABS exposure was observed despite the presence of a competing attractant.

Discussion

These experiments demonstrate that DABS can strongly impact the mortality of female *Ae. aegypti* under laboratory and semi-field conditions. In these settings, we show that mortality occurs within the first 48 hours of exposure to our devices. In addition, DABS attract and kill *Ae. aegypti* even in the presence of an alternative sugar source. To the best of our knowledge, this device is the only known "dry" ATSB. The simple and economic design lends itself to in-home use in resource limited settings where *Ae*. *aegypti* target human hosts and transmit dangerous arboviruses.

Our assessment of the biological action of the devices provides an insight into the mechanism by which low concentrations of boric acid affect *Ae. aegypti*. We determined that boric acid enters the insect´s body by ingestion, further supporting the notion that this inorganic pesticide acts as a stomach poison, as previously suggested (Xue R-D, Kline DL, Ali A, Barnard DR. Application of boric acid baits to plant foliage for adult mosquito control. J Am Mosq Control Assoc. 2006;22: 497–500. Xue R-D, Barnard DR. Boric acid bait kills adult mosquitoes (Diptera: Culicidae). J Econ Entomol. 2003;96: 1559–1562). Based on our electron microscopy analysis, we hypothesize that the ingestion of boric acid disrupts the integrity of the gut epithelium.

Considering that the proposed mechanism by which boric acid exerts its toxic effect (gut disruption) is markedly different from the neurotoxic mechanism by which most traditional pesticides cause mortality, we propose that our devices have the potential to act as efficient complementary tools to combat the spread of resistance to traditional pesticides. By combining the use of DABS with traditional pesticides in the same areas, it would be possible to target two

different and crucial systems (namely, the nervous and digestive systems) in the insect´s body simultaneously, thereby reducing the mosquito´s probability of survival.

We observed significant mortality of blood fed female *Ae. aegypti* exposed to the DABS device, albeit at lower rate than starved females. Interestingly, the largest drop in survival probability in blood fed females is observed between 48h and 72h post-exposure to the device (Fig 5), suggesting that after 48h, females have already used imbibed blood for the development of eggs and are keen to search for further meals. Based on this evidence, it is plausible to suggest that if employed in the field, DABS devices may be efficient in killing female mosquitoes of various physiological statuses, including females that have already ingested blood – a particularly important group for disease transmission.

Novel vector control methods have the potential to serve as critical tools in the public health effort to control persistent and emerging vector borne diseases. Various designs of ATSBs have had promising field trials for potential control of *Aedes albopictus*, *Anopheles* spp. and *Culex* spp. [15–17,20,21,24]. Unfortunately, this success has not yet been achieved in efforts to control *Ae. aegypti*, a vector of major public health importance. Previous research shows that several formulations of ATSBs can achieve *Ae. aegypti* mortalities above 80% in laboratory settings [16,25], but these ATSBs are ineffective in semi-field or field settings [26,27]. The principle barrier to their success was an inability to sufficiently attract *Ae. aegypti* to ATSBs, rendering them ineffectual. Particularly, ATSBs using synthetic floral-based attractants, while attractive to male *Ae. aegypti* in a laboratory setting, do not function well when targeting *Ae. aegypti* in field evaluations [26]. An ATSB with a host-based attractant (*i.e.* CO_2) was unable to meaningfully reduce adult populations [27].

We hypothesize that our device attracts *Ae. aegypti* with strong visual cues (as opposed to a chemical) as an attractant. *Ae. aegypti* are container breeders [31,32], that utilized tree holes in their natural forested habitat before adapting to life in human civilization. The DABS device has a high-contrast (black and white) 28-inch^2 surface to simulate a refuge for *Ae. aegypti* [33]. High contrast

coloring has similarly been integrated into prior trap designs and has been shown to improve capture rates of *Ae. aegypti* [34]. We believe the high-contrast coloring of DABS draws *Ae. aegypti* to land on the device.

These experiments have demonstrated the effectiveness of DABS on *Ae. aegypti* in laboratory and semi-field experimental conditions. Our approach differs from most ATSB approaches in two important ways: firstly, we use a dried sugar solution to elicit an ingestion response while other ATSBs typically use liquid nectar-simulants [12,15,17,26]. We hypothesize that the dried sugar solution is a key element in the effectiveness of DABS. Similar to other diptera [35], *Ae. aegypti* are able to evaluate surfaces with their feet, and the "taste" of a landing surface can either lead the mosquito to feed and ingest, or reject, the surface [36]. Additionally, the dried sugar solution provides two operational advantages over liquid solutions: liquid solutions are more difficult to manufacture, ship, and distribute than dried solutions, and the dried device can be smaller and more easily deployed. Secondly, we use a visual rather than chemical attractant to lure *Ae. aegypti* to the device. Chemical attractants add to the cost and decrease the shelf life of any device. Previous research has questioned the ability of sugar solutions alone to attract mosquitoes [26,37], leading to research on chemical attractant additives for ATSBs, but the use of chemical attractants in ATSBs targeting *Ae. aegypti* have been unsuccessful [26,27]. We demonstrate that a simple black-and-white visual attractant is a sufficient motivator for female *Ae. aegypti* to land on the surface of DABS even in the presence of a competing oasis and alternative sugary food. Taken together, we hypothesize that the visual cues attract the *Ae. aegypti* to land on the device, upon which the presence of the dry sugar on the device's surface entices the insect to ingest it. When this sugar solution is mixed with boric acid, ingestion results in insect mortality.

We propose that these encouraging results justify larger field trials of DABS in open-air environments. We show that 48 hours of DABS exposure leads to high mosquito mortality when used in the laboratory and in experimental houses reminiscent of peri-urban tropical housing. Furthermore, we have established that the effectiveness of DABS for killing *Ae. aegypti* is

maintained even after prolonged storage periods, a characteristic that would facilitate their use in semi-field and field conditions.

Semi-field trials are a crucial step to bring a scalable, marketable product to in-home field testing. An in-home approach is ideal for control of *Ae. aegypti*, as the vector has an extremely limited flight range, often spending its entire life within a single household [5,32,38]. Other research with ATSBs has shown that end-users of these products prefer to have them placed indoors [14]. The successful design and placement strategy of DABS used in our experiments indicate that the device is ideal for in-home field testing.

Limitations

These experiments were conducted under laboratory and semi-field conditions, which can only moderately emulate real-world/field conditions. Semi-field experiments were limited to nulliparous females and we cannot be certain how DABS will affect gravid or blood-fed females or males in an open-air environment—though it should be noted that DABS were equally effective in attracting and killing blood-fed and nulliparous females under laboratory conditions. It is also unclear if DABS would impact non-target insect species, such as butterflies or other pollinators, though if DABS are limited to use inside the home, it is unlikely to affect these species. Although DABS performed well in the presence of a competing attractant (100 grams of apples), it is unlikely that the attractant used in our experiments are a realistic substitute for open-air field conditions. An actual home will contain many competing attractants, including human hosts. It is difficult to know if the success of DABS in semi-field conditions will be replicated in occupied homes in the field; the number and placement of DABS may need to be modified. In addition, it is unclear how end users will react to placement of DABS in their homes, although there is evidence that residents in areas of high *Ae. aegypti* burden are willing to utilize numerous home-based mosquito control products [39].

Conclusions

With careful design and device placement consideration, we have created a promising vector control device ready for large-scale trials to test its ability to control *Ae. aegypti* in natural conditions. We demonstrated that DABS are capable of attracting and killing female *Ae. aegypti* in experimental houses, and that 48 hours in the presence of DABS leads to high mortality among female *Ae. aegypti*. Importantly, DABS were efficient at killing female mosquitoes of diverse physiological statuses, and can attract and kill female *Ae. aegypti* even in the presence of a competing attractant. These devices have promising effectiveness and this semi-field evaluation of DABS is an important step forward in the development of novel devices for the control of *Ae. aegypti*.

Materials and methods

Aim and design

The general aim of our study was to assess the effectiveness of DABS at killing female *Ae. aegypti* under laboratory and semi-field experimental conditions. Our specific aims were (a) to determine the timing of mosquito mortality (Series 1), (b) to assess the relationship between DABS exposure time and mosquito mortality (Series 2), and (c) to demonstrate these effects in the presence of competing attractants (Series 3). These aims were tested in three series of six trial replicates to compare control and treatment experimental conditions.

Study setting

Laboratory experiments

Laboratory experiments were conducted at the Center for Research on Health in Latin America (CISeAL, by its Spanish acronym), where they were reared and maintained under standard insectary conditions: 28 ± 1°C temperature, 80 ± 10% relative humidity, and a 12h:12h (L:D) light cycle. Larvae were fed finely ground fish food. When required, mosquitoes were sexed during the pupal stage. Adults were kept in 15x15x15 cm cages. For maintenance, adult mosquitoes were fed 10% sucrose solution *ad libitum*. For blood feeding, female adult mosquitoes were offered access to a restrained female mouse. All mosquitoes were maintained under insectary conditions between 0 and 14 days after adult emergence before they were used for experiments. Mosquitoes referred to as "starved" in this manuscript were deprived of access to sugar or blood (but not water) for 48h prior to their use in experiments.

Semi-field trials

Assays were performed in experimental houses meant to emulate typical housing found in areas with active dengue transmission. Photographs of the houses are available in an additional file (see Additional file 1). The houses are constructed of wood and cane and are raised on a 1-meter platform with walkways to improve structural integrity and facilitate window access; one window on each house is equipped with window escape traps with sleeves to monitor escape behavior. The dimensions of the houses are 3.85 meters (m) wide x 4.85 m long x 3 m high. Each house has three windows (0.9 m wide × 0.6 m high) and one door (1.03 m wide × 3 m high). The house frames are made of wood, untreated wooden plank flooring, walls of untreated cane, and a roof of zinc panels. The window traps are 0.45 m long x 0.66 m wide x 0.45 m high. The houses are located on campus at the Universidad Técnica de Machala in the city of Machala, Ecuador (3°15' S, 79°57' W), a region with abundant wild populations of *Ae. aegypti* and endemic arbovirus transmission. Experiments were conducted under ambient climate conditions (temperature range: 23.1—35.6° C, mean temperature: 28.4° C, relative humidity range: 43.9—95.0%, mean relative humidity: 75.8%).

Each trial replicate was conducted with one control and one experimental house; the specific house used as the experimental or control house was alternated upon each replicate.

Biological material

Aedes aegypti eggs were provided by the Center for Research on Health in Latin America (CISeAL, by its Spanish acronym) at the Pontificia Universidad Católica del Ecuador. All strains used in this work originated from Ecuador, and had been maintained in laboratory conditions since 2015. Laboratory work was performed with strains originally collected in Ecuador from the cities of Guayaquil and Puerto Francisco de Orellana. Semi-filed work was performed with a strain originally collected in the city of Machala.

Semi-field experiments

Hatching and rearing of *Ae. aegypti* for semi-field experiments were performed at the Laboratory of Entomology at the Universidad Técnica de Machala. Considering this laboratory is located in a region where *Ae. aegypti* actively reproduces and thrives, environmental conditions (temperature: 28-32 °C; relative humidity: 60-80%) were not artificially controlled in our mosquito-rearing facilities. A vacuum pressure system was used to synchronize egg hatching (one hour exposure to obtain first stage larvae). Larvae were fed with finely ground fish food. At the pupal stage, males and females were separated. Adults were kept in 15x15x15 cm cages. Adults were fed on 10% sugar solution *ad libitum*. Each experimental semi-field experiment series used nulliparous females aged 1—5 days and starved for 24 hours prior to experimental release.

Dried Attractive Bait Stations (DABS)

The DABS device consists of two concentric foam disks (an inner white disk one centimeter in diameter, and an outer black disk eight centimeters in diameter). Experimental DABS were impregnated with a 10% sucrose solution containing 1% boric acid as a lethal agent. Control DABS were impregnated with 10% sucrose solution without boric acid. Photographs of the devices are available in an additional file (see Supplementary Figure 2, Additional file 2).

Laboratory experiments

Series 1.1: Survival assessment of mosquitoes exposed to the device

To determine whether exposure to the DABS devices has an influence on adult mosquito survival probability, we conducted an experiment in which groups of 30 adult female mosquitoes, placed in a 15x15x15cm cage, were exposed during 48 hours to either an DABS device or a control device (sugar solution but no boric acid). We replicated each experiment four times. The assessment was repeated using each of the two laboratory strains described previously.

Series 1.2: Appraisal of biological mode of action of the device

To establish whether the toxic component of DABS needs to be ingested by the mosquitoes in order to exert its effect, we presented the devices to cohorts of adult females that were unable to ingest food due to the surgical ablation of their mouthparts. To establish these cohorts, individual mosquitoes were first anesthetized by placing them at 4°C during 10-15 minutes. Anesthetized specimens were individually placed under a dissection microscope and, using a human hair, we tied a knot at the proboscis' proximal end in order to create a constriction that would impede the flow of food. Subsequently, the part of the proboscis anterior to the knot was removed using micro-dissection scissor (Supplemental Figure 3). Following the surgery, mosquitoes were left to rest for 24 hours before being used in any experiment. To control for the potential negative effect of the

anesthetizing procedure in mosquito survival, non-ablated mosquitoes used in control groups were also placed at 4°C during 10-15 minutes, and allowed to recover during 24 hours before experimental set-up.

We conducted the experiment with four separate cages, each with 20 starved mosquitoes (Table 2 shows different treatments). We treated cage 1 with toxic DABS devices and used 20 ablated mosquitoes; cage 2 held non-toxic control devices and 20 ablated mosquitoes. We treated cage 3 with toxic DABS devices and non-ablated mosquitoes; cage 4 held a non-toxic control device and non-ablated mosquitoes. We assessed mortality in all groups at 24 and 48 hours of exposure to the devices. We replicated the experiment three times.

We then conducted an experiment wherein 30 adult starved female mosquitoes were introduced to a cage with a DABS device, and 30 adult starved female mosquitoes were introduced to a cage with a non-toxic control device. We monitored cages for 24 hours, and removed dead mosquitoes by aspiration every hour from the cages. Using a dissection microscope, we removed the legs, head, and wings of every dead specimen onto a drop of 70% ethanol. Through this process we gently disrupted the abdominal cuticle to permit the exposure of internal tissues to the fixative. Afterwards we fixed individual mosquitoes in a solution containing 2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1M cacodylate buffer (pH 7.4), and stored them at 4°C for 72 hours. We then washed specimens in cacodylate buffer with 0.1M sucrose overnight. Post-fixing was achieved by leaving the specimens for two hours at 4°C in 2% osmium tetroxide in 0.1 cacodylate buffer (pH 7.4). Subsequently, individuals were stained using 2% uranyl acetate and left to rest for three hours in the dark at room temperature. Tissues were later dehydrated through a series of ethanol baths (50%, 70%, 95%, 100%). Afterwards, they were placed in propylene oxide for 30 minutes, then in a 1:1 volume propylene oxide resin mixture (Epon 812, Araldite 502, dodecenyl succinic anhydride, benzyl dimethylamine) for one hour and later, one more volume of resin was added and left on a rotator overnight. Finally, mosquitoes were embedded in resin and incubated at 60°C for 24 hours.

Resin were stained using 2% uranyl acetate. We then utilized a transmission electron microscope to observe specimens and obtain micrographs of interesting tissues.

Series 1.3: Effects of the physiological status of the mosquitoes on the performance of DABS

We examined two different physiological statuses using mated starved female adult mosquitoes, namely blood fed and parous. We established females deemed as “blood fed” by selecting blood-engorged individuals immediately after a blood meal. We established females deemed as “parous” by first blood feeding and subsequently maintaining mosquitoes for 7 days under insectary conditions in order to ensure that they had oviposited before being used for experimentation. We set up two cages for each of the defined physiological statuses, each with 30 mosquitoes. One cage exposed the mosquitoes to an ATSB device, and the other held a control non-toxic device. We gathered survival data at 24 and 48 hours following introduction to the cages, and replicated these experiments three times.

Series 1.4: Shelf-life of the device

In order to determine the shelf life of ATSB devices, toxicity tests were performed using devices which had been stored for 38, 80, and 118 days after their production. For storage, devices were individually wrapped inside a sealed plastic bag and placed in an incubator at 28 ± 2°C and 80 ± 10% relative humidity. We conducted three replicates of previously described experiments for each storage time.

Semi-field trials

Series 2.1: 24 hours of DABS exposure in experimental houses

Each house contained four DABS devices (control or treatment DABS as appropriate) suspended on strings attached to the roof of the house at a height of 30—50cm above ground and approximately 30cm from the nearest wall. Diagrams of the experimental layout are available in an additional file (see Supplementary Figure 3, Additional file 2). For each trial replicate, 50 female *Ae. aegypti* were released into each house through the escape window sleeve (release time 11:00a.m.—2:00p.m.). Twenty-four hours after release, dead mosquitos were collected from the floor and window escape traps in each house, and the remaining live mosquitoes were captured with a hand-held aspirator (Prokopack, John W. Hock Company, Gainesville, USA). All live mosquitoes were labeled by experimental group and observed for 48 additional hours in laboratory cages (under laboratory conditions with food available). Mortality was calculated for 24 hours, 48 hours, and 72 hours. Six trial replicates were performed for Series 1.

Series 2.2: 48 hours of DABS exposure in experimental houses

Each house contained four DABS devices (control or treatment DABS as appropriate) and two sources of water (wet cotton in a black plastic bucket). Photographs of the water source are available in an additional file (see Supplementary Figure 3, Additional file 2). For each trial replicate, 50 female *Ae. aegypti* were released into each house through the escape window sleeve (release time 8:00a.m.—11:00a.m.). Forty-eight hours after release, dead mosquitos were collected in each house and remaining live mosquitoes were captured with an aspirator. Mortality was calculated for 48 hours. Six replicates were performed for Series 2.

Series 2.3: 48 hours of DABS exposure in experimental houses with competing attractant

Each house contained four DABS devices (control or treatment DABS as appropriate), two sources of water (wet cotton in a black plastic bucket), and 100 grams of peeled, cut apples in a dish placed on a chair in the center of the house as a competing attractant. Photographs of the competing

attractant are available in an additional file (see Supplementary Figure 3, Additional file 2). Recently emerged female *Ae. aegypti* rely on sugar meals for energy, these meals may include aging fruit and female *Ae. aegypti* will feed on fructose (as is found in apples). For each trial replicate, 50 female *Ae. aegypti* were released into each house through the escape window sleeve (release time 9:00a.m.—12:00p.m.). Forty-eight hours after release, dead mosquitos were collected in each house and remaining live mosquitoes were captured with an aspirator. Mortality was calculated for 48 hours. Six replicates were performed for Series 3.

Statistical analyses

For each replication of the Series 1.1 experiments, we plotted and analyzed interval censored survival data and non-parametric likelihood estimates. We then used a log-rank hypothesis test to compare the survival distributions of the two treatments. In Series 1.2, we examined the normal distribution of the data with Kolmogorov-Smirnov and Shapiro-Wilk tests. We assessed treatment group using an analysis of variance and a post-hoc's test to determine ranks. For Series 1.3 and 1.4, we plotted and analyzed censored survival data with a logrank hypothesis test to compare the survival distributions of experiment and control treatments. In Series 2.1—2.3, mosquito mortality data from each series were compared using a two-tailed paired t-test (paired by replicate). Mean mosquito mortality was compared across series using a two-tailed t-test. Data were analyzed using Excel (Microsoft, Redmond, USA) and R (R Foundation for Statistical Computing, Vienna, Austria) with the survival package [42,43].

List of abbreviations

Ae.=Aedes

ATSB=attractive toxic sugar bait

DABS=dried attractive bait stations

cm =centimeters

m=meters

Declarations

Ethics approval:

These study protocols were found to be exempt from Institutional Review Board review by Syracuse University Institutional Review Board.

Consent for publication:

Not applicable

Availability of data and material:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

ASI, DL, and MN hold a patent on the DABS technology.

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Author contributions:

MN, ASI, and DL designed the study.

MN, ASI, DL, and EBA provided study materials.

GR, ML, AA, BM VS and FHH performed the experiments.

RH performed electron microscopy work

RS and GR conducted data analysis.

RS, GR, MN, VS, and FHH drafted the manuscript.

All authors reviewed and approved the final draft of the manuscript.

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Additional files

Additional file 1 (pdf)

Supplementary Figure 1: Experimental Houses

Additional file 2 (pdf)

Supplementary Figure 2: Experimental Layout

Additional file 3 (pdf)

Supplemental Figure 2: Feeding disruption procedure. A) Anesthetized individual with whole proboscis. B) Human hair tied at the proximal end of the proboscis. C) Micro-dissection scissor removal of proboscis' segment anterior to the knot. D) Feeding disrupted individual.

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Figures

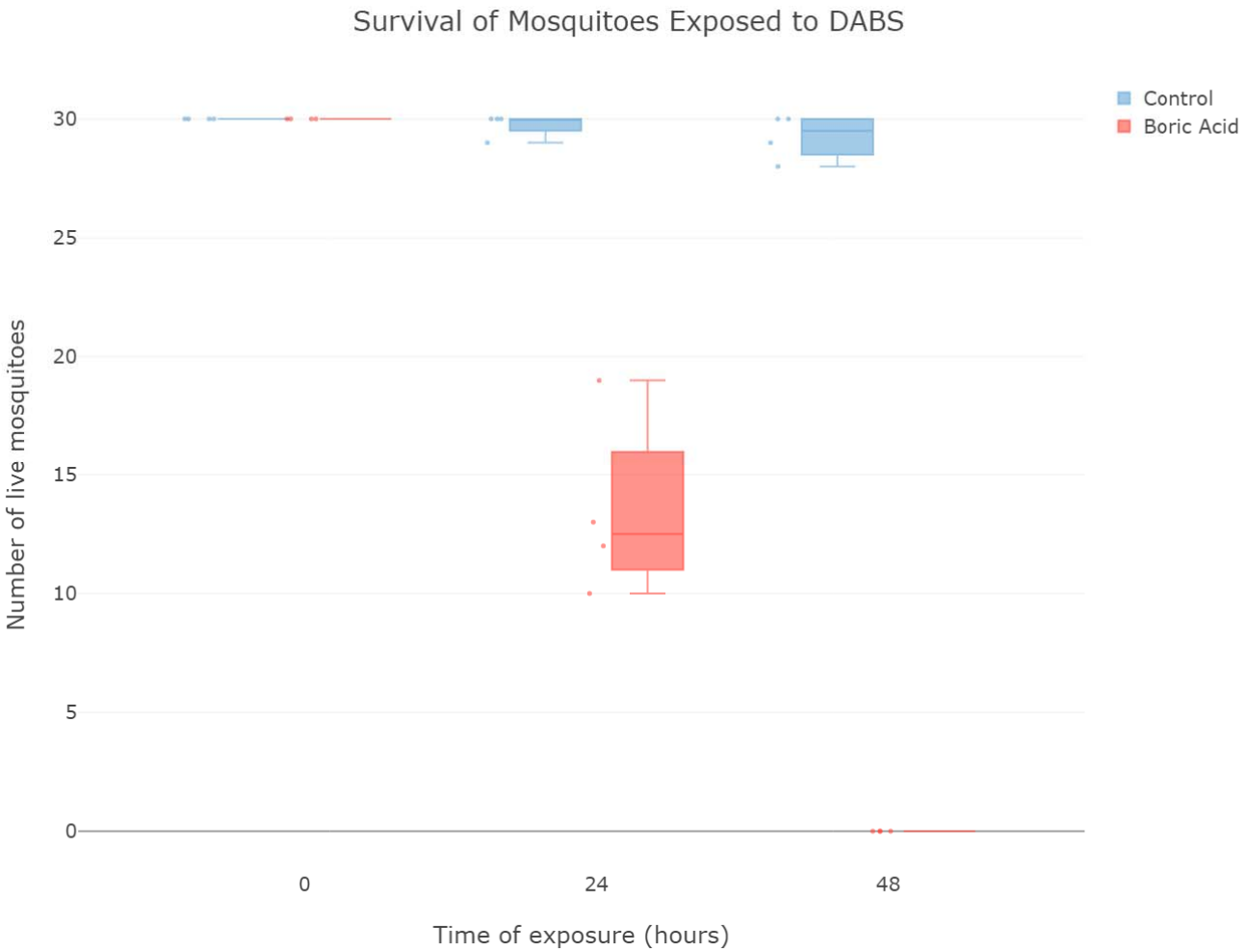

Figure 1. Attractive toxic sugar bait devices. A) 3D model of the DABS devices. Diameters for each of the foam sheet circles are shown.B) Photography of a DABS device used in the study.

Figure 2. Survival assessment of mosquitoes exposed to the device. All mosquitoes (n=30) exposed to toxic devices die after 48 hours of exposure. When presented with non-toxic device almost all survive. Box plot indicating median 25% and 75% quartiles. Error bars indicate max and min values. Each dot indicates an independent count of mosquitoes (y axis) exposed to control devices (blue) or toxic devices (red) at different time points (x axis).

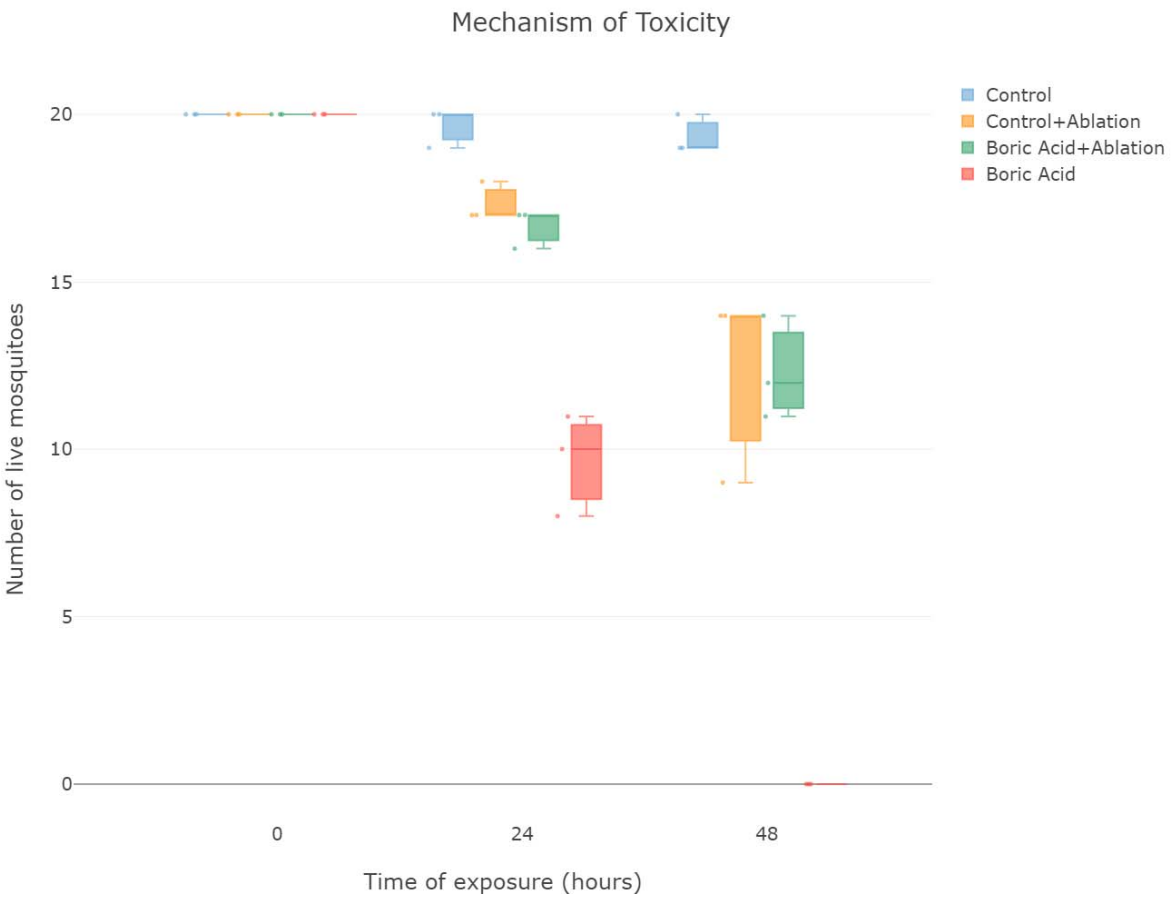

Figure 3. Uptake mechanism of the toxic component. Toxic effect is dependent on the ability of mosquitoes to ingest the toxic component. When mosquitoes are able to ingest the toxic component all mosquitoes (n =20) die after 48 hours (red). Mosquitoes with ablated mouthparts die equally regardless of the toxic or non-toxic condition of the device (green and yellow). Almost all mosquitoes with no ablation or toxic devices survive. Box plot indicating median 25% and 75% quartiles. Error bars indicate max and min values. Each dot indicates an independent count of mosquitoes (y axis) exposed to control devices (blue), control devices + mosquito mouthparts ablation (yellow), toxic devices + mosquito mouthparts ablation, or toxic devices (red) at different time points (x axis).

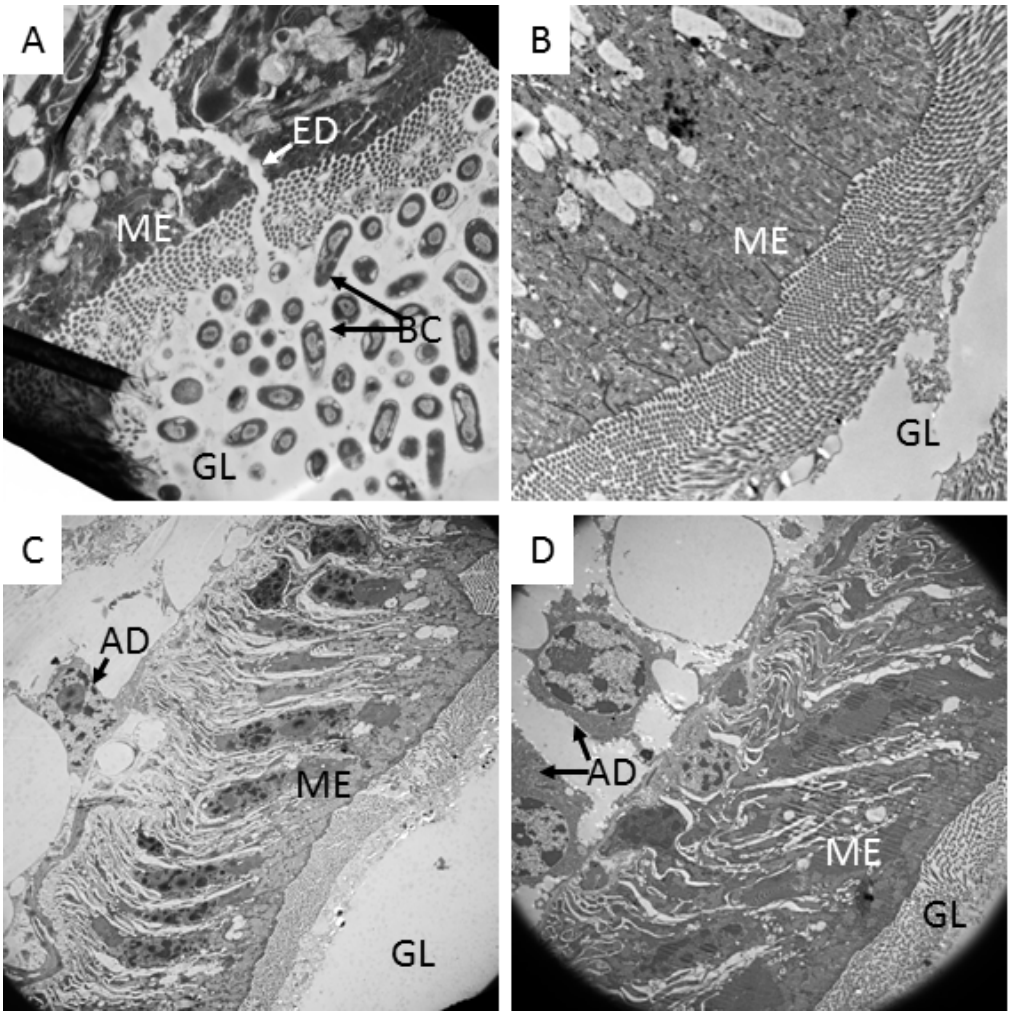

Figure 4. Histopathological effects on the midgut. Longitudinal sections of Ae. aegypti posterior midgut. Panels A, C, D: Mosquitoes exposed to toxic devices. Panel B: Mosquito exposed to non-toxic device. Specimens exposed to toxic devices showed disruptions in the gut integrity (ED, panel A). Because of the even distribution of adjacent bacterial cells in the gut lumen, this disruption is unlikely to be the result of sample processing for electron microscopy. Abbreviations: AD: Adipocite; BC: Bacterial cells in gut lumen; ED: Epithelial disruption; GL: Gut lumen; ME: Midgut epithelium. Magnifications: A: 15,000x; B: 10,000x; C: 3,000x; D: 5,000x.

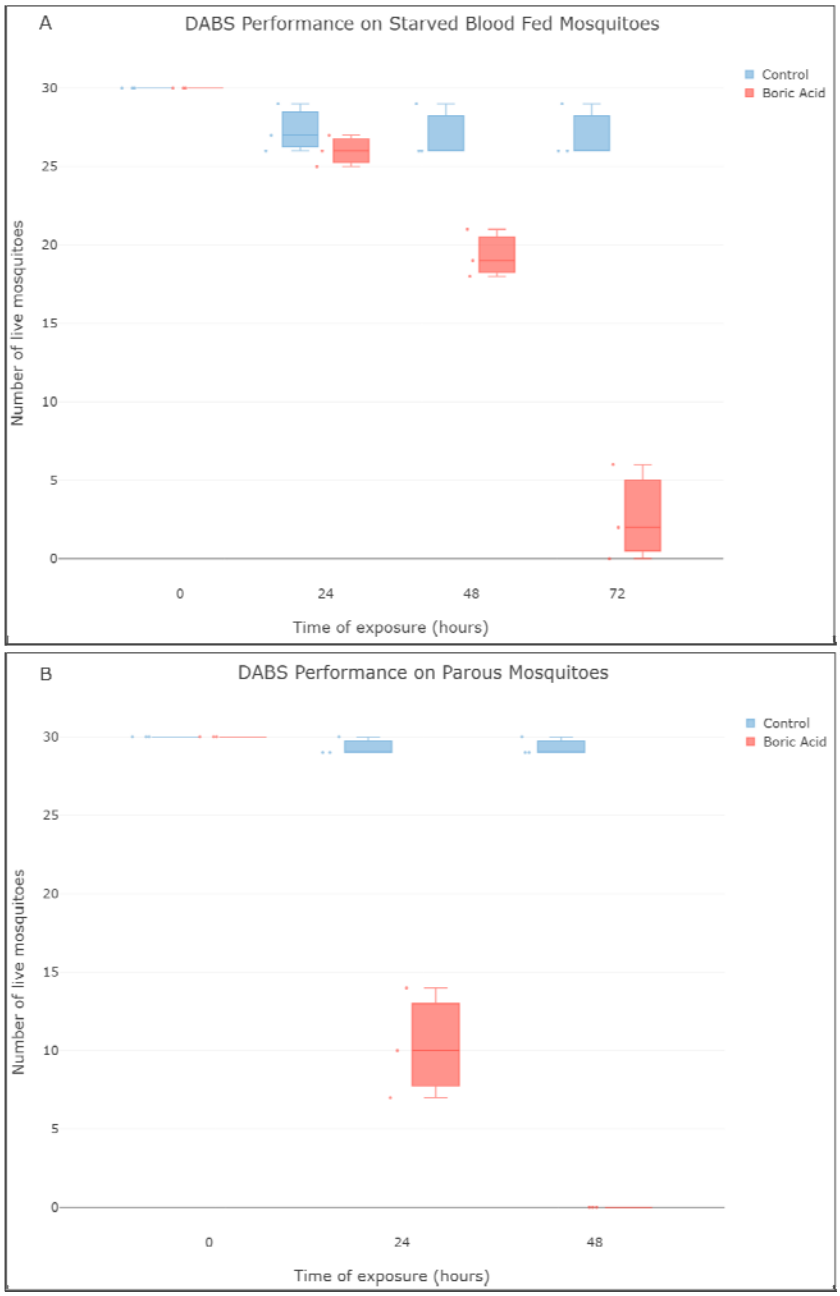

Figure 5. Effects of the physiological status of the mosquitos on the performance of the device. DABS are efficient at killing mosquitoes through different physiological stages. (A) A significant majority of blood fed mosquitoes exposed to toxic devices die after 72 hours of exposure. When presented with non-toxic device almost all survive. (B) All parous mosquitoes exposed to toxic devices die after 48 hours of exposure. When presented with non-toxic device almost all survive. Box plot indicating median 25% and 75% quartiles. Error bars indicate max and min values. Each dot indicates an independent count of mosquitoes (y axis) exposed to control devices (blue) or toxic devices (red) at different time points (x axis).

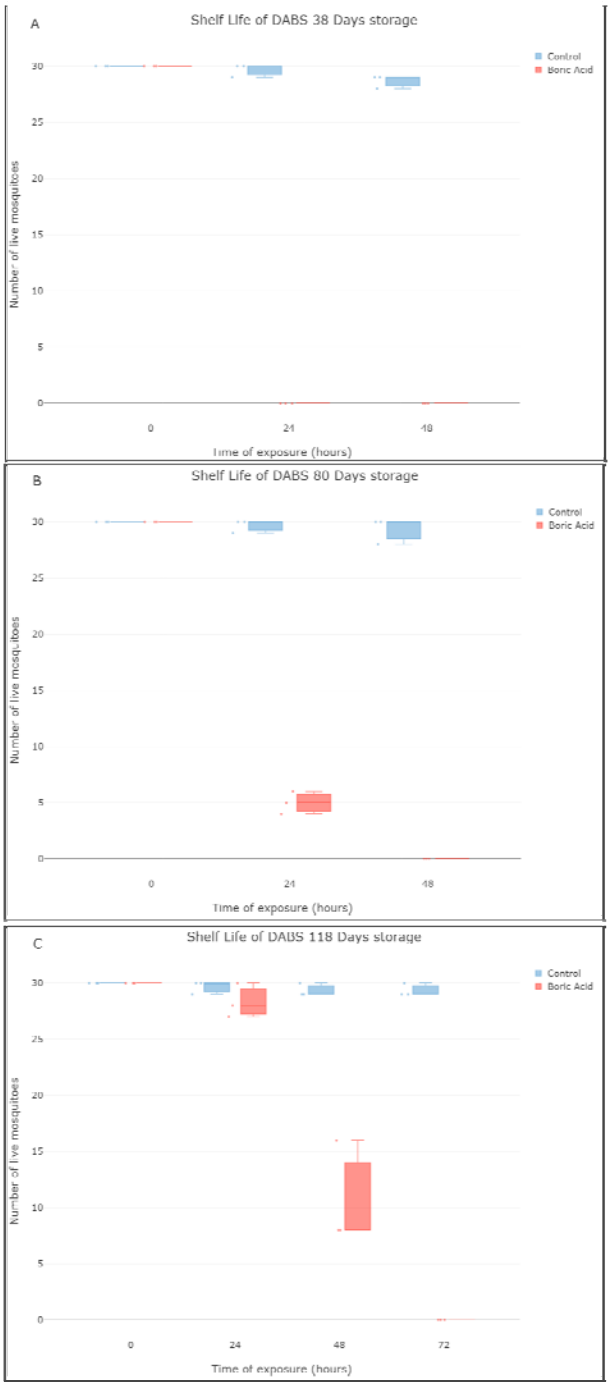

Figure 6. Shelf life of the device. DABS are still effective after (A) 38 days, (B) 80 days and (C) 118 days of storage in insectary conditions. Box plot indicating median 25% and 75% quartiles. Error bars indicate max and min values. Each dot indicates an independent count of mosquitoes (y axis) exposed to control devices (blue) or toxic devices (red) at different time points (x axis).

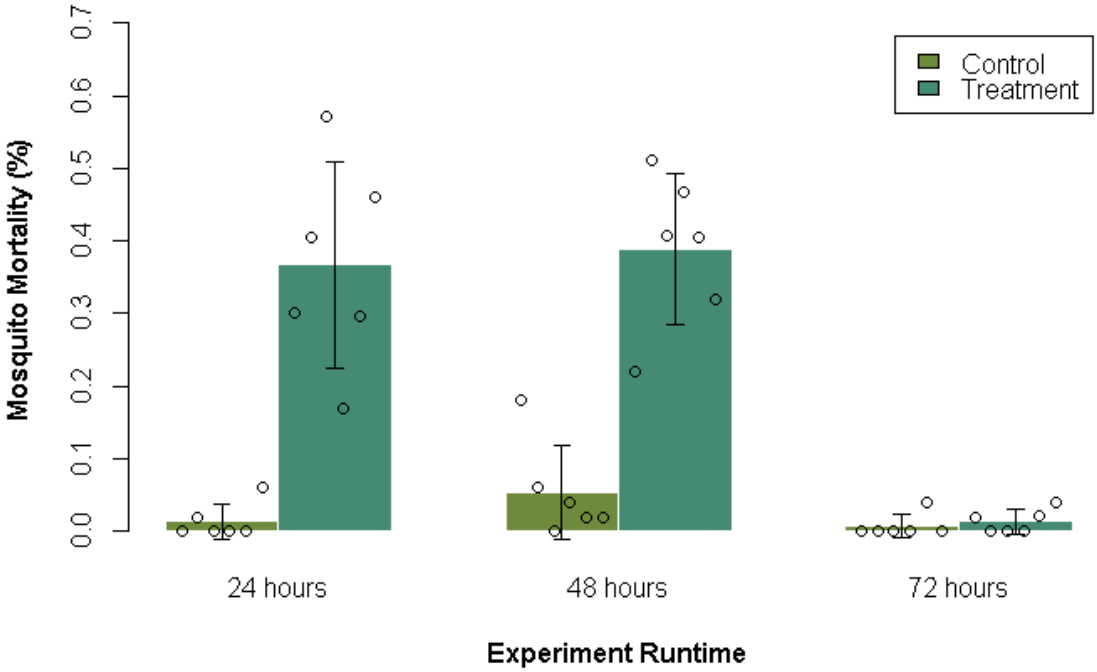

Figure 7: Results of Series 2.1. Mosquitos were exposed to DABS for 24 hours and observed afterwards; mosquito mortality was calculated immediately after the exposure period, and during laboratory observation at 48 hours and 72 hours. Mean control and experimental house mortalities are shown as bars, and standard deviation as error lines. Points indicating the mortality from each individual replicate are overlaid on each experimental condition.

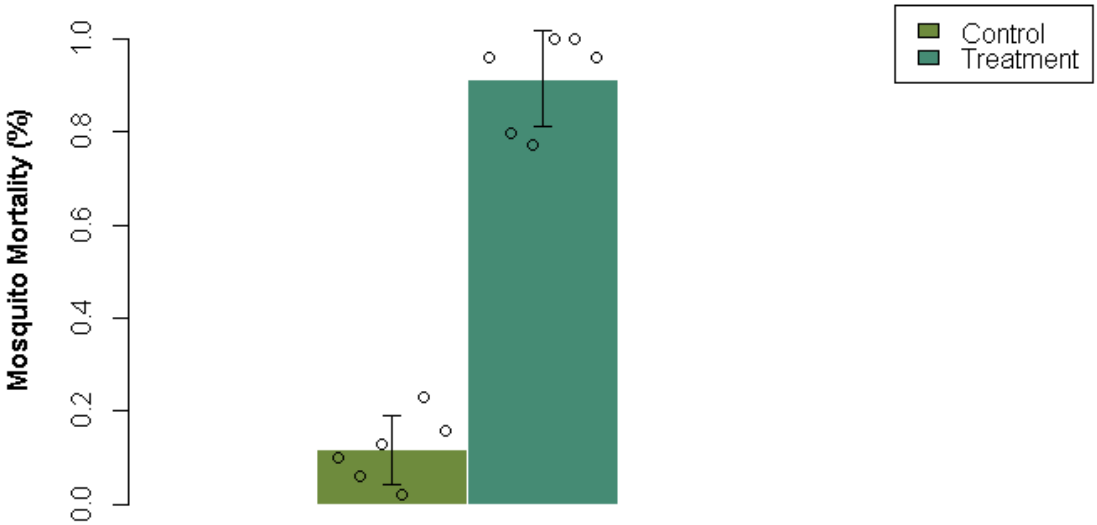

Figure 8: Results of Series 2.2. Mosquitos were exposed to DABS for 48 hours; mosquito mortality was calculated immediately after the exposure period. Mean control and experimental house mortalities are shown as bars, and standard deviation as error lines. Points indicating the mortality from each individual replicate are overlaid on each experimental condition.

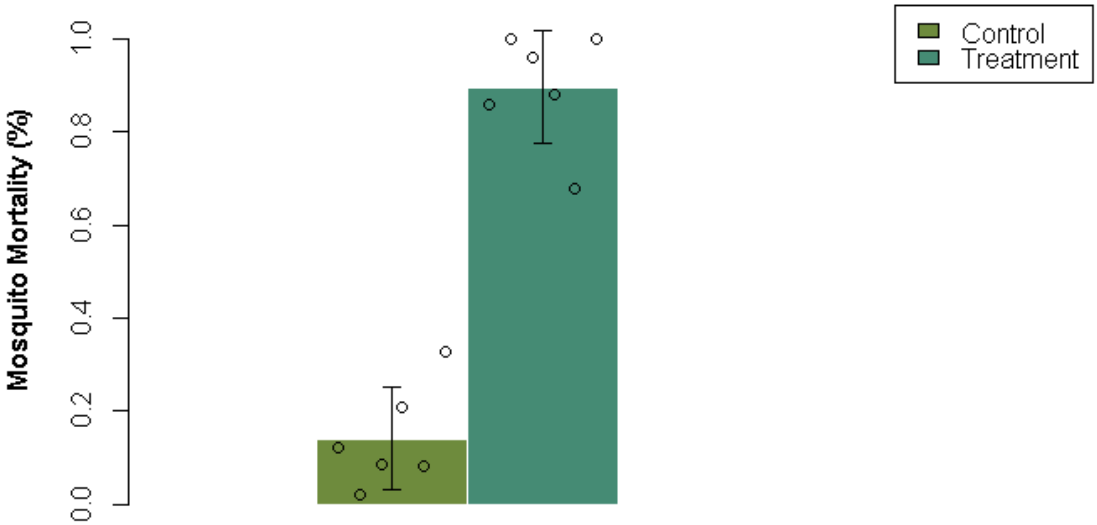

Figure 9: Results of Series 2.3. Mosquitos were exposed to DABS for 48 hours in the presence of a competing attractant; mosquito mortality was calculated immediately after the exposure period. Mean control and experimental house mortalities are shown as bars, and standard deviation as error lines. Points indicating the mortality from each individual replicate are overlaid on each experimental condition and time point.