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Title

Spartan Mosquito Eradicator Pro Tech
EPA Reg. No. 93813-R
Anopheles Species Weight of Evidence Bridging Rationale

Data Requirements

Invertebrate Control Agent Product Performance Testing Guidelines
(OCSPP Guideline 810.3400)
Mosquito, Black Fly, and Biting Midge (Sand Fly) Treatments

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Anopheles Mosquito Species Bridging Rationale

Introduction

The Spartan Mosquito Eradicator Pro Tech product is intended to be used and effective against all mosquitoes that may inhabit an area to be treated with the product, and it is understood that efficacy data are needed on multiple mosquito species (i.e. *Culex, Aedes,* and *Anopheles*) in order to claim product effectiveness against the general class of mosquitoes. During its evaluation of the product, the registrant has conducted various field efficacy trials with different geographical features (i.e. rural wooded areas, residential wooded areas, coastal areas, and areas proximate to water bodies) that are representative of mosquito habitats. In addition to the field efficacy studies that demonstrate 98%-100% efficacy consistent with the product's labeled claims, the registrant also conducted laboratory efficacy evaluations to demonstrate that varying concentrations of boric acid are effective at controlling both *Culex* and *Aedes* mosquitoes. Coupled with the study data developed by the registrant, there is a wealth of published literature on the effectiveness of attractive toxic sugar bait products in mosquito control (of which boric acid is a popular active ingredient). Significant scientific investigation has determined that such products are widely effective across all mosquito genera, including *Culex, Aedes*, and *Anopheles* mosquitoes. It is proposed in this rationale that there is sufficient information in published literature that boric acid-based mosquito attract-and-kill products are effective in the control of all three mosquito genera. Specifically, the registrant requests that the Agency consider the weight of evidence in the public literature in determining that the Spartan Mosquito Eradicator Pro Tech product will perform in the same manner against *Anopheles* mosquitoes as it has been demonstrated to perform against *Culex* and *Aedes* mosquitoes.

Product Background

The Spartan Mosquito Eradicator Pro Tech is a unique pesticide product for the control and suppression of mosquito populations. It is an attractive insecticide bait product containing 9.04% boric acid as the toxicant; however, in-use diluted concentration is 2.3% boric acid once warm water is added to the product. Other inert ingredients in the product are used to attract mosquitoes and once the product is ingested, boric acid will kill the target pest. Mosquito populations will begin to decline and, within two weeks, effective control will last for 30 days. Prior to placement of the product tubes along the perimeter of the area to be treated, warm water is added to the product to initiate attractancy. A specialized cap is placed onto the tube with openings large enough only for mosquitoes to gain access and enter but small enough that other non-target organisms, such as honeybees, butterflies, or hummingbirds, cannot access the product. This specialized cap also affords the ability for the product tube to naturally replenish with rain water over the life of the product as initial water levels may decrease due to evaporative losses.

Demonstrated Laboratory Efficacy Against *Culex* and *Aedes* Species and Field Efficacy

In a laboratory study (MRID No. 50904503), the registrant evaluated the effectiveness of differing concentrations of boric acid and sugar solutions in the control of *Culex quinquefasciatus* and *Aedes aegypti* mosquitoes. Several boric acid formulations that represented diluted versions of the Spartan Mosquito Eradicator Pro Tech product were evaluated. A 1.0% boric acid in 10% sugar/water solution was tested to represent an overly diluted product used in the field, and a 3.0% boric acid in 10% sugar/water solution was tested to represent an evaporated product used in the field along with a 10% sugar/water solution to serve as control. Mosquito mortality data were collected over the course of a 14-day test period. Both test concentrations of boric acid (1.0% and 3.0%) yielded very effective outcomes. For *Culex quinquefasciatus* mosquitoes, 100% control was achieved within 3 days for both

test groups; and for *Aedes aegypti*, 100% control was achieved within three days for the 1.0% group with greater than 95% control being achieved within three days followed by 100% control within six days for the 3.0% group.

The registrant also evaluated the Spartan Mosquito Eradicator Pro Tech product in varied geographic locations representative of mosquito habitat in the Southeastern United States (MRID No. 50904504). While it is understood that the species of collected mosquitoes were not identified from collected control groups of those studies, it is considered justified that a predominant mosquito species that inhabits the Southeastern United States is *Anopheles quadrimaculatus*. Considering that treated areas in the field efficacy studies achieved 98%-100% efficacy, it is reasonable to presume that any *Anopheles* mosquito that was attracted to and consumed the boric acid solution was also controlled by the product.

General Effectiveness of Attractive Toxic Sugar Bait Products

Mosquitoes readily feed on carbohydrates as a nutrient source and multiple investigations have been conducted to determine the effectiveness of attractive sugar bait products – both using fruit derivations and sucrose solutions. Various oral toxicants have been added to those sugar bait solutions to produce attractive toxic sugar baits (ATSBs) on which mosquitoes readily feed and then are killed. Boric acid (typically at 1.0%) has been evaluated extensively in these ATSBs in various locations under differing climatic and geographic conditions over the past fifteen years. Across a variety of geographic locations, ATSB products demonstrate consistent performance in the attract-and-kill mechanism for controlling mosquito populations. Sugar bait products readily attract 90% of more of the native mosquito populations across multiple genera (i.e. *Culex, Anopheles,* and *Aedes*) (Qualls, 2012). When boric acid at 1.0% is added to make an ATSB product, high mortality rates have been consistently demonstrated against *Aedes* mosquitoes (Hossain, 2014; Xue, 2011; Rivera, 2016; Xue, 2006). Similar high mortality rates were observed across multiple genera (*Aedes, Culex, Anopheles,* and *Ochlerotatus*) using 1.0% boric acid attractive toxic sugar bait products as either spray formulations onto foliage or stationary bait stations (Xue, 2006).

Specific Effectiveness of Boric Acid and Sugar Bait Products against *Anopheles* Mosquitoes

In addition to investigations performed to determine effectiveness of ATSB products across multiple genera of mosquitoes, additional studies support its specific effectiveness against several species of *Anopheles* mosquitoes. Early in the research of ATSB products, lethal concentrations were determined for boric acid ATSBs across several mosquito species. At 24 hours, LC_{50} values for male and female *Anopheles quadrimaculatus* mosquitoes were 0.317% and 0.885%, respectively. At 48 hours, LC_{50} values for male and female *An. quadrimaculatus* mosquitoes were measured to be 0.101% and 0.395%, respectively (Xue, 2003). Compared to the in-use concentration of the registrant's product (2.3%), high mortality of *Anopheles* mosquitoes is expected. Studies conducted in Israel and Africa, the latter where many malaria-causing *Anopheles* species are prevalent, demonstrated high morality using 1.0% boric acid ATSB products applied as surface sprays or as stationary bait stations. Greater than 90% control was achieved in *An. gambiae* (Qualls, 2015), *An. sergentii* (Beier, 2012), and *An. gambiae* and *An. arabiensis* (Muller 2010) following 1.0% boric acid ATSB spray or placement (for stationary bait stations).

Summary

It is reasonable to conclude that the Spartan Mosquito Eradicator Pro Tech product is effective in the control of *Anopheles* mosquitoes in a similar way that it is effective in the control of *Culex* and *Aedes* mosquitoes therefore supporting a general effectiveness claim against mosquitoes. The registrant's

conducted research confirming control of *Culex* and *Aedes* mosquitoes coupled with the field trial research on native mosquitoes substantiates that all mosquito species will be controlled by the product, particularly due to the 98%-100% observed rate of control during the field trials. In addition, study results of investigations by previous researchers concludes that boric acid-based ATSB products are highly effective in the control of multiple genera of mosquitoes including multiple species of *Anopheles* mosquitoes, including *An. gambiae, An. sergentii,* and *An. arabiensis*).

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Susceptibility of adult mosquitoes to insecticides in aqueous sucrose baits

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ABSTRACT: Mosquitoes characteristically feed on plant-derived carbohydrates and honeydew just after emergence and intermittently during their lives. Development of toxic baits focusing on this carbohydrate-seeking behavior may potentially contribute to localized control. In the present study, ten insecticides were fed to female *Culex quinquefasciatus, Anopheles quadrimaculatus,* and *Aedes taeniorhynchus* in a 10% sucrose solution. Active ingredients representative of five classes of insecticides (pyrethroids, phenylpyroles, pyrroles, neonicotinoids, and macrocyclic lactones) were selected for comparison with commercial formulations used to facilitate incorporation of active ingredients into aqueous sucrose solutions. Sucrose as a phagostimulant significantly enhanced mortality to toxicants. In general, the most effective active ingredients were fipronil, deltamethrin and imidacloprid, followed by spinosad, thiamethoxam, bifenthrin, permethrin, and cyfluthrin. The least effective ingredients were chlorfenapyr and ivermectin. For some of the ingredients tested, *Cx. quinquefasciatus* was the least susceptible species. One-day-old male *Cx. quinquefasciatus* were more susceptible than females; however, no differences existed between one- and seven-day-old mosquitoes. There were no differences in susceptibility between unfed and gravid ten-day-old female *Cx. quinquefasciatus* to bifenthrin. In conclusion, several pesticides from different classes of compounds have potential for use in development of toxic baits for mosquitoes. ***Journal of Vector Ecology* 36 (1): 59-67. 2011.**

Keyword Index: Mosquito, insecticide, sugar bait, *Culex, Aedes, Anopheles.*

INTRODUCTION

Plant-derived sugars and honeydew provide important components of mosquito nutrition and contribute to energy for survival, flight duration, maintaining nutritional reserves and may enhance fecundity (Nayar and Sauerman 1971, Nayar and Sauerman 1975a, 1975b, Foster 1995, Breigel 2003). Obtaining a sugar meal is critical for male mosquitoes of all species and occurs frequently, possibly several times a day (Gary and Foster 2006). Female mosquitoes typically sugar-feed just after emergence with a stronger dominance of attraction to sugar sources over host responses, and then intermittently as needed (Foster 1995, Reisen et al. 1986, Foster and Takken 2004, Gary and Foster 2006). Nectar is considered a more readily available source of energy than blood and mosquitoes sugar feed more frequently than blood feed (Nayar and Van Handel 1971). For some anthropophilic species such as *Aedes aegypti* L. and *Anopheles gambiae* Giles, sugar feeding may play a lesser role in female nutrition compared to human blood (Foster 1995, Breigel 2003).

This predisposition of mosquitoes to seek and return to carbohydrate sources through their life presents an opportunity for use of an attractant/toxicant bait for localized population reduction. Lea (1965) first reported on the utilization of mosquito sugar-feeding behavior of mosquitoes as a control method when he observed enhanced mortality of *Ae. aegypti* after feeding upon a malathion-sugar solution. Incorporation of *Bacillus sphaericus* Meyer and Neide spores with the sucrose/dye solutions provided further evidence with *Culex pipiens* L. (Schlein and Pener 1990) and phlebotomine sand flies (Robert et al. 1997) that this approach could be used to cause mortality as well as provide a pathway for dissemination of mosquito-borne pathogens. Feral mosquitoes feeding on a dried sucrose solution mixed with dye and spinosad applied to flowers on trees at desert oases has resulted in reduction of populations compared to control locations (Müller and Schlein 2006). Using this same approach but including fruit juice as an attractant and spraying the solution on vegetation around a larval habitat provided control of local *Cx. pipiens* (Müller et al. 2010). Similar applications of a sucrose solution combined with boric acid and applied to vegetation resulted in reduced populations and landing rates of *Aedes albopictus* (Skuse) and *Culex nigripalpus* (Xue et al. 2006). Lastly, use of aqueous sugar solutions in bait stations containing boric acid, fipronil (Xue et al. 2008), or spinosad (Muller and Schlein 2008) resulted in significant reductions of local mosquito populations.

While numerous insecticides have been evaluated for their toxicity to mosquitoes and other biting flies by means of external contact, relatively little is known about the oral toxicity of different active ingredients. Previous studies have reported efficacy of ingested solutions of malathion (Lea 1965), boric acid (Xue and Barnard 2003), spinosad (Müller and Schlein 2006), and fipronil (Xue et al. 2008), however, comparative studies of active ingredients or target pest species are few. As part of an overall strategy of providing alternative methods for mosquito control, we evaluated the efficacy of active ingredients for potential use in toxic aqueous baits for adult mosquitoes.

MATERIALS AND METHODS

Mosquitoes

Culex quinquefasciatus Say, *Aedes taeniorhynchus* (Wiedemann) and *Anopheles quadrimaculatus* (Say) were reared in the laboratory following the methods of Gerberg et al. (1994). Adults were maintained in screen cages with a 10% sucrose solution provided continuously. Cages were held at 27-29° C and 70-85% RH under a photoperiod of 14:10 (L:D).

Assays

Assays were conducted using disposable plastic cups (100 ml) covered by a piece of fabric screening fastened by an elastic band. Mosquitoes were immobilized on a chill table (4° C), sexed, and counted with ten mosquitoes placed in each cup. Mosquitoes were allowed to recover for at least one h before the test. Baits were presented as a 1 cm length of cotton dental wick (Unipack Medical Corp., Commerce, CA) saturated with 1 ml of solution and placed on top of the screen lid of each cup. Cups were held at 70-85% RH and 25-27° C during the tests. Mortality was difficult to determine with mosquitoes that were moribund so knockdown (inability to stand) (KD) was determined as an index of mortality. Mosquitoes that were knocked down were not observed to revive.

Enhanced knockdown with sucrose

To determine if mortality was enhanced with the addition of sucrose in an aqueous solution of pesticide, knockdown of five to ten-day-old female *Cx. quinquefasciatus* to bifenthrin (Table 1) in solutions of sucrose was compared to that in solutions of water. Solutions of bifenthrin were prepared using 10% sucrose (w/v) or water over the range of 0.1-1000 mg/liter of active ingredient (AI). Knockdown was evaluated at four and 24 h. Bifenthrin was selected as a representative insecticide for this assay because of the lack of phagorepellency at high doses.

Effect of sex and physiological condition

To determine if sex and physiological condition affected response, bifenthrin-sucrose solutions were first used to compare susceptibility of teneral (one-day-old) *Cx. quinquefasciatus* males and females not exposed to sucrose. Additionally, comparisons were made between seven-day-old males and females (exposed to sucrose) and between ten-day-old gravid (five days post blood-feeding), and non-blood-fed females. Mosquitoes were exposed to a range of concentrations and sucrose controls as described above and KD_{50} and KD_{90} values estimated.

Evaluation of insecticide efficacy

Toxicants were selected to represent a range of active ingredients belonging to several classes of insecticides (Table 1). As many pesticides have low solubility in water, commercial formulations were used to facilitate the incorporation of the AI into the aqueous sugar solution. Stock solutions (1000 mg/liter AI) and subsequent dilutions of insecticides were made in 10% sucrose/water (w:v) solutions with 10% sucrose as the negative control (following Pridgeon et al. 2008). At least five concentrations of AI were tested of each insecticide and each test was replicated at least five times. Observations of knockdown were made at one, four, and 24 h after initial exposure. Females of *Cx. quinquefasciatus*, *Ae. taeniorhynchus*, and *An. quadrimaculatus* that were seven to 14-days-old with constant access to sucrose solution were used for bioassays.

Analysis

Data for comparisons between bifenthrin solutions made with sucrose or water were arcsine transformed and compared by paired *t*-test. Data for comparisons between active ingredients were analyzed by probit regression analysis (PoloPlus, LeOra Software 2003) after correction for control responses (if needed) using Abbot's formula (Abbott 1925). Data were presented at KD_{50} and KD_{90} values with (95% confidence levels) for different materials or life stages. Values were considered significantly different ($P < 0.05$) if confidence intervals did not overlap.

RESULTS

Mortality of *Cx. quinquefasciatus* females was significantly greater when exposed to bifenthrin-sucrose concentrations of >1 mg/liter compared with bifenthrin-water mixtures at four h (Figure 1). At 24 h, similar trends were observed, but there was no difference in bifenthrin mortality between sucrose or water at 1000 mg/liter. With many of the toxic doses, hyper-extended abdomens were observed (Figure 2). These mosquitoes did not recover and were dead at 24 h.

Age and sex of *Cx. quinquefasciatus* did affect susceptibility to bifenthrin in sucrose solutions (Table 2). Male mosquitoes were more susceptible than females at one day of age (KD_{50}) but not at 7 days. Unfed and gravid ten-day-old mosquitoes were equally susceptible (Table 2). There was no difference in the KD_{50} values between one-, seven- and ten-day-old females. One-day-old males were more susceptible than seven-day-old males based on KD_{50} values.

Because mortality at one and four h was generally low, KD_{50} and KD_{90} values were difficult to estimate with any accuracy, therefore only 24 h data are presented. Based on KD_{50} values, *Cx. quinquefasciatus* were most susceptible to fipronil > imidacloprid > deltamethrin, spinosad, thiamethoxam, and permethrin > bifenthrin > cyfluthrin > chlorfenapyr and ivermectin (Table 3). Females of *An. quadrimaculatus* were most susceptible to imidacloprid, fipronil, and deltamethrin > thiamethoxam, cyfluthrin, bifenthrin, spinosad, and permethrin > chlorfenapyr and ivermectin (Table 4). Females of *Ae. taeniorhynchus* were most susceptible to imidacloprid, permethrin, thiamethoxam, deltamethrin, and fipronil > bifenthrin and spinosad > cyfluthrin > chlorfenapyr and ivermectin (Table 5). These rankings were generally similar when KD_{90} values were used, except that some repellency was observed

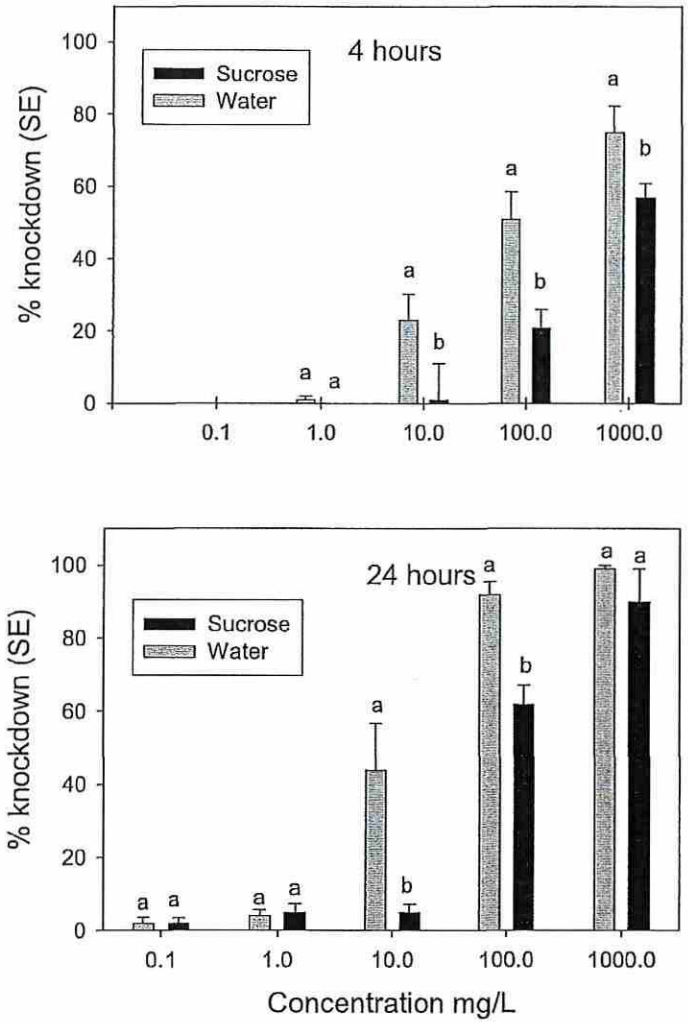

Figure 1. Knockdown of *Cx. quinquefasciatus* females exposed to bifenthrin combined with 10% sucrose or water (control). At each concentration, bars with similar letters were not significantly different (*t*-test, $P < 0.05$).

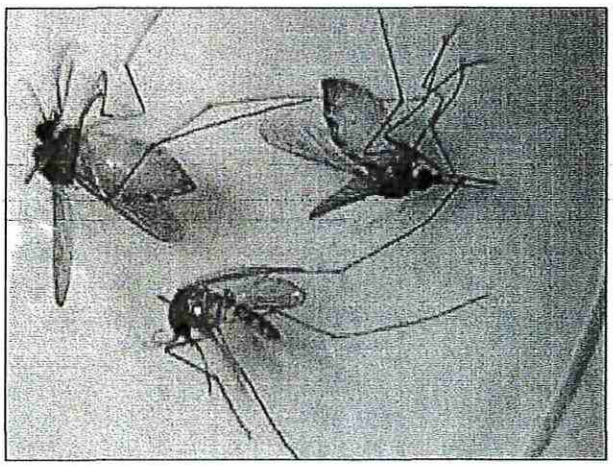

Figure 2. Characteristic hyper-extended abdomens of mosquitoes fed on toxic sugar baits.

Table 1. Active ingredients and commercial formulations used for toxic baits.

Class	Active ingredient	Formulation	% AI	Source
Pyrethroid	Bifenthrin	Talstar®	7.9%	FMC Corporation, Philadelphia PA
	Cyfluthrin	Tempo®	11.8%	Bayer Corporation, Kansas City MO
	Deltamethrin	Suspend®SC	4.75%	Bayer Environmental Health Sciences, Research Triangle Park NC
	Permethrin	Dragnet®	36.8%	FMC Corporation, Philadelphia PA
Phenylpyrazole	Fipronil	Termidor®SC	9.1%	BASF, Research Triangle Park NC
Pyrrole	Chlorfenapyr	Phantom®	21.45%	BASF, Research Triangle Park NC
Neonicotinoid	Imidacloprid	QuickBayt™	0.5%	Bayer HealthCare, Shawnee Mission KS
	Thiamethoxam	Platinum®	21.6%	Syngenta Crop Protection Inc. Greensboro NC
Macrocyclic lactone	Spinosad	Elecktor®	2.46%	Elanco Animal Health, Indianapolis IN
	Ivermectin	Ivomec®	0.1%	Merial Limited, Duluth GA

Table 2. Effect of age and stage of *Culex quinquefasciatus* on efficacy of bifenthrin in 10% sucrose solutions.

Age	Stage	KD_{50} (CL) [a]	Slope (SE)	N
1 day	Female	2.1 (1.8-2.6)a	2.66 (0.16)	900
	Male	1.5 (1.4-1.6)b	5.90 (0.56)	800
7 day	Female	3.0 (2.0-4.4)a	1.56 (0.08)	700
	Male	4.2 (2.0-7.4)a	1.77 (0.14)	900
10 day	Unfed female	1.9 (1.4-2.4)a	3.70 (0.37)	600
	Gravid female	1.8 (1.4-2.2)a	1.52 (0.12)	700

Means within each column and age followed by different letters are significantly different.
[a]Values are presented as mg of active ingredient per liter of sucrose solution.

at higher doses with some compounds and species resulting in high variation.

In general, of the pyrethroids, deltamethrin appeared to be the most toxic with cyfluthrin the least toxic. Fipronil, deltamethrin, and imidacloprid were the most toxic compounds across the three species. The neonicotinoid compounds thiamethoxam and imidacloprid were generally equivalent, although the former was more toxic in the case of *Ae. taeniorhynchus*. For all species, the least toxic compound was ivermectin, which was 49-, 3- and 387- fold less toxic than permethrin for *Cx. quinquefasciatus*, *An. quadrimaculatus*, and *Ae. taeniorhynchus*, respectively. The second least toxic compound was chlorfenapyr.

For the three species tested, there was no difference in susceptibility to spinosad or fipronil. However, *Cx. quinquefasciatus* was less susceptible than both *Ae. taeniorhynchus* and *An. quadrimaculatus* to bifenthrin, cyfluthrin, chlorfenapyr, thiamethoxam, and ivermectin.

DISCUSSION

This study provides a list of potential pesticides for use in a toxic bait approach for mosquitoes and other biting flies. With concerns of resistance in field populations, availability of effective toxicants with different modes of action may expand the potential for development of a toxic bait system. Other concerns with the selection of such a delivery system include the environmental impact of the toxicant, effects on non-target organisms, and stability under delivery conditions in the field. Development of a successful toxic bait system involves several components including visual and olfactory lures to attract mosquitoes to the bait. Moreover, mechanical exclusion of larger non-target insects, phagostimulants to maximize uptake of toxicant, a pesticide effective against target species formulated to optimize delivery of the active ingredient, minimal environmental impact, and avoidance of UV degradation will need to be addressed. The Environmental Protection Agency has designated some active ingredients

Table 3. Toxicity of active ingredients presented in sucrose solution for *Culex quinquefasciatus* females after 24-h exposure.

Active ingredient	N	KD_{50} (CL)[a]	KD_{90} (CL)[a]	Slope (SE)
Pyrethroid				
Bifenthrin	550	12.0 (9.1-15.5)	54.6 (33.6-88.3)	1.94 (0.15)
Cyfluthrin	750	23.9 (18.5-31.5)	124.5 (84.0-218.3)	1.79 (0.11)
Deltamethrin	500	1.5 (0.7-2.5)	77.8 (43.8-168.9)	1.57 (0.15)
Permethrin	550	7.8 (2.2-12.9)	36.5 (24.4-74.4)	1.91 (0.32)
Phenylpyrazole				
Fipronil	500	0.1 (0.1-0.2)	2.7 (1.2-11.4)	0.90 (0.11)
Pyrrole				
Chlorfenapyr	500	204.6 (146.4-300.4)	1103.1 (653.0-2588.3)	1.75 (0.15)
Neonicotinoid				
Imidacloprid	500	0.8 (0.3-1.9)	494.2 (150.9-3472.2)	0.46 (0.05)
Thiamethoxam	550	5.1 (2.2-7.9)	316.01 (120.9-1636.9)	0.71 (0.09)
Macrocyclic lactone				
Spinosad	790	1.7 (1.1-2.7)	15.3 (8.4-38.8)	1.37 (0.18)
Ivermectin	500	382.9 (291.7-587.9)	1254.4 (755.4-3621.5)	2.48 (0.35)

[a]Values are presented as mg of active ingredient per liter of sucrose solution.

as "reduced risk" compounds on the basis of factors such as hazards to humans and other animals, environmental fate, and photostability. In the current study, the compounds that fit into that category were fipronil, imidacloprid, thiamethoxam, and spinosad. Insecticide baits for mosquitoes using a phagostimulant such as sucrose can be effective in eliciting mortality. Based on toxicity, fipronil, imidacloprid, thiamethoxam, and spinosad appear to have potential for such an approach.

Pyrethroids are commonly registered for mosquito control with permethrin being the most widely used and available in the most formulations. Pyrethroids are sodium channel modulators and although highly toxic to mosquitoes, concerns of resistance may mitigate their potential use in toxic baits. Of the insecticides evaluated in our study, all pyrethroids were highly toxic with deltamethrin and permethrin the most toxic, bifenthrin moderately toxic, and cyfluthrin the least toxic. In a comparison of 19 active ingredients applied topically, Pridgeon et al. (2008) reported that permethrin was the second most toxic compound against adults of the three species. *Culex quinquefasciatus* was the least susceptible species to permethrin with *Ae. aegypti* the most susceptible species. Liu et al. (2004) reported that deltamethrin was more toxic than permethrin against adults of a susceptible strain of *Cx. quinquefasciatus*. Cyfluthrin and permethrin were equally toxic against a susceptible strain of *Cx. quinquefasciatus* (Nanzi et al. 2005).

Age and sex of mosquito did affect susceptibility to bifenthrin, with one-day-old males more susceptible than one-day-old females and more susceptible than seven-day-old males. This difference may be related to the larger size of females compared to male *Cx. quinquefasciatus* upon emergence and the lower glycogen reserves of teneral males compared with older males[1]. Differences in susceptibility to insecticides also existed between mosquito species. Similar to Pridgeon et al. (2008), we observed differences between species with some compounds less toxic to *Cx. quinquefasciatus* than to *An. quadrimaculatus* and *Ae. taeniorhynchus*. This observation was also supported by Corbel et al. (2004) who reported that dinotefuran was less toxic to *Cx. quinquefasciatus* than to *Ae. aeygpti* and *An. gambiae*. Our results demonstrate that the general tolerance of *Cx. quinquefasciatus* to pesticides compared to other species previously noted for tarsal contact (Curtis et al. 1996) and to dorsal application (Pridgeon et al. 2008), can

[1]Vrzal, E.M. 2009. The effects of various carbohydrate sources on longevity and nutritional reserves of *Culex quinquefasicatus* Say, *Culex nigripalpus* Theobald and *Culex salinarius* Coquillett. M.S. thesis. University of Florida, Gainesville. 93 pp.

Table 4. Toxicity of active ingredients presented in sucrose solution for *Anopheles quadrimaculatus* females after 24-h exposure.

Active ingredient	N	KD_{50} (CL)[a]	KD_{90} (CL)[a]	Slope (SE)
Pyrethroid				
Bifenthrin	500	2.8 (1.5-4.4)	9.2 (5.5-29.4)	2.47 (0.24)
Cyfluthrin	550	2.0 (0.7-5.6)	81.5 (24.1-621.7)	0.80 (0.05)
Deltamethrin	550	0.2 (0.1-0.6)	49.1 (10.1-103.5)	0.54 (0.05)
Permethrin	500	4.6 (3.1-9.3)	25.7 (11.7-160.9)	1.71 (0.23)
Phenylpyrazole				
Fipronil	500	0.2 (0.1-0.3)	0.7 (0.5-1.8)	2.35 (0.28)
Pyrrole				
Chlorfenapyr	500	24.4 (19.2-31.2)	90.2 (65.5-141.8)	2.26 (0.19)
Neonicotinoid				
Imidacloprid	500	0.03 (0.001-0.1)	2.1 (0.6-14.3)	0.70 (0.07)
Thiamethoxam	550	0.8 (0.6-0.9)	3.6 (2.9-4.8)	1.90 (0.23)
Macrocyclic lactone				
Spinosad	650	3.6 (1.7-7.6)	37.6 (15.6-172.6)	1.25 (0.09)
Ivermectin	500	14.2 (9.5-20.3)	141.6 (86.3-293.5)	1.28 (0.11)

[a]Values are presented as mg of active ingredient per liter of sucrose solution.

Table 5. Toxicity of active ingredients presented in sucrose solution for *Aedes taeniorhynchus* females after 24-h exposure.

Active ingredient	N	KD_{50} (CL)[a]	KD_{90} (CL)[a]	Slope (SE)
Pyrethroid				
Bifenthrin	550	2.1 (1.1-3.8)	13.5 (7.1-47.6)	1.60 (0.13)
Cyfluthrin	550	12.7 (10.4-15.6)	56.7 (42.2-83.8)	1.97 (0.16)
Deltamethrin	700	0.2 (0.1-1.3)	110.8 (17.4-443.9)	0.49 (0.03)
Permethrin	500	0.1 (0.1-0.2)	0.8 (0.5-1.9)	1.71 (0.13)
Phenylpyrazole				
Fipronil	400	0.3 (0.2 -0.5)	0.6 (0.4-1.2)	4.98 (0.52)
Pyrrole				
Chlorfenapyr	500	25.2 (21.5-29.4)	43.9 (36.5-58.9)	5.31 (0.62)
Neonicotinoid				
Imidacloprid	750	0.06 (0.001-0.2)	47.6 (12.5-523.5)	0.44 (0.04)
Thiamethoxam	550	0.1 (0.05-0.2)	0.9 (0.7-1.5)	1.44 (0.24)
Macrocyclic lactone				
Spinosad	650	4.1 (1.3-10.8)	32.2 (13.7-302.4)	1.25 (0.09)
Ivermectin	500	38.7 (29.3-51.1)	131.3 (92.2-224.1)	2.41 (0.20)

[a]Values are presented as mg of active ingredient per liter of sucrose solution.

be extended to oral toxicity. These results underscore the need for testing across species when evaluating the efficacy of toxicants for mosquitoes.

Neonicotinoid insecticides are agonists of the nicotinic acetylcholine receptor and have low toxicity to mammals, birds, and fish (Tomizawa and Casida 2005). Because they are generally stomach poisons, many have low contact toxicity and are most effective against piercing-sucking insects. Corbel et al. (2004) evaluated neonicotinoid dinotefuran as a topical against three species of mosquitoes and reported lower toxicity than deltamethrin but concluded that neonicotinods were potential candidates for disease vector control, particularly in areas where resistance to other insecticides is high. In our study, we evaluated two neonicotinoids, imidacloprid and thiamethoxam. The known tolerance of imidacloprid in some insect predators is advantageous with respect to non-target concerns (Bozsik 2006). Pridgeon et al. (2008) reported that imidacloprid applied topically was moderately toxic and lower in toxicity than permethrin for the three mosquito species tested. Paul et al. (2006) reported low mortality to imidicloprid treatments of *Ae. aegypti* in treated bottle assays. Liu et al. (2004) reported that susceptible strains were as susceptible to imidacloprid as to permethrin in larval assays. In our studies, imidacloprid was highly effective and as toxic as deltamethrin and permethrin for *Cx. quinquefasciatus*; it was also one of the most toxic compounds for *An. quadrimaculatus* and *Ae. taeniorhynchus*. Thiamethoxam was as toxic as permethrin for all species and one of the most toxic compounds for *An. quadrimaculatus*. Thiamethoxam combined with sugar has also been reported as highly toxic to house flies (Kristensen and Jespersen 2008) and eye gnats (Jiang and Mulla 2006). Thiamethoxam-treated spheres have been reported to be effective against blueberry maggot flies (Ayyappath et al. 2000). These neonicotinoid compounds are effective as both contact and stomach poisons and appear to be promising toxicants in an ingested bait system.

Macrocyclic lactones are products or chemical derivatives of soil micro-organisms and include the spinosyns and avermectins, both of which have contact and stomach effects. Spinosad is considered a naturally-derived biorational insecticide with low toxic effects for mammals, avians, predatory beneficial insects, and for the environment in general (Liu et al. 1999, Williams et al. 2003, Galvan et al. 2006). Spinosad is used commercially with sucrose for control of various tephritid fruit flies (Prokopy et al. 2003, Yee and Chapman 2009). Lui et al. (2004) reported relatively low toxicity of a susceptible strain of *Cx. quinquefasciatus* to spinosad, but when used against field strains resistant to permethrin and other insecticides, spinosad was one of the most toxic compounds tested. These authors concluded that spinosad could be important for mosquito vector control, particularly for mitigation of development of resistance. Pridgeon et al. (2008) reported that spinosad was slightly less toxic than permethrin against all three species of mosquitoes tested, with *Cx. quinquefasciatus* being the least susceptible. Spinosad has also been reported as effective against mosquito larvae (Darriet and Corbel 2006, Jiang and Mulla 2009).

Previous studies have reported on effective delivery of spinosad in sucrose as oral baits. A combination of sucrose and spinosad evaluated by Jiang and Mulla (2006) was toxic to eye gnats but over 25-fold less toxic than thimethoxam and half as toxic as imidacloprid. Dry sugar baits containing spinosad have also been found to be eight times more toxic than imidacloprid baits against house flies (White et al. 2007). Romi et al. (2006) reported oral toxicity of spinosad in a 5% sucrose solution with 100% mortality at 24 h of female *Ae. aegypti* at 100 ppm and for *Anopheles stephensi* Liston and *Culex pipiens* L. at 1000 ppm. In field studies, dried as well as aqueous sucrose and spinosad baits have reduced local populations (Müller and Schlein 2006, 2008, Müller et al. 2010).

The second macrocyclic lactone in our study, ivermectin (chloride channel activator), at systemic doses in mammalian blood is clearly toxic to mosquitoes (Pampiglione et al. 1985, Jones et al. 1992, Bockarie et al. 1999, Chaccour et al. 2010) producing high mortality at 24 h for high doses. In our study, ivermectin as a component of a sugar bait was the least toxic of compounds tested.

Chlorfenapyr, as a pyrrole, is an uncoupler of oxidative phosphorylation and acts as a contact and stomach poison. In laboratory and field trials, it has been reported to be moderately efficacious against pyrethroid-resistant mosquitoes (N'Guessan et al. 2009, Oliver et al. 2010) and as such was recommended to be considered as a resistance management tool to circumvent or slow development of resistance. Paul et al. (2006), using tarsal contact assays, concluded that chlorfenapyr was moderately toxic to adult mosquitoes compared with permethrin. Our results with sucrose and chlorfenapyr mixtures indicated that chlorfenapyr was less toxic than many of the other compounds examined. Fipronil is a phenylpyrazole acting as a gamma amino butyric acid (GABA)-gated chloride channel agonist with both contact and stomach effects (Cole et al. 1993).

Fipronil was one of the most efficacious compounds in our study, 78-fold more toxic than permethrin for *Cx. quinquefasciatus*, and 23-fold for *An. quadrimaculatus*, but not different for *Ae. taeniorhynchus*. Liu et al. (2004) reported similar toxicity of fipronil and permethrin for *Cx. quinquefasciatus* larvae. Based on topical application of adults, higher efficacy of fipronil than permethrin was reported by Pridgeon et al. (2008) for *Ae. aegypti* (106-fold) and *Cx. quinquefasciatus* (ca. 4,800-fold), but not for *An. quadrimaculatus*. While direct comparisons between studies are difficult because of differences in methods of delivery and doses absorbed are different, the general trend remains that fipronil appears to be at least as toxic as permethrin. Use of fipronil in sugar bait stations reduced landing counts of *Ae. aegypti* and *Ae. taeniorhynchus* in screened cages but did not impact field populations of mosquitoes (Xue et al. 2008).

Sucrose enhanced uptake of solutions containing insecticide as seen with higher mortality from ingestion of

solutions containing sugar. Mortality was not considered to be a result of the lack of ingestion as a result of repellency due to high survival of water-deprived control mosquitoes at 24 hr and the abundance of abdomens bloated with sugar solution in all treatments. At high doses, mortality did not always reach 100%, possibly because of repellency. Jiang and Mulla (2006) also provide evidence of sucrose as an important phagostimulant for the ingestion of insecticide solutions.

These results indicate that there are several compounds from different classes of insecticides that are effective in eliciting high levels of mosquito mortality through ingestion of the insecticide as a sucrose bait. Formulations of insecticides used in this study were not developed for delivery as aqueous sucrose baits and therefore are not optimized for this delivery method. However, delivery of formulated compounds was uniform throughout the test and a relative comparison of compounds is presented. A primary factor determining efficacy of an insecticide is dose. Most studies use standardized methods (i.e., topical application) that ensure an exact dose is delivered to each individual being evaluated. In contrast, it is very difficult to determine the exact amount of insecticide reaching the target in bait studies. Although this uncertainty introduces more variation in the resulting data, it provides a realistic indication of the doses necessary to kill the target mosquitoes and the degree to which the mosquitoes will ingest a lethal dose.

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MALARIA JOURNAL

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Attractive toxic sugar bait (ATSB) methods decimate populations of *Anopheles* malaria vectors in arid environments regardless of the local availability of favoured sugar-source blossoms

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Abstract

Background: Attractive toxic sugar bait (ATSB) methods are a new and promising "attract and kill" strategy for mosquito control. Sugar-feeding female and male mosquitoes attracted to ATSB solutions, either sprayed on plants or in bait stations, ingest an incorporated low-risk toxin such as boric acid and are killed. This field study in the arid malaria-free oasis environment of Israel compares how the availability of a primary natural sugar source for *Anopheles sergentii* mosquitoes: flowering *Acacia raddiana* trees, affects the efficacy of ATSB methods for mosquito control.

Methods: A 47-day field trial was conducted to compare impacts of a single application of ATSB treatment on mosquito densities and age structure in isolated uninhabited sugar-rich and sugar-poor oases relative to an untreated sugar-rich oasis that served as a control.

Results: ATSB spraying on patches of non-flowering vegetation around freshwater springs reduced densities of female *An. sergentii* by 95.2% in the sugar-rich oasis and 98.6% in the sugar-poor oasis; males in both oases were practically eliminated. It reduced daily survival rates of female *An. sergentii* from 0.77 to 0.35 in the sugar-poor oasis and from 0.85 to 0.51 in the sugar-rich oasis. ATSB treatment reduced the proportion of older more epidemiologically dangerous mosquitoes (three or more gonotrophic cycles) by 100% and 96.7%, respectively, in the sugar-poor and sugar-rich oases. Overall, malaria vectorial capacity was reduced from 11.2 to 0.0 in the sugar-poor oasis and from 79.0 to 0.03 in the sugar-rich oasis. Reduction in vector capacity to negligible levels days after ATSB application in the sugar-poor oasis, but not until after 2 weeks in the sugar-rich oasis, show that natural sugar sources compete with the applied ATSB solutions.

Conclusion: While readily available natural sugar sources delay ATSB impact, they do not affect overall outcomes because the high frequency of sugar feeding by mosquitoes has an accumulating effect on the probability they will be attracted to and killed by ATSB methods. Operationally, ATSB methods for malaria vector control are highly effective in arid environments regardless of competitive, highly attractive natural sugar sources in their outdoor environment.

Keywords: Sugar feeding, Vectorial capacity, Malaria, Attractive toxic sugar baits (ATSB), Outdoor mosquito control, *Anopheles sergentii*

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Background

Attractive toxic sugar bait (ATSB) methods are a new form of vector control that kill female and male mosquitoes questing for essential sugar sources in the outdoor environment [1-7]. ATSB solutions consist of fruit or flower scent as an attractant, sugar solution as a feeding stimulant, and oral toxin to kill the mosquitoes. ATSB solutions that are sprayed on small spots of vegetation or suspended in simple removable bait stations attract mosquitoes from a large area and the mosquitoes ingesting the toxic solutions are killed. The ATSB methods developed and field-tested in Israel demonstrate how they literally decimate local populations of different anopheline and culicine mosquito species [1-5]. Similar successful ATSB field trials have also controlled *Culex quinquefasciatus* from storm drains in Florida, USA [6] and *Anopheles gambiae* s.l. malaria vectors in Mali, West Africa [7]. The new ATSB methods are highly effective, technologically simple, low-cost, and circumvent traditional problems associated with the indiscriminate effects of contact insecticides [8] by narrowing the specificity of attraction to sugar-seeking mosquitoes and by using environmentally safe oral toxins such as boric acid, that is considered to be only slightly more toxic to humans and other vertebrates than table salt [9].

ATSB methods work by competing with available natural plant sugar sources, which are an essential source of energy for females and the only food source for male mosquitoes [10,11]. Mosquitoes are highly selective in their attraction to locally available flowering plants and other sources of sugar including fruits, seedpods, and honeydew [12-14] and the availability of favourable natural sugar sources strongly affects mosquito survival [13]. All of the above-noted ATSB field trials used juices made from local natural fruits to successfully divert sugar-seeking mosquitoes from their natural sources of plant sugars. Between 50 and 90% of the local female and male mosquitoes feed on ATSB solutions within the first few days after applications, as inferred from data at control sites where the same attractive bait solutions are applied without toxin but containing coloured food dye markers, which are readily apparent in sugar-fed mosquitoes [1].

The present study is on *Anopheles sergentii*, the most common and abundant *Anopheles* species in Israel and the main vector of malaria in the Afro-Arabian zone [15,16]. This mosquito species was the main vector responsible for malaria outbreaks [17-19] before the elimination of malaria parasite transmission from Israel in the 1960s [20-22].

The objective of this study was to determine the relationship between the efficacy of ATSB control and the availability of natural plant sugar sources. As demonstrated in a recent comparative study of *An. sergentii* in sugar-rich and sugar-poor oases in Israel, the availability of natural plant sugar sources affects mosquito fitness, population dynamics, and malaria vector capacity [23]. Accordingly, a single application of ATSB was made in the same two relatively small, isolated, and uninhabited sugar-rich and sugar-poor oases. Another larger oasis with high densities of *An. sergentii* was not sprayed and served as a control site for the ATSB field trial.

Methods

Study sites

The study was conducted at three oases located within the depression of the African-Syrian Rift Valley, in the northern part of the Arava Valley, about 25 km south of the Dead Sea. The shoreline of the Dead Sea is about 400 m below sea level while the central Arava Valley rises to about 200 m above sea level before it is again descending towards the Red Sea. The region belongs to the Sahara-Arabian phyto-geographical zone [24]. The area is an extreme desert with occasional natural oases centred on springs and artificial agricultural oases created by irrigation; the conditions in these sites are tropical [25]. The climate is arid with an average humidity of 57% and annual winter rains averaging of 50-100 mm. The average temperature ranges from 20°C from the end of September to early April and to 30°C from May to August [26,27]. The area is known for its rich mosquito fauna dominated by *An. sergentii, Ochlerotatus caspius* and *Culex pipiens* [28].

Field experiments were conducted at three oases. Two of the oases included small, unnamed and uninhabited oases, 5 km apart in the Arava Valley. As recently described [23], the environments of the two oases are very similar except for the availability of sugar sources. In one of the oases (termed "sugar-rich oasis"), there were two flowering *Acacia raddiana* trees that were the preferable source of sugar for the mosquitoes [14]. In contrast, there were no flowering plant blossoms in the other oasis (termed "sugar-poor oasis"). Both sites covered areas of about 5 ha, included small fresh-water springs surrounded by dense non-flowering vegetation which was largely grazed out by camels and donkeys with no visible plant sugar sources during the period of field experiments [23,24]. Neot Hakikar oasis served as an untreated control site. It is located about 20 km north of the small oases and is the largest natural oasis in southern Israel and the Dead Sea region. In the eastern more agricultural part of the oasis a small settlement is located with gardens, vast fields and greenhouses. The western, much more natural part is a nature reserve with a mixture of salt marshes, wet and dry Salinas (ie areas high in salt content with specialized plant communities) and fresh-water springs surrounded by riparian vegetation

largely dominated by *Phragmites australis* L. Gramineae and *Carex* sp. L. Cyperaceae. This natural vegetation, crossed by a drainage canal, is partially overgrown by reeds and sedges. On the dry banks of the canal vegetation is dominated by groves and thickets of trees and bushes like *Tamarix nilotica* and *Tamarix passerinoides* (Tamaricaceae), *Prosopis farcta* (Mimosaceae), *Nitraria retusa* (Nitrariaceae) and chenopod bushes like *Atriplex halimus, Atriplex leucoclada, Suaeda asphaltica, Suaeda fruticosa* (Chenopodiaceae). At the time of the experiment some *T. nilotica*, and *P. farcta* bushes were flowering.

Preparation of ATSB solutions

The ATSB bait solution used in the sugar-rich and sugar-poor oases consisted of ~75% juice of over-ripe to rotting prickly pear cactus (*Opuntia ficus-indica*, Cactaceae), 5% V/V wine, 20% W/V brown sugar, 1% (W/V) BaitStab™ concentrate (a product containing antifungal and antibacterial additives produced by Westham Innovations LTD, Tel Aviv, Israel) and boric acid 1% (W/V) [29]. The solution was ripened outdoors for 48 h in covered buckets before adding the BaitStab™ and the boric acid. In this study, prickly pear cactus fruit (*Opuntia ficus-indica*) was used because it was locally abundant and known to be highly attractive for both sand flies [29] and mosquitoes (Schlein and Muller, unpublished).

Field application of ATSB solutions

The ATSB solution was sprayed with a 16-l back-pack sprayer (Killaspray, Model 4526, Hozelock, Birmingham UK) in aliquots of ~80 ml on 1 m^2 spots at distances of ~3 m on the vegetation surrounding the fresh-water springs of the two isolated oasis (sugar-rich and sugar-poor). Predominant types of non-flowering plants sprayed at the two sites were *P. australis, Atriplex* sp. and *Suaeda* sp. As a strategy to minimize potential harm to non-target insects, the predominant natural sugar source for *An. sergentii*, the flowering *A. raddiana* trees, present in the sugar-rich oasis were not sprayed. One sprayer completed the applications in less than 1 h per site. No bait solution was sprayed at the control site Neot Hakikar.

Study design and methods for the ATSB field trial

The field trial was conducted over a period of 47 days, from 1 November to 17 December, 2009. During this period, at each of the three study sites, adult mosquitoes were sampled at two-day intervals (a total of 24 times) using six CDC UV traps (Model 1212, John W. Hock, Gainesville, FL) without attractants in fixed positions surrounding the available fresh-water springs. ATSB bait solutions were sprayed on day 12 of the field experiment. Collected mosquitoes were sexed, identified to species, and the physiological age of female mosquitoes was determined by dissecting ovaries and counting the number of dilatations [30].

Statistical analysis

To evaluate impacts of ATSB on mosquito populations, captures of *An. sergentii* were examined at four intervals (1-12, 13-24, 25-36, and 37-47 days). A logistic regression was used to examine the proportion of females with three or more gonotrophic cycles in each oasis over time. Contrasts were used to test for significant changes from the pre-treatment period in each oasis. Separate Poisson regressions were used to analyse the number of male and female *An. sergentii* caught in the light traps over time in the three oases. Contrasts were used to compare the control oasis with the poor and rich oases at each time.

Estimation of vectorial capacity

Vectorial capacity (VC). defined as the average number of infectious bites the mosquito could potentially deliver over her lifetime, was used to estimate the impact of ATSB on the potential for malaria parasite transmission:

$$VC = \frac{mp^{EIP}}{-T^2 \log(p)}$$

Where m was the number of mosquitoes per person, T was the estimated duration of the gonotrophic cycle [23]. *EIP* was the extrinsic incubation period of malaria parasites in mosquitoes assuming to be 10 days [31]. p was the survival rate estimated based on parous rates r.

$$p = \sqrt[T]{r}$$

Following Dye [32], VC was compared before and after the intervention. Therefore, only m and p were separately estimated for the two periods. m was estimated as the average number of female mosquitoes caught per trap night.

Results

At both the sugar-poor and sugar-rich oases, a single application of ATSB on day 12 reduced densities of female *An. sergentii* by over 95% and practically eliminated male *An. sergentii* (Figure 1). Densities of female and male *An. sergentii* in the sugar-poor oasis were immediately reduced by ATSB treatment compared with the more gradual decreases observed in the sugar-rich oasis.

Densities of female *An. sergentii* in the sugar-poor and sugar-rich sites from the pre-treatment period (days 1-12) to the post-treatment period (days 13-47) decreased over 75-fold and 20-fold, respectively, compared to less than a two-fold natural decrease at the control site that did not receive ATSB treatment. At the control site,

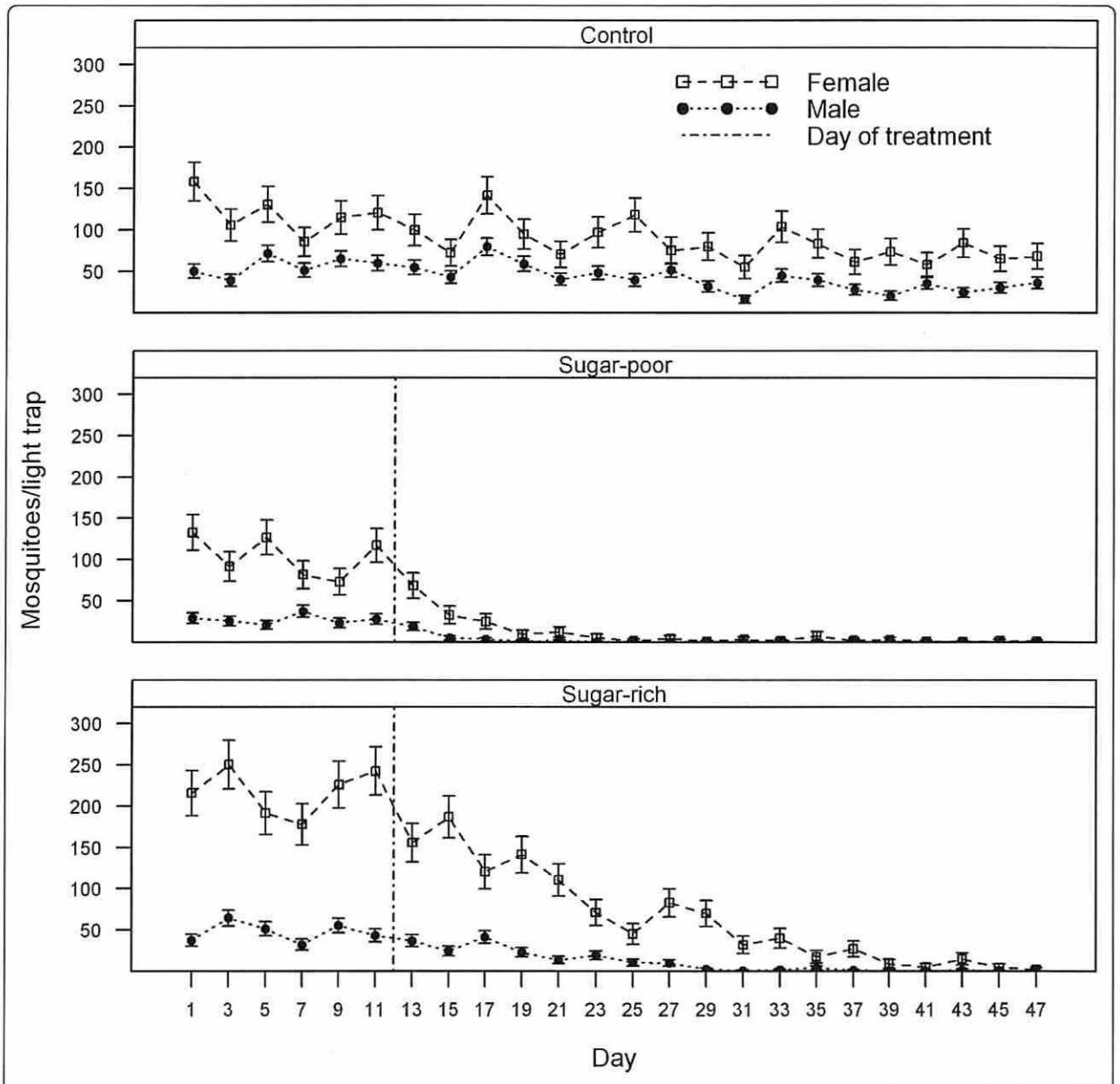

Figure 1 Averages (± 1 standard error) of light trap captures of female and male *Anopheles sergentii* in three oases (sugar-rich, sugar-poor, and control) from 1 November to 17 December, 2009, in Israel (vertical dot lines in panels indicate the date of implementation of ATSB).

densities of female *An. sergentii* averaged 119.42 ± 9.98 before day 12 and 83.52 ± 5.30 from days 13-47. At the sugar-poor oasis, densities of female *An. sergentii* averaged 103.81 ± 10.20 before ATSB treatment and 9.97 ± 4.02 post-treatment. At the sugar-rich oasis, densities of female *An. sergentii* averaged 217.19 ± 11.54 before ATSB treatment and 63.07 ± 13.63 post-treatment. For all but two comparisons of the control oasis with either rich or poor oases the differences were significant at $p < 0.001$ for females after the treatment was applied: control was significantly higher than poor and lower than rich.

Similarly, for male *An. sergentii*, densities decreased about 15-fold and four-fold from the pre-treatment to the post-treatment period in the sugar-poor and sugar-rich sites, respectively, compared to only a one-fold decrease at the control site. At the control site, densities of male *An. sergentii* averaged 56.22 ± 4.77 before day 12 and 40.18 ± 3.59 from days 13-47. At the sugar-poor oasis, densities of male *An. sergentii* averaged 27.36 ±

2.37 before ATSB treatment and 1.75 ± 1.05 from post-treatment. At the sugar-rich oasis, densities of male *An. sergentii* averaged 47.42 ± 4.88 before ATSB treatment and 10.64 ± 3.10 post-treatment. After treatment, males were significantly lower in both poor and rich oases compared with the control oasis at $p < 0.001$.

Table 1 shows, according to pre-treatment days 1-12 and the three post-treatment periods, how ATSB treatment in the sugar-poor and sugar-rich oases affected the proportion of females classified according to gonotrophic cycles (0, 1, 2, 3, and > 3). ATSB treatment reduced the proportion of older more epidemiologically dangerous mosquitoes (three or more gonotrophic cycles) by 100% and 94.9%, respectively, in the sugar-poor and sugar-rich oasis. In the control group the proportion of females with three or more gonotropic cycles increased slightly but not significantly over time. At the sugar-poor site, the proportion of females with three or more gonotropic cycles was significantly reduced compared to pre-treatment levels at 13-24 days ($p = 0.011$), at 25-35 days ($p = 0.014$), and at 36-47 days ($p < 0.001$). At the sugar-rich site, the number of females with three or more gonotropic cycles was significantly reduced in the first week post-treatment ($p = 0.001$) and at the subsequent measurement times ($p < 0.001$ for both times).

Table 1 also shows how ATSB treatment markedly reduced female *An. sergentii* densities, parous rates, survival rates and vectorial capacity. Compared with the control site, while female *An. sergentii* densities decreased less than two-fold as indicated above, parous rates, survival rates, and vectorial capacity remained fairly constant throughout the monitoring period. From the pre-treatment period (days 1-12) to the last period of post-treatment monitoring (days 37-47), the parous rates decreased from 0.59 to 0.12 at the sugar-poor site and decreased from 0.73 to 0.26 at the sugar-rich site. During the same periods, the survival rates decreased from 0.77 to 0.35 at the sugar-poor site and decreased from 0.85 to 0.51 at the sugar-rich site. Malaria vectorial capacity was reduced from a pre-treatment level of 11.2 to 0.0 (last two monitoring periods, days 25-35 and days 37-47) at the sugar-poor oasis and from a pre-treatment level of 79.0 to 0.03 (last monitoring period, days 37-47) at the sugar-rich oasis. Reduction in VC to negligible levels was observed days after ATSB application in the sugar-poor oasis but not until after 2 weeks in the sugar-rich oasis.

Discussion

This field trial shows that a single application of ATSB solution by plant spraying at the two oases treatment sites markedly reduced the relative abundance of *An. sergentii* populations and their longevity. Densities of adult females and males, and the proportion of "older" more dangerous females were reduced by 95% or more. Not unexpectedly, the impact of the ATSB treatment is comparable to that demonstrated in previous field trials [1-7].

The comparison of ATSB spraying of non-flowering vegetation in the sugar-rich and sugar-poor oases allowed experimental testing of the hypothesis that natural sugar resources compete with the ATSB. As expected, ATSB application in the sugar-poor oasis reduced densities of female *An. sergentii* by 95% within 2 weeks. In contrast, it took 4 weeks in the sugar-rich oasis for ATSB application to reduce densities of female *An. sergentii* by 95%. The difference of 2 weeks to 95% population reduction in the sugar-rich oasis, likely due to a reduced frequency of mosquito exposure to ATSB, represented competition with attractive natural sources.

The finding that, regardless of the available natural sugar resources, ATSB use can substantially reduce mosquito densities in arid environments is likely due to

Table 1 Age structure, population parameters, and vectorial capacity (VC) of female *Anopheles sergentii* before and after ATSB treatment on day 12

Site	Intervals of days	Total dissected	Number of gonotrophic cycles (%)					Density/day	Parous rate	Survival rate	VC
			0	1	2	3	> 3				
Control	1-12	180	30	23	14	11	22	119.42	0.70	0.84	33.99
	13-24	180	36	19	12	9	24	96.08	0.64	0.80	16.85
	25-36	180	29	18	12	10	30	85.97	0.71	0.84	25.69
	37-47	180	24	17	15	12	32	68.50	0.76	0.87	32.00
Sugar poor	1-12	180	41	32	14	6	7	103.81	0.59	0.77	11.19
	13-24	180	73	18	5	2	1	25.42	0.27	0.52	0.08
	25-36	141	81	16	2	1	0	3.14	0.19	0.44	0.00
	37-47	92	88	11	1	0	0	1.36	0.12	0.35	0.00
Sugar rich	1-12	180	27	19	14	9	30	217.19	0.73	0.85	79.00
	13-24	180	39	20	17	8	15	130.83	0.61	0.78	16.33
	25-36	180	67	22	7	3	2	47.89	0.33	0.58	0.37
	37-47	171	74	18	6	1	1	10.50	0.26	0.51	0.03

high frequencies of mosquito sugar-feeding [33,34]. Most female and male mosquitoes likely encounter sprayed ATSB solutions and feed at least once during their lifespan (Figure 1). When ATSB solutions are sprayed on non-flowering vegetation as a strategy to reduce overall impact on non-target insects [7], the sprayed areas largely represent favourable outdoor mosquito resting microenvironments and not sugar-feeding centres containing attractive flowering plants. The probability that mosquitoes encounter and feed on sprayed ATSB solution at their outdoor resting microhabitats is high because these are specific locations where mosquitoes spend most of their time.

This study demonstrates for the first time under experimental field conditions how a single application of ATSB can reduce malaria VC from relatively high to negligible levels. Based on ATSB field trials to date [1-7], it is likely that this new approach can also be used in different malaria endemic environments to impact entomological inoculation rates (EIRs) and epidemiological parameters of malaria in humans. Remaining are challenges in the areas of: 1) product development, to standardize attractive baits; 2) deployment methods, to determine the seasonal timing and coverage needed to maximize efficacy while minimizing potential costs and any potential harm to non-target invertebrates; and 3) controlled field trials, to determine how ATSB strategies can be used in combination with existing vector control methods to additionally impact EIRs, especially in eco-epidemiological situations where the continuing problems of malaria cannot be solved using current vector control methods.

Conclusions

This study provides further evidence that ATSB methods can effectively target and kill sugar-feeding anopheline mosquitoes, and shows how available natural sugar resources used by mosquitoes in arid environments compete with applied ATSB solutions. While abundant sugar resources in the sugar-rich oasis delayed full impacts of ATSB by about 2 weeks, mosquito population reductions of over 95% were none-the-less achieved by a single ATSB application. As well, this study shows for the first time how ATSB can reduce malaria VC from relatively high to negligible levels, with only minimal differences due to sugar-poor and sugar-rich environmental conditions. Overall, this demonstration of how even single applications of ATSB solutions can operationally decimate populations of anopheline mosquitoes and drive their potential for malaria transmission to near zero levels highlights the importance of ATSB as a promising new tool for outdoor vector control.

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Authors' contributions

GCM, JCB, WG, and YS conceived and planned the study, interpreted results, and wrote the paper. GCM directed and performed the field experiments and managed the data. KLA and WG analysed the data. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Mosquito community composition in South Africa and some neighboring countries

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Abstract

Background: A century of studies have described particular aspects of relatively few mosquito species in southern Africa, mostly those species involved with disease transmission, specifically malaria and arboviruses. Patterns of community composition such as mosquito abundance and species diversity are often useful measures for medical entomologists to guide broader insights and projections regarding disease dynamics and potential introduction, spread or maintenance of globally spreading pathogens. However, little research has addressed these indicators in southern Africa.

Results: We collected 7882 mosquitoes from net and light traps at 11 localities comprising 66 species in 8 genera. We collected an additional 8 species using supplementary collection techniques such as larval sampling, sweep-netting and indoor pyrethrum knockdown catches. Highest diversity and species richness was found in the Okavango Delta of Botswana and in South Africa's Kruger National Park, while the lowest diversity and abundances were in the extreme southern tip of South Africa and in semi-desert Kalahari close to the South Africa border with Botswana. Species composition was more similar between proximal localities than distant ones (Linear model *P*-value = 0.005). Multiple arbovirus vector species were detected in all localities we surveyed (proportion of vector mosquito numbers were > 0. 5 in all locations except Shingwedzi). Their proportions were highest (> 90%) in Vilankulo and Kogelberg.

Conclusions: Multiple known arbovirus vector species were found in all study sites, whereas anopheline human malaria vector species in only some sites. The combination of net traps and light traps effectively sampled mosquito species attracted to carbon-dioxide or light, accounting for 89% of the 74 species collected. The 11% remaining species were collected using supplementary collection techniques mentioned above. The diversity of species weas highest in savanna type habitats, whereas low diversities were found in the drier Kalahari sands regions and the southern Cape fynbos regions.

Keywords: Mosquitoes, Vectors, Arboviruses, Malaria, Shannon index, Diversity measures, Mosquito community composition

Background

Historic interest in the mosquitoes of southern Africa has largely been based on their role as vectors of human or animal disease. Malaria-associated morbidity and mortality was very high during the early decades of the 20th century [1–5] and gave rise to a disproportionately large body of still-increasing literature on anopheline mosquitoes [6–13]. Outbreaks of arboviral disease, associated with high mortality in livestock, resulted in sustained research on non-anopheline mosquitoes [14–22], with an associated surge in publications relating to regional surveys commencing in the 1950's and 1960's for arboviruses affecting humans [23–29]. In more recent decades the rapid spread and increasing global challenge posed by mosquito-borne viruses, such as West Nile, Zika and others, spurred further publications on mosquitoes [30–33].

The overwhelming majority of the mosquito literature for southern Africa addresses mainly aspects of identification,

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taxonomy or classification [34–40], vector potential or status in one way or another [14, 31, 41–46], or discussion of specific species in relation to insecticide resistance or other aspects of disease control [47–53]. Aside from the breeding biology of some species [54–57], little work has been done on the general ecology and compositions of mosquito communities in southern Africa. Much of the earlier knowledge is captured in summary overviews in the standard reference volumes on anophelines [58, 59] and culicines [60]. Broadly speaking, the species composition and geographical distribution patterns of anopheline mosquitoes in southern Africa are better documented than for culicines, whilst abundance trends and diversity patterns for all mosquito groups have been largely neglected or undocumented.

The increase in frequency of arbovirus outbreaks and rapid spread of such diseases, as well as scale of the public health consequences [61–65], have given rise to multiple calls for countries globally to raise vigilance regarding arboviruses [66, 67] and an associated need to understand the population status of known or potential vector mosquitoes. This paper provides an initial assessment for understanding broad patterns of mosquito diversity, abundance and distribution in southern Africa. Most of the southern African landscape has been altered, due to human agricultural and settlement influences, but a few pockets of more pristine mosquito diversity attributes should still be found in designated National Parks and wilderness areas. For this study, priority was given to natural reserves so that mosquito catches would represent the historic 'natural' state of populations. Therefore our results would represent baseline species diversity data which future surveys could be compared to for assessing human impact in nearby areas of land use change. These studies are also broadly aimed to develop projections and models of where arboviruses are likely to establish and persist when mosquito vector and vertebrate host data are combined.

Methods

We limited the species diversity comparisons to one season to avoid inter-annual fluctuations by undertaking all the surveys within eight weeks, from multiple habitats, using predominantly net (CO_2 baited) and (white light + CO_2 baited) CDC traps [68].

Collection period

Our surveys ran from late-January until early-April 2015, averaging three to four nights per locality. Much of the southern African region experiences summer rainfall from November to April [69]. Mosquito breeding also peaks during these hotter and wet months, so that most mosquito populations are at their highest levels from about January to mid-April. The Kogelberg Nature Reserve in the Western Cape is the exception, falling within a winter rainfall region. However, all the trapping locations in this Reserve were close to the Palmiet River and its fringing fynbos vegetation, which would be the primary source of mosquitoes independent of rain.

Geographical distribution of study sites

A priori selection of localities was aimed at sampling the widest range of biomes, land cover types and geographical spread within the available time and resource constraints, with an emphasis on South Africa, which is the focal country for studies on zoonoses by the University of Pretoria. Georeferenced locations and land-cover types of these localities are provided in Table 1 and Fig. 1, respectively. Locations where mosquitoes were captured were mainly savanna and grassland habitats, except for Vilankulo where some land cover consisted of croplands (Fig. 1).

We moved trap locations each night to cover as many different habitat types as possible and therefore get the widest possible range of species. However, within each locality, all the different trap locations clustered within an arbitrary 10 km distance (Euclidean distance) were collectively designated with one locality name (e.g. Kogelberg, Tswalu or Shingwedzi, etc.), whereas trap sites more than 10 km apart were recorded and named separately (e.g. Skukuza, Lower Sabie and Tshokwane).

Collection methods

We placed three net traps ([70], Fig. 2a) and three CDC white fluorescent light traps (Fig. 2b) each night, each baited with CO_2 in the form of dry ice as attractant. Light traps were not deployed at the Mozambican sites, where we used CO_2-baited net traps and also pyrethrum knockdown catches within rural dwellings (the latter results not used for analyses here, but mention is made of species collected for context). At other sites, traps were usually paired consisting of one net trap and one CDC trap that were placed within 100 m from each other, and each pair of traps was placed several hundred metres or several kilometres apart. CDC light traps were not used in Vilankulo and in Lower Sabie the CDC light traps failed because of battery charging problems and the catch nets falling off the traps.

Traps were placed and baited late afternoon, before dusk, and emptied before first light each morning. Mosquitoes were removed from within net traps using hand held aspirators and transferred into mesh-topped polystyrene cups, and the light traps by tying off the neck-sleeve of the collection bucket. All collections were killed within a few hours thereafter by freezing and immediately examined microscopically for species identification. Representative specimens were pinned as reference material and the remaining specimens grouped by species and frozen in liquid nitrogen for subsequent virus isolation assays.

Table 1 Mosquito composition and diversity data from collections performed in wildlife reserves in southern Africa 2017. Lettering in the first column corresponds to the letter in the map insert in Fig. 1

	Locality	Latitude, Longitude	Survey period	N_{TT} ± SD[a]	N_{LT} ± SD[a]	Net *vs* light trap *P*-value[b]	% vectors[c]	CCM[d]	CCM_{av}[d]	CCM_{NT}[d]	CCM_{LT}[d]	H
	Mozambique											
A	Vilankulo	21°57.1'S, 35°18.8'E	21–28 January	784 ± 662	na	na	93.7	2353 (13) [5]	2205 (5) [3]	2353 (13) [5]	na	0.89
	Botswana											
B	Moremi Game Reserve	19°07.1'S, 23°23.2'E	20–23 February	115 ± 93	163 ± 130	< 0.001	76.2	1339 (27) [8]	1020 (7) [4]	689 (15) [6]	650 (25) [8]	1.74
C	Khwai Community Conservancy Area	19°07.3'S, 23°52.1'E	20–23 February	186 ± 128	399 ± 336	< 0.001	64.2	1356 (18) [8]	870 (4) [3]	559 (12) [4]	797 (16) [8]	1.79
	Kruger National Park, South Africa											
D	Shingwedzi	23°06.7'S, 31°27.4'E	18–21 March	17 ± 9	33 ± 10	< 0.001	23.2	168 (20) [5]	39 (6) [3]	68 (15) [4]	100 (16) [5]	2.12
E	Tshokwane	24°47.1'S, 31°51.3'E	24 March	116 ± 56	33 ± 18	< 0.001	68.4	414 (20) [6]	283 (7) [4]	348 (20) [6]	66 (7) [4]	1.71
F	Skukuza	24°59.1'S, 31°34.8'E	22–23 March	17 ± 6	19 ± 8	< 0.001	74.3	109 (14) [4]	81 (5) [3]	52 (7) [3]	57 (11) [4]	1.80
G	Lower Sabie	25°7.2'S, 31°55.6'E	25 March	46 ± 8	na	na	76.9	91 (13) [4]	70 (8) [3]	91 (13) [4]	na	1.76
	Other South Africa											
H	Lapalala Nature Reserve	23°53.5'S, 28°16.0'E	7–10 April	30 ± 20	17 ± 11	< 0.001	56.2	297 (19) [5]	2,205 (5) [3]	213 (15) [5]	84 (13) [4]	1.99
I	Tswalu Game Reserve	27°17.8'S, 22°28.1'E	14–17 February	2 ± 0	9 ± 9	0.01391	73.3	60 (6) [3]	44 (3) [2]	6 (2) [2]	54 (6) [3]	1.13
J	Rooipoort Nature Reserve	28°56.2'S, 24°10.1'E	7–11 March	115 ± 58	29 ± 20	< 0.001	62.6	1000 (13) [4]	626 (9) [2]	689 (10) [3]	311 (11) [4]	1.40
K	Kogelberg Nature Reserve	34°19.3'S, 18°58.1'E	9–11 February	66 ± 34	73 ± 36	< 0.001	96.7	695 (11) [4]	672 (3) [2]	331 (5) [2]	364 (9) [4]	0.24

Abbreviations: *H*, Shannon's index, *na*, not available

[a]"N_{NT} ± SD" and "N_{LT} ± SD" denote the average number of mosquitoes captured each night in net and CDC light traps, respectively

[b]The net *vs* light trap species composition significance test results analysis

[c]"% vectors" indicates the percentage proportion of mosquitoes that are known human disease vectors out of the total number of specimens

[d]"CCM" denotes community composition measures (number of mosquito specimen captured followed by the number of unique species collected in parenthesis followed by the number of unique genera collected in square brackets) for combined net and CDC light traps (CCM), for combined net and CDC light traps of known human disease vector species, for net trap collections only (CCM_{NT}) and CDC-light trap collections only (CCM_{LT})

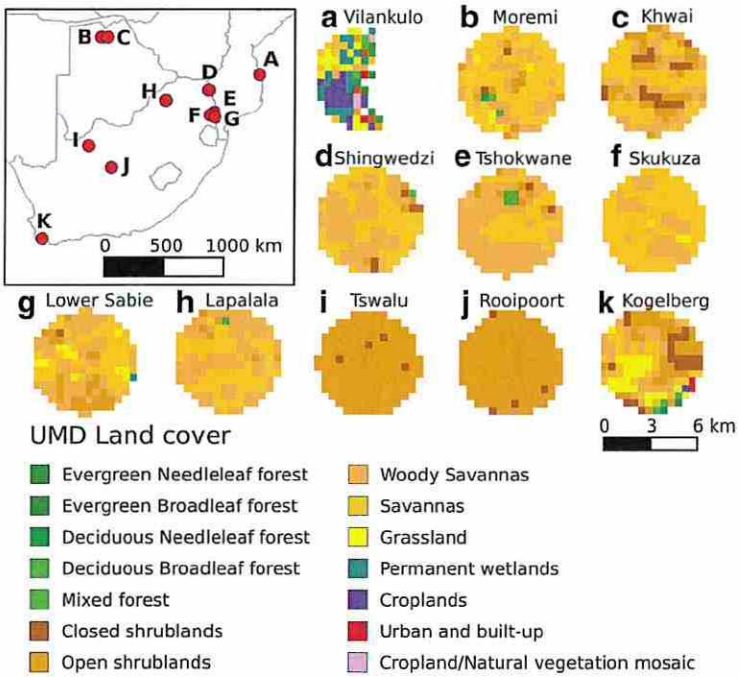

Fig. 1 Map of collection sites and their land cover type within a 3 km radius area (**a-k**). The latest (2013) University of Maryland Land (UMD) cover type data was used. Snow/Ice and Barren land cover type are omitted from UMD legend as our study localities do not include any of those categories. Capital letters superimposed on the map insert correspond to equivalent small letter landcover charts

Supplementary mosquito collections were performed on an *ad-hoc* basis using larval dipping for larvae and pupae, (method described in [71]), and sweep netting and pyrethrum knock down catches for adults. After capture the larvae and pupae were reared individually to adults in single tubes provided with fish flakes (Tetra-Min™- Tetra Holdings, VA, USA) as a food source. Pyrethrum knock down catches were performed in homes in Mozambique.

Species identification methods

Morphological species identification was carried out using keys and descriptions [59, 60, 72, 73]. In cases of

Fig. 2 Traps used for this study. **a** Mosquito net trap baited with polystyrene box containing dry ice placed on the ground in centre of trap. **b** Center for Disease Control (CDC) light trap with white light baited with dry ice

doubt in the morphological identifications, pinned specimens were compared with specimens in the reference collections of the South African National Institute for Communicable Diseases. For members of the *Anopheles gambiae* complex and *Anopheles funestus* group, laboratory PCR identifications were done on individually-tubed specimens preserved in tubes containing silica gel (*funestus* group) or 80% ethanol (*gambiae* complex) following protocols in [74–76], respectively. *Anopheles funestus* group members from Moremi and Khwai were not identified to group member because the DNA of these specimens was too degraded for preservation by the time we got back to the base camp.

In southern Africa, *Culex quinquefasciatus* and *Culex pipiens* do not hybridize and are easily distinguishable as adults [77]. Some adult female species cannot be reliably distinguished and in these instances they were identified to the two possible species they could be.

Data analysis

Net and light trap mosquito species counts were averaged across all the nights of mosquitoes captured at each location. Species composition comparisons between net and light traps were done using Wilcoxon rank sum test ($P > 0.5$) using the R statistical package [78].

Pie charts depicting the percent proportions of catches represented by each of the major species (CDC light and net trap counts combined) in each location were created in Microsoft Excel (Version 2010) based on the raw capture data provided in Additional file 1: Table S1. CDC light traps were not performed in Vilankulo and Lower Sabie so the pie charts relevant to these locations represent only net trap data species compositions. All species that contributed relative percentage catches below 0.5% of the total catch were represented as "other spp." pertaining to their specific genera in the pie charts. Color coding for each species was kept consistent for all pie charts.

We selected two measures for depicting taxonomic richness and species diversity. To reflect taxon richness we developed a simple indicator that provides a 'one-glance catch-all' measure of the number of individuals caught in a trap, followed by the number of species and then genera in brackets. For example, if 300 mosquitoes representing 12 species and 5 genera were caught in a particular trap then this catch is summarized as '300 (12) [5]'. For ease of reference in the discussion below, we refer to the simple numeric 'catch-all' measure as the 'Community Composition Measure' (CCM). As a measure of species diversity we used the Shannon's index (H) [79], which takes into account not only the number of taxa (species in our case) but also the relative abundance in which the different species are represented in the catch. For example, a trap catch having 100 individuals made up of 2 species and each of the 2 species represented by 50 individuals will have a higher score (H = 0.693) than a trap catch of 100 mosquitoes made up of 2 species but of those, one species is represented by 90 individuals (H = 0.325). This index is commonly used in ecology to provide information about community composition reflecting not only the unique number of species but also its abundance [80].

QGIS version 2.18.4 was used for generating the land cover maps based on the University of Maryland (UMD) Year 2013 Land Cover Classifications. The 2013 UMD Land cover data was extracted from MCD12Q1 MODIS Land Cover Type product available from Land Processes Distributed Active Archive Center (https://lpdaac.usgs.gov/dataset_discovery/modis/modis_products_table/mcd12q1) using the HDF-EOS to GeoTIFF Conversion Tool (HEG) version 2.14.

Morisita-Horn index [81] was calculated as measure of species composition similarity following the recommendation by Wolda [82]. Morisita-Horn index of 0 means no similarity and its value close to 1 means high similarity in species composition. A dendrogram based on the Morista-Horn index similarity matrix was generated using Scipy python library (https://www.scipy.org/).

Results

A total of 7882 mosquitoes were collected. Sixty-six species from 8 genera were collected in either or both the net and CDC traps and an additional 8 species, thus bringing the overall total to 74, were collected as larvae and other supplementary collections. Supplementary collections were not included in the diversity indices calculations. Additional file 1: Table S1 provides a full list of species collected, and includes a summary of geographical localities each species was found in, the total number and percentage of total catch each species comprised, the known pathogens vectored by each of the species, and reference to the publication confirming their role as a vector. Pie charts representing species composition percentages for combined net and CDC light traps are provided in Fig. 3.

In Kogelberg, a specimen keyed out as *Uranotaenia hopkinsi*, a species that according to Jupp [60] occurs in Mozambique and not in South Africa. However, morphological features such as, (i) broad band of bluish scales along eye margins, (ii) pale scales at base of wing vein R, and (iii) broad patch of bluish white scales at wing root, were noted in the mosquito from Kogelberg, which are characters that differ slightly from that described for *Ur. hopkinsi* [69].

Culex quinquefasciatus, represented by 1708 individuals, was the most abundantly captured mosquito, but > 99% of these were collected in one locality (Vilankulo, Mozambique, see Shannon's H in Fig. 4). *Mansonia uniformis*, with 1505 individuals captured from 8 different trapping locations, was more equitably abundant over the

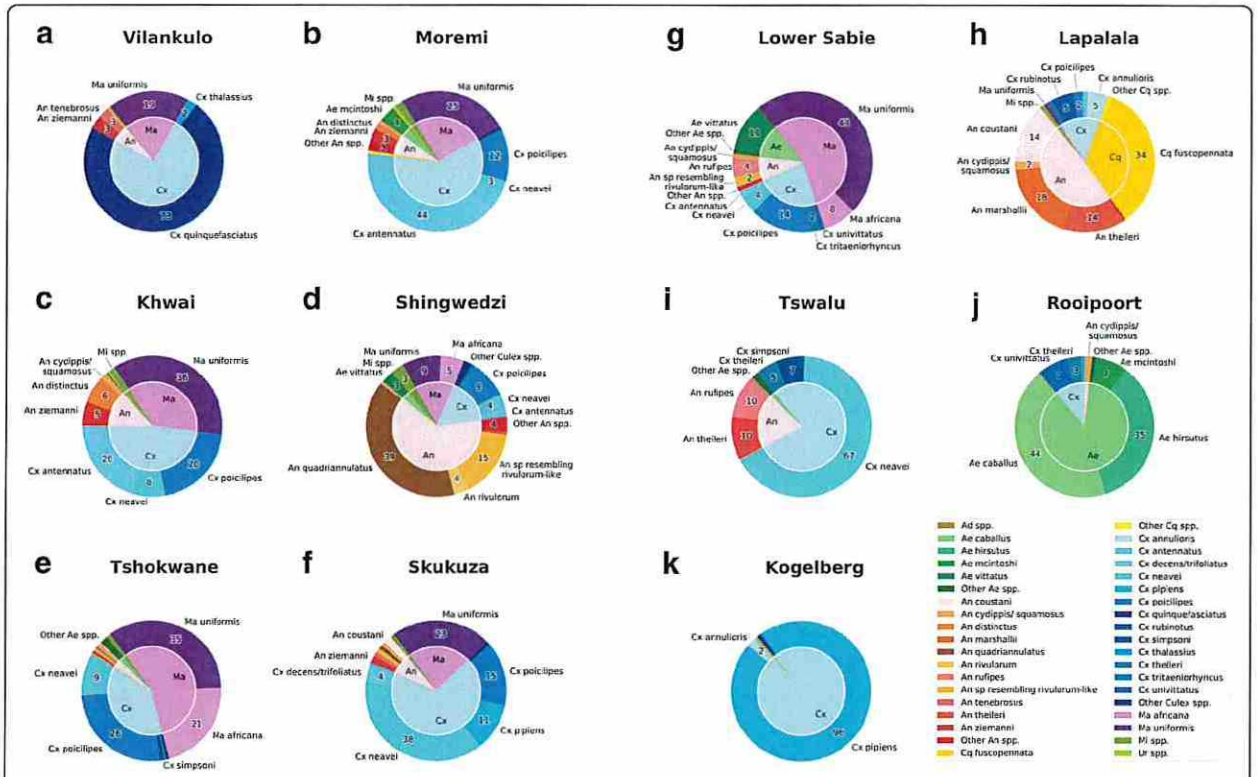

Fig. 3 Mosquito species composition pie charts of combined net and CDC-light traps. **a** Vilankulu, MZ. **b** Moremi, BW. **c** Khwai, BW. **d** Shingwedzi, KNP, SA. **e** Tshokwane, KNP, SA. **f** Skukuza, KNP, SA. **g** Lower Sabie, KNP, SA. **h** Lapalala, Limpopo, SA. **i** Tswalu, Northern Cape, SA. **j** Rooiport, Northern Cape, SA. **k** Kokelberg, Western Cape, SA. Species with densities below 2% were grouped into respective genera as spp. Genus name abbreviations are consistent with [95]. Dark tan color was used for *Aedomyia* species; shades of greens for *Aedes*; shades of red and oranges for *Anopheles*; shades of yellow for *Coquillettidia*; shades of blue for *Culex*; shades of purple for *Mansonia*; olive green for *Mimomyia*; and lime green for *Uranotaenia*. Numbers in outer pie slices indicate proportions of individuals from total net and light trap catches within each locality. Inner pie shows the relative proportions of each genus

southern African region, followed by *Culex antennatus* (n = 876), *Cx. pipiens* (n = 689) and *Cx. poicilipes* (n = 592). Fourteen species were represented by only one specimen having been caught. The species found in the largest number of localities were *Anopheles squamosus*, *Cx. antennatus*, *Cx. neavei*, *Cx. poicilipes*, *Cx. univittatus* and *Mansonia uniformis*. Of all genera, *Culex* was the genus represented by the most species (n = 27).

Malaria vector species were collected mainly in Vilankulo, and a few in Moremi, Khwai and the Kruger National Park.

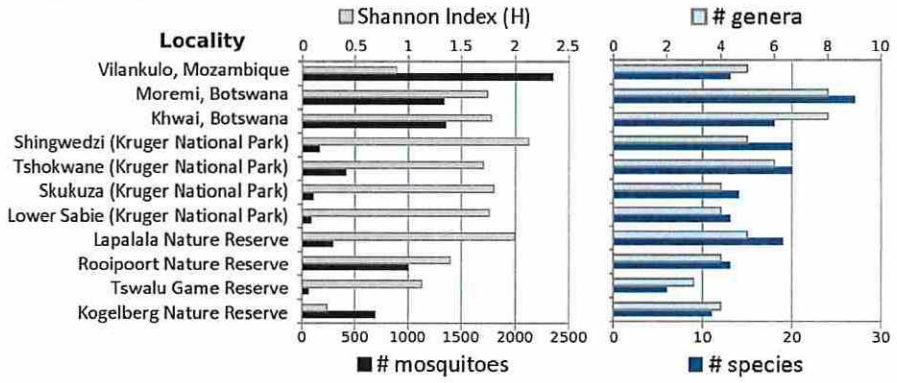

Fig. 4 Species diversity measures for each location. Shannon's index (top X axis) are represented in gray bars, the total number of mosquito specimens collected (bottom X axis) in black, the number of unique genera (top axis) in light blue and the total number of unique species (bottom X axis) are in dark blue

Table 2 Species collected in either net or CDC light traps. Species collected in multiple locations are highlighted in bold

Species only found in net traps	Species only found in light traps
Ae. juppi (*n* = 3)	*Ae. aegypti* (*n* = 1)
Ae. ochraceus (*n* = 8)	*Ae. fowleri* (*n* = 1)
An. longipalpis (*n* = 1)	*Ae. ledger* (*n* = 1)
An. parensis (*n* = 3; 3 locations: Shingwedzi , Vilankulo and Lower Sabie)	*Ae. mixtus/microstictus* (*n* = 1)
Coq. maculipennis (*n* = 3)	*Ae. simpsoni* (*n* = 1)
Cx. aurantapex (*n* = 2; 2 locations: Moremi and Tshokwane)	*Ae. subdentatus* (*n* = 1)/*Aedeomyia africana* (*n* = 1); ***Ae. furfurea*** (*n* = 10; 3 locations: Moremi, Khwai and Rooipoort)
Cx. decens/trifoliatus (*n* = 4)	*Cx. chorleyi* (*n* = 2)
Cx. quinquefasciatus (*n* = 1708; 2 locations: Vilankulo and Rooipoort)	*Cx. duttoni* (*n* = 1)
Cx. salisburiensis (*n* = 1)	*Cx. nebulosus* (*n* = 1)
	Cx. pulchrithorax (*n* = 1)
	Mi. splendens (*n* = 25; 3 locations: Moremi, Khwai and Shingwedzi)
	Ur. hopkinsi (*n* = 1)
	Ur. mashonaensis (*n* = 1)

However, a significant proportion of mosquitoes that vector arboviruses were collected at all locations in combined net and CDC light traps (Table 1: % vectors and CCM_{av}). Their numbers of individuals comprise more than half of the total mosquito catches, except in Shingwedzi (KNP).

A significant difference in species captured between net and CDC light traps was found in all locations (Table 1, in bold print), except for in Tswalu, (Wilcoxon-rank sum test, V = 36–630, *P*-value < 0.0008). There was no significant advantage of CDC light traps over net traps in capturing higher numbers of different species (Wilcoxon-rank sum test *P*-value > 0.5). There were 9 species that were captured only in net traps and 14 species only in the CDC light traps (Table 2).

The Community Composition Measure (CCM) of mosquito numbers (Table 1, Fig. 4), indicated the highest species richness (31 species in 6 genera) along the Sabie River and Tshokwane Picnic Site in the southern KNP. CCM indicated the lowest species richness at Tswalu Game Reserve in the arid Kalahari (6 species in 3 genera). The Moremi Game Reserve and Khwai Conservancy in the Okavango Delta of Botswana had fewer species than in the southern KNP but had more genera represented (29 species in 8 genera), but catch sizes were also much larger in Okavango.

With Shannon's index greater than 1.7, Kruger National Park (KNP) and Botswana localities had greater mosquito diversities than other localities (Table 1, Fig. 4). The number of species captured in Vilankulo and Kogelberg was comparable to Kruger National Park sites but Shannon's diversity index was lower than 1, indicating less evenness of species composition (Fig. 4). The number of species collected in Tswalu Game Reserve was lowest, but its diversity index (Shannon's H) was higher than Vilankulo and Kogelberg, indicating the more evenness in species composition (Fig. 4). The species diversity represented in Shannon's index (H) correlated with latitude (R^2 = 0.4415, linear model *P*-value = 0.0257). Correlation between H and historic precipitation (WorldClim version 2.0) was not significant (linear model *P*-value > 0.05).

Similarity in species composition between localities, measured as Morisita-Horn Index [81], show that southern Kruger National Park (KNP; Lower Sabie, Skukuza and Tshokwane) and Botswana (Moremi and Khwai)

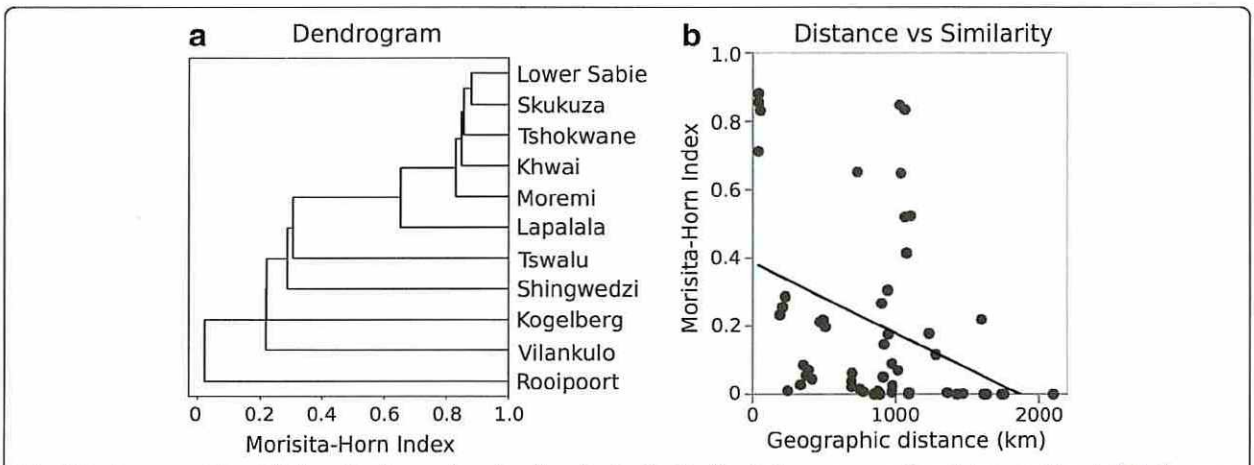

Fig. 5 Species composition similarity. **a** Dendrogram based on the pair-wise Morisita-Horn Index, a measure of species composition similarity between any two localities. **b** Relationship between species composition similarity measured as Morisita-Horn Index and geographical distance (Linear model slope = -0.0002, R^2 = 0.1399, *P*-value = 0.005)

were similar (Morisita-Horn Index > 0.8) as shown in Fig. 5a. Generally, the species composition similarity decreased as geographical distance between two localities increased (Fig. 5b). Botswana and KNP were exceptions to this rule as they are almost 1000 km apart but yet had similar species compositions. Hence, other factors such as land cover types and availability of blood source may be affecting species composition. However, due to the limited number of sites we surveyed, we did not have enough power to detect any significant relationship between land cover types and species diversity and/or composition.

Discussion

In a country or region where relatively little is known about mosquito populations, or presence of arboviruses, understanding the potential for arbovirus transmission is dependent on answering the following key questions "What species occur here?" and "How abundant are they and how do they vary in time and space?". Given the potential for rapid spread of arboviruses at global scale [83, 84], and their alarming public health impacts and little capacity to prevent this [85], answers to these key questions are becoming increasingly important. These questions are even more important on the African continent where many of the current crop of emerging and re-emerging viruses have originated. Africa also hosts many relatively quiescent viruses that remain confined to sylvatic cycles.

Trapping was not done within five km of the rest camps within the wildlife reserves, to avoid collections of mosquitoes associated with human habitats. However, it should be noted that at the rest camps in Tswalu and Rooipoort nature reserves, *Ae. aegypti* and *Culex quinquefasciatus* were profound nuisances. Both of these mosquitoes are arbovirus vectors and potentially pose a risk of arbovirus transmission to guests if the viruses were inadvertently introduced.

Our CCM metric provides an intuitive sense of mosquito population attributes, that enables one to instantly get a 'sense' of species diversity represented in a given area (Table 1). Our trapping methods were proven to be effective in capturing potential vector species as indicated in Table 1. All locations have more than three species and two genera that are known to be arbovirus vectors (Table 1). The high numbers of one vector species, namely *Cx. pipiens* and *Cx. quinquefasciatus* (> 90%) in Kogelberg and Vilankulo, respectively, is concerning in terms of transmission potential.

Our survey also demonstrated the high degree of consistency using a combination of net traps and light traps to quickly sample mosquito species richness in a particular locality. For instance, southern Kruger National Park sites (Skukuza, Tshokwane and Lower Sabie) all had H around 1.7 (Table 1, Figs. 3 and 4) and their species compositions were very similar (Morisita-Horn Index > 0.8; Fig. 5). We collected most of the species (89.2%, of 74 species) using these two trap types and additional sampling of mosquitoes by way of collecting larvae in tree-holes, rock-pools and various other habitats, and sweep-netting through tall and dense grass along riverbanks, and conducting pyrethrum knockdown sprays in Vilankulo yielded relatively fewer species. Because of the significant differences in species captured between the net and CDC-light traps at almost all locations, we recommend using both trap types for species diversity and for outdoor biting arbovirus and malaria vector surveillance.

Despite the relative efficiency of our sampling design to survey the diversity of carbon-dioxide-attracted mosquitoes within the space of three to four days at a particular locality, clear shortfalls have also become apparent. Two years after this survey, we collected mosquitoes at Shingwedzi (KNP) in March 2017 for ten days following two months of sustained good and regular rainfall, which created excellent breeding conditions for many mosquito species. This survey yielded several additional species not found during the 2015 survey. These included *Aedes sudanensis*, *Anopheles arabiensis* and *Culex tigripes*. The reverse was also true, that despite highly favourable conditions, some species were not collected during March 2017 but were caught under very average conditions of March 2015. Furthermore, copious numbers of *An. arabiensis* were collected by Cornel, Lee and Braack, in Lapalala in February 2017, a species that had not been collected in this region for many years. There were also multiple reported cases of malaria in this region which had also been malaria free for many years previously, indicating that species distributions contract and expand periodically. This emphasises the obvious though, that a single visit even during a 'good season' will not yield an exhaustive catch of all species and that repeat visits in different seasons over several years are necessary to achieve a 'complete' picture of mosquito diversity in a region or to monitor trends in mosquito community composition.

Despite most of our sampling localities being situated in hot, summer rainfall areas, in the bushveld or savanna habitats, with abundant surface water available and similar mix of plentiful wildlife (blood meal sources) and despite the known presence of both members of the *Anopheles gambiae* complex being present in the Okavango Delta (Moremi, Khwai), and KNP, only *Anopheles arabiensis* was captured in the Okavango traps and only *Anopheles quadriannulatus* in the traps at KNP. However, larvae collected in one elephant footprint pool at Lower Sabie in southern KNP and reared to adulthood yielded a mix of *Anopheles arabiensis* and *A. quadriannulatus*, suggesting

that the absence of one or the other species in the traps was simply an artefact of chance. The ecological determinants underpinning local dominance and abundance of these two partially sympatric species are poorly understood, with one species predominating in one region of sympatry, such as *Anopheles arabiensis* apparently more common than in the Okavango Delta than *An quadriannulatus* [86], the reverse apparently applying in the Mpumalanga Province of South Africa [87, 88], all or which likely plays a role in the presence of these species in trap catches.

Although our survey is limited in geographical and trap coverage, we found high species richness and diversity in extensive wildlife conservation areas. These areas retain historic ecological integrity and habitat diversity and are moderately or well supplied with good quality surface water for breeding substrate and have readily available sources of blood meals in the form of birds and mammals. These conditions exist in the Okavango Delta, Kruger National Park, and Lapalala Game Reserve, which all show Shannon's indices above 1.7, but lower levels of diversity are present at localities where essential elements of favourable habitat are lacking, such as in the Kogelberg which had few medium to large birds and mammals, and Rooipoort and Tswalu Nature Reserve, which have adequate birds and mammals but very little surface water appropriate for mosquito breeding.

Subsets of the data can be usefully compared with findings of other studies. For example, Ngomane et al. [89] found that in a sample of 319 *Anopheles funestus* group mosquitoes collected from eight sites in Mpumalanga Province of South Africa between 2002–2005, 7.8% were *Anopheles funestus* (*sensu stricto*), 60.2% *An. rivulorum* (presumably includes *An. rivulorum*-like), 10.7% *An. vaneedeni*, 10.9% *An. parensis* and 10.3% *An. leesoni*. Our collective sample of 63 *Anopheles funestus* group captured over a two-week visit (includes a few individuals collected from traps not reported on here) at five collection areas in Kruger National Park comprised 77.7% specimens resembling *An. rivulorum*-like, 17.5% *An. rivulorum*, 3.2% *An. parensis* and 1.6% *An. leesoni*. These differences in species assemblage across geographically-adjoining areas are likely due to different sampling methodologies, Ngomane et al. [89] obtaining most of their specimens from night-time human landing catches and day-time catches of mosquitoes resting in natural shelters, compared to our net trap and light trap techniques. These differences also emphasize the need to standardize trapping techniques to allow for valid comparisons.

Steyn et al. [90] spent 15 days sampling mosquitoes at multiple sites along the Limpopo River valley, covering some 300 miles from Vaalwater (very close to Lapalala Game Reserve) eastwards to the northern Kruger National Park at Pafuri (not far from Shingwedzi). Similar to our sampling surveys at Lapalala and Shingwedzi, their survey was also in March in late wet-season. Their survey yielded 538 mosquitoes comprising 21 species in three genera. Their catches are of the same general scale as ours, where we record 20 species (5 genera) at Shingwedzi and 19 (5 genera) at Lapalala in much shorter collection periods. Steyn et al. [90] based their publication mostly on larval collections (409 larvae) from tree-holes, pools at quarries and borrow-pits, rock pools and even large snail shells, supplemented with adult catches (129). Importantly however, despite the similarity in species richness, nearly 50% of the species they caught or reared were different to those in our collections, with a predominance of *Aedes* species as can be expected from the types of breeding sites they sampled. Once again, the need to standardize collection techniques is emphasized. Long term monitoring of mosquito populations and comparisons between different sites are reliant on quick efficient trapping techniques directed at sampling important species, whilst ecological studies aimed at understanding species richness will require a wide a range of collection methods.

The Shingwedzi River and Sabie River collection areas roughly bisect the northern and southern regions of the Kruger National Park in South Africa. The habitat along these rivers is fairly representative of these two regions, especially as it relates to mosquito breeding site types. In March 2015, we collected 20 species (5 genera) in the north and 31 species (6 genera) in the south of KNP. In April 1953, Schultz et al. [91] surveyed culicine mosquitoes by way of larval collections throughout the KNP, and collected a total of 907 mosquitoes (799 larvae, 108 adults) made up of 25 species in four genera; three species were *Anopheles*, one *Orthopodomyia*, 12 *Aedes* and nine *Culex*. Schultz et al. [91] collected 12 species and one genus (*Orthopodomyia*) over 18 days that we did not collect in our stay in eight days. Conversely, we collected 15 species and three genera (*Coquellittidia*, *Mansonia* and *Mimomyia*) that they did not collect. Clearly each collection method has its own limitations and there is a need to clearly identify the purpose of the survey and to design appropriate sampling strategies to optimize appropriately targeted outcomes, especially if time and manpower are limited. Our combination of net traps and light traps proved efficient in yielding a good range of species and genera in very limited time, and were good at collecting a wide range of *Culex* and *Anopheles* species not well represented in the Schultz et al. [91] and Steyn et al. [90] collections, but were poor at trapping *Aedes* in comparison with the larval collections in the Steyn et al. [90] and Schultz et al. [91] collections.

To give some sense of what kind of species richness can be expected after very intensive and continuous sampling; van der Linde et al. [92] placed four light traps at weekly intervals for three years in a rural area near Bloemfontein

in the central region of South Africa. They collected 143,438 mosquitoes representing 25 species in four genera, of which 85% were of the three species *Aedes juppi*, *Aedes durbanensis* and *Culex theileri*. Our Rooipoort site is within a couple of hundred kilometres from Bloemfontein, and we collected 1000 mosquitoes representing 13 species in 4 genera during five days of sampling using net traps and light traps. This again suggests that even relatively short sampling periods of three to five days using CO_2 baited net traps and light traps can be effective in providing broad insight to species composition and abundance if done at the appropriate seasonal time.

Conclusions

For disease epidemiological or surveillance aimed at collecting mosquitoes of as broad a range of species as quickly as possible, our findings suggest that a combination of night-operated CO_2 baited net traps and light traps provides good representation of mosquito diversity and abundance in an area even during relatively short sampling visits lasting 3–5 nights. *Anopheles*, *Culex*, *Mansonia*, *Coquellittidia* and *Mimomyia* are well represented in our collections, but *Aedes* appeared to be underrepresented. This may be because many *Aedes* are predominantly day-biting and, at least in some cases, also have shorter periods of seasonal abundance. For *Aedes* collections it is therefore probably better to do larval collections from container habitats, supplemented with day-operated odour-baited BG traps or similar devices [93]. We also find a simple Community Composition Measure (CCM) which combines numbers of mosquitoes captured, number of species, and number of genera, a far more useful indicator of mosquito community status and structure at a particular sampling site than the Shannon's index, although the two measures do complement each other and together provide a more informed assessment. Limited time and resources constrained our ability to develop a finer-grained understanding of mosquito communities across South Africa, yet the selected broad coverage of the sites represented in this study does provide good initial insight as to where high diversity and numbers are likely to be found, such as in the extensive untransformed conservation areas located in warm climates and having a combination of diverse mammals and birds as blood meal source and good stands of surface water of different types; arid environments and areas poor in birds and animals appear to support lower species richness. This may seem intuitively obvious, but is useful confirmation.

Additional file

Additional file 1: Table S1. Mosquito species capture data and disease vector status for each of the mosquito species captured. (XLSX 31 kb)

Abbreviations

BAGV: Bagaza virus; BANV: Banzi virus; BATV: Batai virus; BBKV: Babanki virus; BUNV: Bunyamwera virus; BWAV: Bwambwa virus; CCM: Community Composition Measure; CDC: Centers for Disease Control and Prevention; CHIKV: Chikungunya virus; CYV: Chaoyang virus; DENV1-4: Dengue virus serotypes 1 to 4; GERV: Germiston virus; H: Shannon Index; KNP: Kruger National Park; MIDV: Middelburg virus; NDUV: Ndumu virus; NRIV: Ngari virus; ONNV: O'Nyong-Nyong virus; PGAV: Pongola virus; QBV: Quang Binh virus; RVFV: Rift Valley fever virus; SFV: Semliki Forest virus; SHUV: Shuni virus; SINV: Sindbis virus; SPOV: Spondweni virus; UGSV: Uganda S virus; USUV: Usutu virus; WESV: Wesselsbron virus; WITV: Witwatersrand virus; WNV: West Nile virus; YFV: Yellow fever virus; ZIKV: Zika virus

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Availability of data and materials

The data supporting the conclusions of this article are included within the article and Additional file 1: Table S1.

Authors' contributions

AC and LB did the fieldwork, assisted at times by PA. AC did the mosquito identifications, assisted at times by JM, YL and PA. LB, PA and YL conducted data analysis. LB and AC conceptualized and wrote the manuscript drafts, with subsequent additions and editorial comments by all other authors. LB conceptualized and developed the community composition measure. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Modelling and observing the role of wind in *Anopheles* population dynamics around a reservoir

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Abstract

Background: Wind conditions, as well as other environmental conditions, are likely to influence malaria transmission through the behaviours of *Anopheles* mosquitoes, especially around water-resource reservoirs. Wind-induced waves in a reservoir impose mortality on aquatic-stage mosquitoes. Mosquitoes' host-seeking activity is also influenced by wind through dispersion of CO_2. However, no malaria transmission model exists to date that simulated those impacts of wind mechanistically.

Methods: A modelling framework for simulating the three important effects of wind on the behaviours of mosquito is developed: attraction of adult mosquitoes through dispersion of CO_2 (*CO_2 attraction*), advection of adult mosquitoes (*advection*), and aquatic-stage mortality due to wind-induced surface waves (*waves*). The framework was incorporated in a mechanistic malaria transmission simulator, HYDREMATS. The performance of the extended simulator was compared with the observed population dynamics of the *Anopheles* mosquitoes at a village adjacent to the Koka Reservoir in Ethiopia.

Results: The observed population dynamics of the *Anopheles* mosquitoes were reproduced with some reasonable accuracy in HYDREMATS that includes the representation of the wind effects. HYDREMATS without the wind model failed to do so. Offshore wind explained the increase in *Anopheles* population that cannot be expected from other environmental conditions alone.

Conclusions: Around large water bodies such as reservoirs, the role of wind in the dynamics of *Anopheles* population, hence in malaria transmission, can be significant. Modelling the impacts of wind on the behaviours of *Anopheles* mosquitoes aids in reproducing the seasonality of malaria transmission and in estimation of the risk of malaria around reservoirs.

Keywords: Malaria transmission, Water-resource reservoirs, Environmental conditions

Background

Malaria transmission is an intricate function of environment. Alternation in environment may exacerbate malaria risks, with global warming being an example [1–4], and the construction of dam-related reservoirs and irrigated fields being another [5–13]. Understanding the environmental determinants of malaria transmission helps in predicting the seasonality and the future risks of transmission, and hence in designing efficient control programs.

Wind conditions, as well as many other environmental conditions, are likely to influence malaria transmission through the behaviours of *Anopheles* mosquitoes—the vectors of malaria. The responses of adult mosquitoes to wind have been poorly understood. Controversy over field observations exists regarding mosquitoes' flight responses; some suggest mosquitoes fly upwind [14, 15], and others downwind [16, 17]. Results from laboratory experiments support upwind flights, especially in the presence of odour and heat, but also in the absence of

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them [18–20]. Within some tens of meters from human bait, mosquitoes are generally believed to fly upwind guided by CO_2 plume originating from the humans (upwind flight leads mosquitoes towards higher concentration of CO_2) [21–24]. Downwind flight behavior may be prominent for long-distance migration [16, 25].

The influence of wind on the population of *Anopheles* mosquitoes may be especially significant around reservoirs [26]. Aquatic-stage mosquitoes that breed at reservoir shorelines face additional mortality through surface waves in reservoirs. Like turbulence during rainstorms, high waves at a reservoir shoreline provide an unfavourable condition for aquatic-stage mosquitoes [5, 27, 28]. Because surface waves become higher in larger and deeper bodies, the mortality from waves is often unique to large water bodies such as reservoirs, but not to small rain-fed puddles.

This paper aims (1) to model the role of wind in the behaviours of *Anopheles* mosquitoes based on physics and physiology known to date, and (2) to quantify the role of wind using observations. To the best of the authors' knowledge, no mechanistic model exists that incorporates the effect of wind on malaria transmission, except for site-to-site deductive correlation-based models. The *Anopheles* population data come from a village adjacent to the Koka Reservoir in Ethiopia [26]. The impacts of wind on mosquitoes behaviours are incorporated in a malaria transmission model, HYDREMATS—one of the most detailed mechanistic malaria models to date [29]. The role of wind in *Anopheles* population was analysed combining simulations and observations.

Methods

Field observations

Multi-year extensive field surveys were conducted near a village adjacent to Koka Reservoir (N8° 25′; E39° 05′) in Ethiopia. The village, named Ejersa, is located north-west of the Koka Reservoir. Its elevation is around 1600 m, and its mean annual temperature is about 21.1 °C. The annual malaria incidence rate in this village was 55 [cases/1000 persons/year] between 2009 and 2014 (personal communication). Of the cases, approximately two-thirds are caused by *Plasmodium falciparum* (*P. falciparum*) and one third by *Plasmodium vivax* (*P. vivax*). The major *Anopheles* (*An.*) vectors present at Ejersa are *An. arabiensis*, *An. pharoensis*, *An. funestus*, and *An. coustani* [9, 26]. Malaria in this area is classified as hypo-endemic, and the incidence rate is declining owing to control measures in place (free diagnosis and medicines, distribution of insecticide-treated bed nets, occasional use of indoor residual spraying).

The field campaigns span from Jul. 2012 to Apr. 2015, monitoring environmental and entomological conditions [26]. Detailed information on local wind profile (wind speed and wind direction) were obtained from an *in situ* weather station at 30-min resolution, as well as other climatological data (Fig. 1a–d). The daily water levels of the Koka Reservoir were obtained from the Ethiopian Electric Power Corporation (EEPCo) (Fig. 1e). *Anopheles* population dynamics were monitored through weekly or bi-weekly adult sampling surveys using six CDC miniature light traps deployed in Ejersa (Fig. 1f).

This area experiences three climatological seasons: a main rainy season from June to September, locally known as *Kiremt*; a dry season from October to February, *Bega*; and a secondary rainy season from March to May, *Belg* (Fig. 1b). During the main rainy season, the temperature becomes lower than the other two seasons (Fig. 1a). Wind profile also shifts between the rainy seasons and the dry season (Fig. 1c, d). The reservoir water levels have the lowest and highest peaks around the beginning and the end of the main rainy seasons (Fig. 1e). The *Anopheles* population peaks once or twice a year (Fig. 1f). A large increase in population occurs during Sep.–Dec. (hereafter the *major mosquito season*). A small increase may or may not occur during May.–June (hereafter the *minor mosquito season*). How the *Anopheles* population is influenced by the environmental conditions in Ejersa is described in Endo and Eltahir [26].

Modelling mosquitoes' flight behaviours

The locomotion of adult mosquitoes ($\overrightarrow{v}$) is modelled as a combination of active dispersal ($\overrightarrow{v_{active}}$) and passive dispersal ($\overrightarrow{v_{passive}}$). Active dispersal ($\overrightarrow{v_{active}}$) is also called appetential dispersal, where mosquitoes fly around by themselves in search of blood sources and oviposition sites, for example. Passive dispersal ($\overrightarrow{v_{passive}}$), on the other hand is called non-appetential dispersal, where mosquitoes move with wind through advection.

$$\overrightarrow{v} = \overrightarrow{v_{active}} + \overrightarrow{v_{passive}} \quad (1)$$

Modelling the role of wind

Three important effects of wind on the behaviours of mosquitoes are modelled: attraction of adult mosquitoes through dispersion of CO_2 (hereafter, CO_2 *attraction*), advection of adult mosquitoes (hereafter, *advection*), and aquatic-stage mortality due to wind-induced surface waves (hereafter, *waves*).

CO_2 attraction

Mosquitoes active flight component ($\overrightarrow{v_{active}}$) can be modelled as a summation of random flight ($\overrightarrow{v_{random}}$) and directed flight ($\overrightarrow{v_{directed}}$).

$$\overrightarrow{v_{active}} = \overrightarrow{v_{random}} + \overrightarrow{v_{directed}} \quad (2)$$

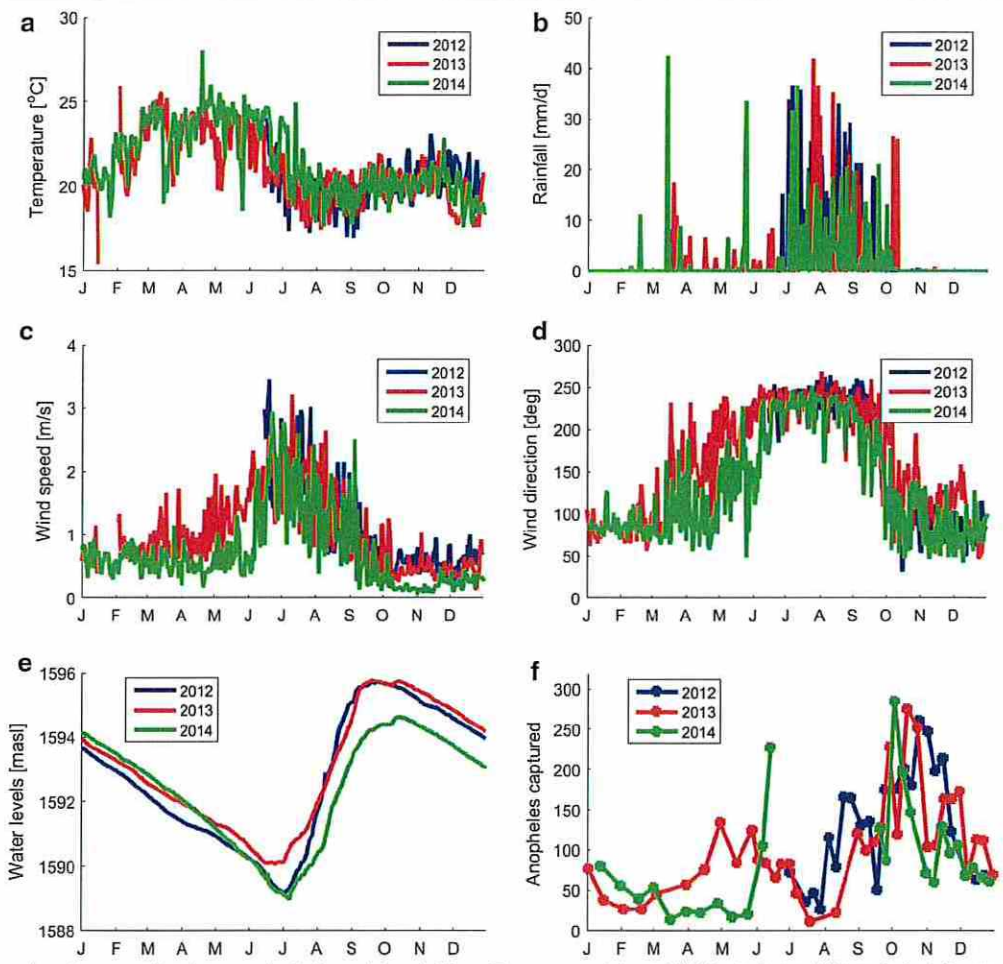

Fig. 1 Observed environmental and entomological conditions in Ejersa. Temperature (**a**), rainfall (**b**), wind speed (**c**), and wind direction (**d**) were observed at an in situ weather station at 30-min resolution, and the daily-average data are presented here. Wind direction was measured with respect to the north, increasing clockwise. The operation of the weather station started in Jul. 2012. The daily reservoir water levels were obtained from EEPCo and presented in meters above sea level [masl] (**e**). Observed *Anopheles* population in the six light traps are shown in square, and the data points are connected with simple linear interpolation (**f**). Observational *Anopheles* population data are not available during Jan.–June 2012 and Jul.–Aug. 2014. Through **a**–**f** the data in 2012, 2013, and 2014 are presented blue, red, and gree, respectively

Mosquitoes use various olfactory sensors, visual sensors, and thermal sensors to identify the locations of hosts; the signal that travels the farthest is CO_2. A CO_2 plume moves downwind with turbulent diffusion [22, 30]. When mosquitoes sense elevated CO_2 concentration (activation), they become activated and generally fly upwind, towards the source of CO_2 [18–24]. In the absence of the clues of hosts, mosquitoes fly randomly.

The relative importance of $\overrightarrow{v_{random}}$ and $\overrightarrow{v_{directed}}$ is determined by the CO_2 concentration and the concentration gradient. Studies show that mosquitoes can sense the fluctuation of CO_2 concentration by as little as 40 ppm [31] or 100 ppm [20]. In the experiment by Healy and Copland [20], approximately 60% of mosquitoes were activated and flew upwind when they encountered pulses of 100 ppm or more CO_2 above the background level (350–370 ppm). It can be assumed that mosquitoes' active dispersal is fully random, unless mosquitoes sense at least 40 ppm higher CO_2 concentration than the background; with the concentration difference of 40 ppm, 60% of their flight is directed towards the higher concentration of CO_2, while the other 40% remaining as the random-direction flight. The weight of the directed flight component was assumed to increase linearly with the concentration gradient, such that mosquitoes located 10 m downwind from a source of CO_2 fly directly to the source (i.e., the directed component is 100%), assuming a typical house in Ejersa with five inhabitants and one

cow. Using the weight of the directed flight component (a, $0 \leq a \leq 1$) and the average flight velocity of mosquitoes v, the magnitude of $\overrightarrow{v_{random}}$ and $\overrightarrow{v_{directed}}$ are modelled as:

$$|\overrightarrow{v_{random}}| = (1-a)v \tag{3}$$

$$|\overrightarrow{v_{directed}}| = av. \tag{4}$$

The direction of $\overrightarrow{v_{directed}}$ is toward the steepest CO_2 concentration gradient, and that of $\overrightarrow{v_{random}}$ is random.

The concentration of CO_2 is simulated for time-averaged mean values using the Gaussian dispersion equation [32]:

$$c(x,y,z) = \frac{Q}{2\pi\sigma_y\sigma_z u} exp\left(\frac{-y^2}{2\sigma_y^2}\right) \left(exp\left(\frac{-(z-h)^2}{2\sigma_z^2}\right) + exp\left(\frac{-(z+h)^2}{2\sigma_z^2}\right)\right), \tag{5}$$

where: c is the concentration of CO_2 [g m^{-3}] at any position x meters downwind of the source, y meters crosswind of the source, and z meters above the ground level, Q is the carbon dioxide exhalation rate [g s^{-1}], u is the horizontal wind velocity along the plume centerline [g s^{-1}], h is the height of the emission plume centerline above the ground [m], σ_z is the vertical standard deviation of the emission distribution [m], and σ_y is the horizontal standard deviation of the emission distribution [m].

The horizontal and vertical dispersion is a function of atmospheric stability conditions and the downwind distance (x) [30]. During the nighttime periods, when mosquitoes are active, atmospheric conditions are stable due to radiative cooling at the land surface under clear skies. From Smith [30], the horizontal and vertical dispersions for stable conditions are given by:

$$\sigma_y = 0.24x^{0.71} \tag{6}$$

$$\sigma_z = 0.06x^{0.71}. \tag{7}$$

The height of the emission plume (h) was set at 1 m, roughly the level of beds, and the height at which mosquitoes sense the plume (z) at the same height (1 m). The source emission of CO_2 exhaled is set at 275 ml min^{-1} per human and 3925 ml min^{-1} per cow [33]. Based on field surveys, it is assumed every household compound contains five humans and one cow. The concentration of CO_2 at each time step in the model domain is calculated as the sum of the contributions of all exhaling members of the community.

The Gaussian model is a well-established time-averaged model of plume dispersion; however, mosquitoes are known to respond to the instantaneous high concentration of CO_2 that is maintained in a pocket of air due to turbulence, rather than the mean concentrations of CO_2 [21, 22]. Because of turbulence, a CO_2 plume is unevenly distributed in the air with some small eddies containing high concentrations of CO_2. Studies with high-resolution measurements observed many-fold higher than mean concentration of CO_2 at a frequency of 0.1 to a few seconds [21, 22]. Simulating this small-scale structure of CO_2 plumes is computationally expensive. Instead, it is assumed that there exist pockets of CO_2 plume with concentration as high as 10 times the concentration simulated in the Gaussian model, and that mosquitoes can respond to the instantaneous burst of CO_2.

As the Gaussian dispersion equation demonstrates, the concentration of CO_2 depends on the source load of CO_2, distance to the source, wind speed, and wind direction. Assuming that *Anopheles* can sense elevated levels of CO_2 above 40 ppm (simulated mean concentration of 4 ppm), the maximum range over which *Anopheles* is activated was simulated to be about 100, 50, 30 and 15 m downwind of a house (with five inhabitants and one cow) under 0.5, 1, 2, and 5 m/s of wind, respectively. The effective range of CO_2 activation is believed to be within some tens of meters around a host [21, 34–36]. Considering that the ranges simulated are for a house with multiple hosts, simulated ranges agree with literature values.

Advection

In addition to mosquitoes' active flight behaviours, mosquitoes are assumed to move downwind with the wind's advective effect. This mechanism plays an important role in mosquitoes' long-distance migration [16, 17]; however, its role is assumed to be negligible if hosts are found near the breeding habitats. In this simulation, mosquitoes were assumed to move downwind, but only at a small fraction (0.01%) of the wind speed:

$$\overrightarrow{v_{passive}} = 0.0001\,\vec{u}, \tag{8}$$

where $\vec{u}$ is a wind vector pointing downwind.

Waves

High waves exhaust larvae and cause higher mortality [5, 27, 28]. High waves are not likely to occur in small and shallow water bodies, such as rain-fed pools, but could be significant in large and deep water bodies, such as reservoirs. Waves seen at reservoirs are called surface waves and are created by the shear stress generated by wind. The surface waves are larger with higher wind speed, larger fetch, and deeper water. The height of the surface wave (H_w [m]) can be estimated through the shallow-water forecasting model [37] (Fig. 2). The model is based on theoretical assumptions and successive approximations

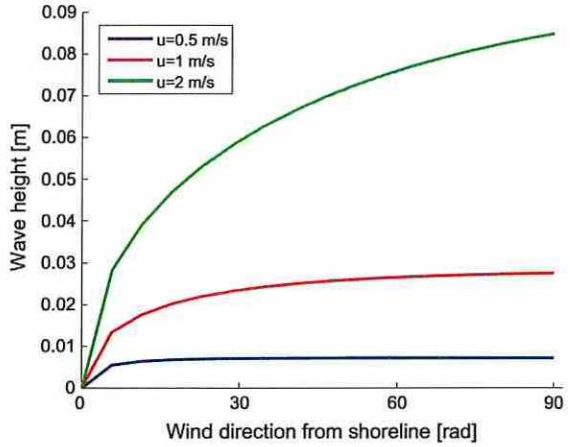

Fig. 2 Predicted wave height for Ejersa. The wave heights calculated for the Koka Reservoir using the shallow-water forecasting model are shown as a function of the wind direction with respect to a shoreline. The wind direction determines the fetch. The estimates are made for different wind speeds: 0.5 m/s (blue), 1 m/s (red), and 2 m/s (green). Larger wind speed yields larger waves. The estimates are made using the average water depth of the Koka Reservoir as *d*

in which wave energy is added due to wind stress and subtracted due to bottom friction and percolation:

$$\frac{gH_w}{u^2} = 0.283 \tanh\left(0.530\left(\frac{gd}{u^2}\right)^{3/4}\right) \tanh\left(\frac{0.00565\left(\frac{gF}{u^2}\right)^{1/2}}{\tanh\left(0.530\left(\frac{gd}{u^2}\right)^{3/4}\right)}\right), \quad (9)$$

where: u is the wind speed [m s^{-1}], d is the depth of water [m], F is the fetch, the length of water over which the wind blows in a single direction [m], and g is the gravitational constant [m^3 kg^{-1} s^{-2}].

For simplicity, the average water depth of a reservoir is used as d. The fetch, F, is calculated assuming that a reservoir is circular.

H_w was then converted into mortality (m_{wave} [h^{-1}]), assuming the mortality linearly increases with wave height:

$$m_{wave} = f \times H_w, \quad (10)$$

where f is the conversion factor [h^{-1} m^{-1}]. f was set at 0.1 as a result of calibration using observational data.

Expected wave height at shorelines of the Koka Reservoir is shown in Fig. 2 for $u = 0.5$ (blue), 1 (red), and 2 m/s (green) and various angles of wind (x-axis). The in situ wind sentry recorded that the daily wind speed in Ejersa varied between 0.5 and 2 m/s in a year. The observed wave heights at the centimetres, which is in good agreement with the predicted results.

Malaria transmission simulator

The role of wind in shaping the behaviours of *Anopheles* mosquitoes was incorporated in HYDREMATS [26, 32] to test the accuracy of the model, comparing with observations. HYDREMATS is a village-scale malaria transmission model that features explicit representation of evironmental conditions and behaviours of *Anopheles* mosquitoes in space and time. Its agent-based approach is suitable for employing the role of wind described above. HYDREMATS was tailored for Ejersa (hereafter, *Ejersa model*) [26].

In order to examine the role of wind in the dynamics of *Anopheles* population, the Ejersa model was also forced with fixed wind speed (*fixed wspd model*) or with random wind direction (*random wdir model*) instead of respective observational values. In the fixed *wspd* model, the observed mean wind speed (0.884 m/s) was employed for every timestep (1 h) throughout the simulation period. The impact of wind speed and direction can be understood by the deviation between the Ejersa model and the respective simulation.

Results

Observation of environment and *Anopheles* population dynamics

Temperature and rainfall are often described as the primary determinants of *Anopheles* population dynamics [3, 38, 39]; however, in Ejersa, reservoir water levels and wind profile are likely to be more important [26] (Fig. 1). Around the temperature range in Ejersa (19–24 °C), the expected longevity of *Anopheles* is not sensitive to temperature [40, 41]. Thus, the influence of temperature on the *Anopheles* population dynamics is limited. Field observations found a handful of rain-fed puddles in Ejersa, but only a few of them were positive breeding sites. This is because rain-fed puddles rarely persist long enough to support the completion of *Anopheles'* aquatic-stage development, which takes around 15–20 days at the cold range of temperature in the field. [26]. The lag in observed *Anopheles* population dynamics was too large to be explained by the rainfall (Fig. 1b, f). On the other hand, the primary determinant of the *Anopheles* population was analysed to be the reservoir water levels (Fig. 1e, f), which determine the location of the reservoir shoreline [26]. The shoreline—the main breeding habitat for *Anopheles* mosquitoes—becomes closer to the village as the reservoir water levels increase, making reproduction more likely.

The observed *Anopheles* population dynamics during the minor mosquito season (around May, more precisely)

were distinctive between 2013 and 2014 (Fig. 1f). The mosquito population increased in 2013 but not in 2014. Neither temperature, rainfall, nor reservoir water level is likely to explain the difference, because the observed data during the same season in the two years were similar. The noticeable differences between the two periods were found only in wind speed and wind direction (Fig. 1c, d, respectively). Whether or not this observational differences in wind profile can explain the observed *Anopheles* population dynamics during the minor mosquito season are examined mechanistically in the next section.

Simulation of *Anopheles* population dynamics

Simulated and observed *Anopheles* population are presented in Fig. 3. The observed dynamics of *Anopheles* population (orange asterisks) were reproduced in the Ejersa model (solid black line), both during the major and minor mosquito seasons, with some reasonable accuracy [26]; however, without the incorporation of wind impacts, models (solid green line and solid blue line) failed to reproduce the observed seasonality of *Anopheles* population. The result from the fixed *wspd* model (solid green line) was somewhat similar to that of the Ejersa model, but the performance of the model declined. In the random *wdir* model (solid blue line), the performance of the model declined even more significantly, and the observed dynamics of the *Anopheles* population was not reproduced with this model. The random *wdir* model failed to simulate the timing of the onset of the major mosquito season. In addition, it failed to simulate the observed difference of the *Anopheles* population dynamics during the minor mosquito season.

The observed *Anopheles* population increased in May in 2013, but not in 2014 (Fig. 4a, b). This difference was simulated in the Ejersa model, and the simulation results suggest that it is accounted for mostly by the wind direction (Fig. 3). Figure 4 demonstrates that the observed wind direction in the minor mosquito seasons in 2013 worked to enhance mosquito population (c, black) slightly, as compared to an assumed condition with random wind direction (c, blue). The observed wind direction in the minor mosquito season in 2014, on the other hand, worked to suppress mosquito population (d, black), as compared to an assumed condition with random wind direction (d, blue).

Discussion

The role of wind in mosquito behaviours was modelled based on the physics of CO_2 dispersion and surface waves and on the physiology of mosquitoes. The mosquitoes' behavioural responses to wind are still not fully understood; however, the model incorporating some of the known effects of wind speed and wind direction was demonstrated to be able to reproduce the observed dynamics of *Anopheles* population in Ejersa, which was not possible without those wind effects. Thus, the impacts of wind on

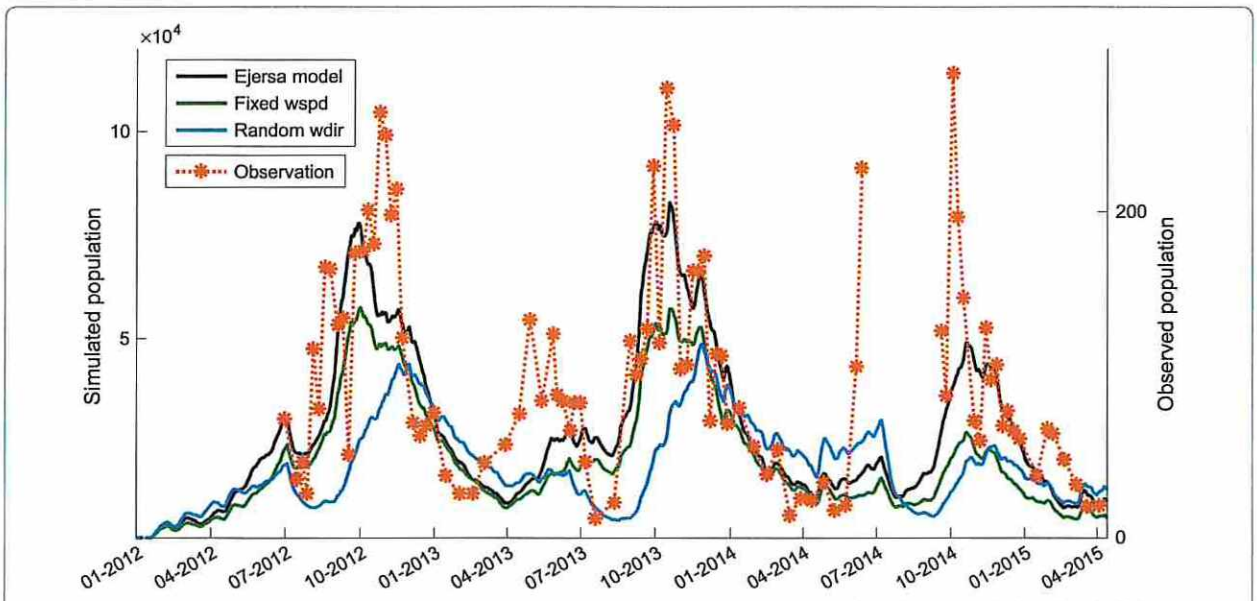

Fig. 3 Simulated and observed *Anopheles* population. The total size of the *Anopheles* population simulated in the simulation domain is shown on the left y-axis for the Ejersa model (black), the fixed *wspd* model (green), and random *wdir* model (blue). The observed numbers of *Anopheles* captured in the six light trap houses are shown on the right y-axis; observed values are presented in orange asterisks and orange dotted lines are simple linear interpolation. Observational data are not available during Jan.–June 2012 and Jul.–Aug. 2014

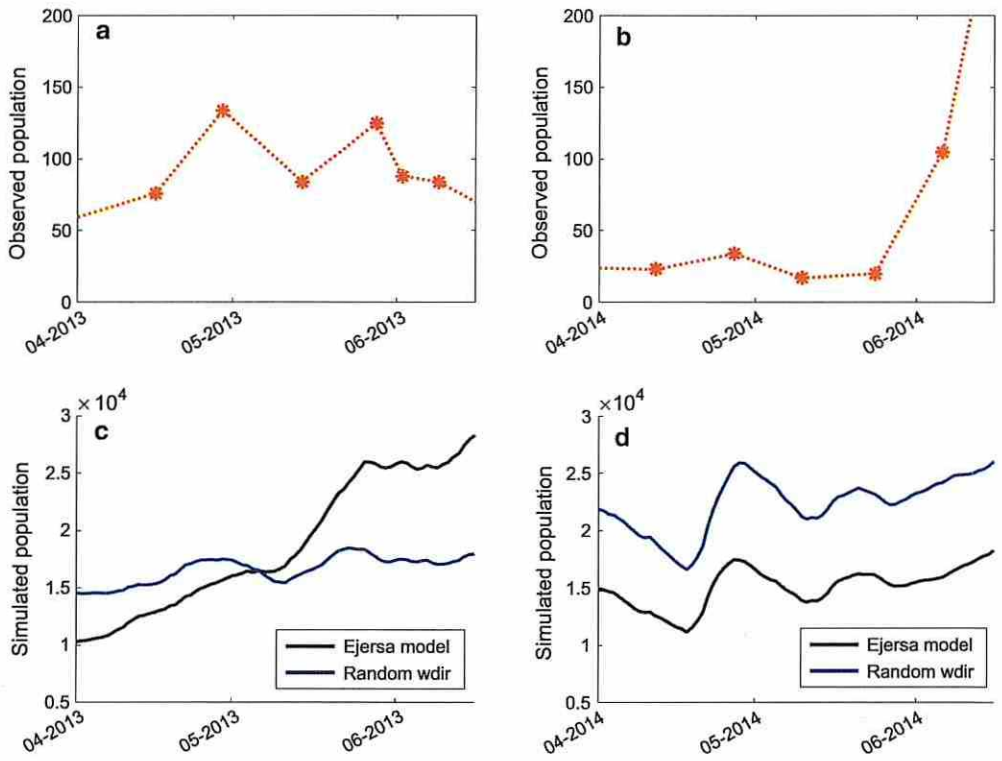

Fig. 4 *Anopheles* population in minor mosquito season. Observed (**a**, **b**) and simulated (**c**, **d**) *Anopheles* populations during the minor mosquito season in 2013 (**a**, **c**) and in 2014 (**b**, **d**). An increase in *Anopheles* population was observed in May 2013, but not in May 2014 (**a**, **b**). This difference was simulated with reasonable accuracy in HYDREMATS (**c**, **d**, black line), which can be attributable to different profiles of wind direction. The observed wind direction in 2013 (**c**, black) worked to enhance mosquito population slightly, as compared to an assumed condition with random wind direction (primary determinan, blue). The observed wind direction in 2014, on the other hand, worked to suppress the mosquito population (**d**, black), as compared to an assumed condition with random wind direction (**d**, blue)

the mosquitoes' behavioural responses are believed to be credibly represented in this analysis. Some of the model parameters may still benefit from further calibration. To the best of the authors' knowledge, HYDREMATS is the only malaria transmission model that mechanistically incorporates the effect of wind.

The effect of wind on the *Anopheles* population dynamics has received limited attention in the scientific community [20, 21, 23, 24], yet it was shown to have a significant contribution around a reservoir. The importance of wind is expected to be particularly significant around reservoirs for two reasons. The first reason is that the waves created by the wind can become fatal to aquatic-stage mosquitoes at large water bodies. The height of the wave increases with the depth and the fetch (~ surface area) of the reservoir. Thus, waves are more likely to influence *Anopheles* mosquitoes' breeding at reservoirs than at small water bodies such as rain-fed pools. The second reason is the heterogeneity in the surrounding environment around reservoirs, where human settlements are located only at one side of the shoreline. Under such environment, the population dynamics of *Anopheles* mosquitoes are likely to be influenced by the wind direction.

As compared to offshore wind, onshore wind creates larger waves because the fetch is large. Thus, aquatic-stage mosquitoes experience larger mortality, leading to smaller *Anopheles* populations. In addition, under the onshore wind, a large part of CO_2 plume emanated from the village moves away from the reservoir, which makes mosquitoes emerging at reservoir shorelines less efficient in identifying the direction of the village for host-seeking. Thus, under onshore wind, *Anopheles* population can further decrease due to limited CO_2 attraction and less efficient host-seeking activity. These two factors conclude that *Anopheles* populations are generally low under the onshore wind condition.

Wind direction in Ejersa shifts from about 90° north in the dry season to about 220° north in the main rainy season. A gradual shift of wind direction is experienced during the secondary rainy season. The wind from 90° and 220° north corresponds to onshore wind and

near-offshore wind for Ejersa. As a results, part of the increase in the *Anopheles* population during the main rainy season (beginning of the main mosquito season, more specifically) can be explained by the wind blowing from near-offshore (Fig. 3). The difference in the *Anopheles* population dynamics during the minor rainy season (which almost corresponds to the secondary mosquito season) between 2013 and 2014 can also be explained by the wind direction. In 2013, the shift in wind direction during the minor rainy seasons occurred earlier than in 2014 (Fig. 1), explaining the observed and simulated difference in the *Anopheles* population dynamics.

The model that replaced the observed wind speed with the averaged wind speed (fixed wind model) consistently simulated smaller *Anopheles* population than the model with observed wind speed (Ejersa model) throughout a year. This unexpected result can be explained by the fact that *Anopheles* mosquitoes are modelled to be active only during the night time, and the "average" wind speed averages the observed profile not only over the seasons but also over day and night. The day-time wind speed (~ 1.0 m/s) was larger than the night-time wind speed (~ 0.65 m/s). Thus, the average wind speed was larger than the night-time wind speed. Larger wind speed enhances waves and does not deliver high concentration of CO_2 plume far enough—both mechanisms contribute to decrease the *Anopheles* population. Thus, the fixed *wspd* model resulted in consistently smaller *Anopheles* population than the Ejersa model throughout the simulation period.

Conclusion

Around large water bodies such as reservoirs, the role of wind in *Anopheles* population dynamics, hence in malaria transmission, can be significant. This paper provided a framework to model the effects of wind in the behaviours of *Anopheles* mosquitoes. The effects important for *Anopheles* behaviours include: attraction of adult mosquitoes through dispersion of CO_2, advection of adult mosquitoes, and aquatic-stage mortality due to wind-induced surface waves. Combining simulation studies and observational data of *Anopheles* population dynamics collected around the Koka Reservoir in Ethiopia, this study demonstrates a substantial role of wind in *Anopheles* population dynamics—hence the dynamics of malaria transmission. It is suggested that malaria is generally suppressed when wind blows from a reservoir to a village.

Authors' contributions

NE conceived and conducted the study. EABE supervised the research. NE wrote the manuscript. NE and EABE edited the manuscript. Both authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All the data used in this study are available upon request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Evaluation of Boric Acid Sugar Baits Sprayed on Plants against the Salt Marsh Mosquito, Aedes taeniorhynchus (Diptera: Culicidae)

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EVALUATION OF BORIC ACID SUGAR BAITS SPRAYED ON PLANTS AGAINST THE SALT MARSH MOSQUITO, *AEDES TAENIORHYNCHUS* (DIPTERA: CULICIDAE)

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The black salt marsh mosquito, *Aedes taeniorhynchus* Wiedemann (Diptera: Culicidae), is an abundant nuisance mosquito distributed throughout coastal regions of the U.S. and Caribbean, and is also an important vector of canine heartworm and Venezuelan equine encephalitis (Apperson 1991). With the increasing human encroachment on coastal habitats, environmentally sensitive control methods are critically needed to balance human needs for mosquito control with those of delicate ecosystems.

Attractive Toxic Sugar Baits (ATSBs) against mosquitoes have been successful (Müller & Schlein 2008). The 'toxic' active ingredient in ATSBs is mostly boric acid, which is an environmentally friendly compound harmless to humans, but toxic to adult mosquitoes (Xue & Barnard 2003; Müller et al. 2010a, b; Beier et al. 2012). Other recent evaluations of ATSBs in tropical environments have shown much promise (Xue et al. 2011, 2013; Qualls et al. 2012; Naranjo et al. 2013).

Aedes taeniorhynchus has been shown to seek and feed on nectar (Van Handel & Day 1990). Concurrently, there is evidence that toxic sugar baits (TSBs) may be used to exploit resting behaviors of *Aedes* mosquitoes (Schlein & Müller 2012). However, there is no information about using these techniques to control *Ae. taeniorhynchus*. TSBs do not contain the additional "attractant", thus focusing on resting and sugar feeding mosquitoes. Resting vegetation commonly found in and around coastal hammocks, such as yaupon holly (*Ilex vomitoria* Sol. ex Aiton; Aquifoliales: Aquifoliaceae) and black mangrove (*Avicennia germinans* (L.) L.; Lamiales: Acantaceae), were selected based on their presence at the field site and other local hammocks. The focus on boric acid toxic sugar baits built upon previous work by Xue et al. (2006, 2008) to advance field-based studies of environmentally friendly adult salt marsh mosquito control technology. The major objective of the present study was to evaluate the efficacy of boric acid sugar baits sprayed on black mangrove and yaupon holly cuttings in the laboratory and common coastal plants in the field against the adult black salt marsh mosquitoes.

The Toxic Sugar Bait (TSB) solution was formulated using 1% boric acid (Sigma-Aldrich, USA) as the active ingredient diluted into a 5% sucrose (Domino Brand, ASR Group, Palm Beach, Florida) solution. The solution was prepared by adding the boric acid to hot (60 °C) tap water until fully dissolved. Eleven liters were mixed for each field application and 100 mL batches were prepared for the laboratory experiments.

Seven to 10-day old adult *Ae. taeniorhynchus* mosquitoes were obtained from the USDA, Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, Florida and used for the laboratory experiments. Conditions in the insectary were maintained at 26-28 °C, 70-80% RH and 12:12 h L:D for the duration of the experiment. Both control and treatment cages of mosquitoes were provided with 59 mL Diamond Daily Mini Cups™ containing approximately four water saturated (25 mL) cotton balls during the observation period, while negative controls received 10% sugar.

Experiment 1

One hundred female mosquitoes were aspirated from the colony into each of the six cages (45 × 45 × 37cm) for the black mangrove laboratory evaluation. Three of the cages comprised the experimental group and the other three cages were for controls. Permission from Anastasia State Park, St. Augustine, Florida was granted to collect cuttings of plants (DEP/AMCD 06-01-13). Anastasia State Park was chosen because insecticidal spraying is prohibited at this location and the plant samples were less likely to be contaminated. We immediately placed the

black mangrove cuttings into small glass cups (500 mL). The cups were supplied with brackish water from the leaf collection site. Aluminum foil was used as a cover and barrier over the cup opening, so the mosquitoes could not reach the water. Each cup contained approximately twelve non-flowering 10-12 cm in length cuttings with 8-10 leaves per cutting. The TSB solution (10 mL/plant) was applied using a handheld 946 mL Zep Professional Sprayers™ (Ace Hardware, St. Augustine, Florida) to the 3 treatment cuttings to the point of run off. The 3 control cuttings were not sprayed. Mortality counts were taken daily until greater than 90% (cumulative) mosquito mortality was observed. The evaluation had 3 repetitions.

Experiment 2

The yaupon holly evaluation was conducted as in experiment 1 with the following modifications: the yaupon holly cuttings were approximately 6 cm long and 4-5 cuttings were placed into small 100 mL glass vials filled with tap water. One vial was placed into each 9.5 L bucket cage in the laboratory. Thirty female mosquitoes were introduced into each of these cages and mortality was recorded at 24, 48 and 72 h. The evaluation had 3 repetitions. Each repetition was composed of 3 treatments and 2 controls.

Experiment 3

Fish Island is an undeveloped peninsula along the Matanzas River (N 29° 51' 42" W 81° 18' 04"), St. Augustine, Florida. This coastal area is surrounded concentrically by tidal salt marsh vegetation at its edge, typical yaupon holly barrier island-type habitat moving inland, and has inner reaches populated by more upland species such as hickory (*Carya* spp.; Fagales: Juglandaceae). Four Mosquito Magnet™ X (MMX) traps were set in different locations in pairs on Fish Island, St. Augustine, FL to verify the presence of *Ae. taeniorhynchus*. Traps were spaced approximately 100 m apart, baited with CO_2 (dry ice), and left overnight. Mosquitoes collected were killed by freezing, identified and counted. The data collected was then used to determine the best sites for the field application of the TSB solution.

TSB was applied from an 11 liter B & G Pest Pro 2050 Back Pack Sprayer in a vertically oscillating pattern from ground coverage to a height of no more than 2 meters on non-flowering vegetation. Effort was made to ensure coverage to a depth of 3-5 m from the trail edge. A section of approximately 280 m in length was sprayed. Following a 200 m buffer zone, another 280 m was considered to be the control length. Landing rate counts by two or three people in the morning were taken at three sites in the sprayed area for 1 min before spraying and post spraying day 1, day 2, day 3, and 1 week (AMCD's IRB protocol#10-13-2005 as approved by the Board of Commissioners for use of human subjects).

Differences between the treatments and controls were assessed using a series of one-way analysis of variance (ANOVA) determinations. All analysis was done using SAS v9.4.

In the mangrove study, overall mortality was greatest at 48 h with a cumulative mortality of 52% (Fig. 1, $F_{3,12} = 6.21$, $P < 0.01$). However, the exposure time did not result in significant differences in the mortality between treatments and controls. We hypothesize that mangrove was not a good nutritional resource for *Ae. taeniorhynchus*. In the yaupon holly experiment, a direct comparison of the treatment and control showed significant mortality (68%) at 48 h post treatment (Fig. 1, $F_{2,2} = 20.72$, $P < 0.05$), but the exposure time did not result in a significant difference in the mortality between treatments and controls. Also, yaupon holly was not a good nutritional resource for this species of salt marsh mosquitoes.

At the field site, there was a very diverse mosquito population (total of 13 species) sampled at Fish Island. The experimental site was chosen because *Ae. taeniorhynchus* was the most prevalent species (> 30%). The field experiment showed that boric acid sugar baits sprayed on plants within a hammock island near salt marshes resulted in the significant reduction of landing rate counts on human subjects (Fig. 2, $F_{1,52} = 4.46$, $P < 0.05$).

In the black mangrove laboratory experiment we found 52% mortality at 48 h. This percent mortality is less than that reported by Xue et al. (2006, 2008), who found > 80% mortality in the application of boric acid to vegetation against a laboratory-reared population of *Ae. taeniorhynchus* in the laboratory and in a semi-field trial. The different species of plants used and experimental conditions may be the reason why different mortalities were induced. Salt excretion by black mangrove leaves may reduce the toxicity of boric acid sugar baits against salt marsh mosquitoes. The impact of plant species and habitants on efficacy of ATSB against adult mosquitoes needs to be further addressed.

Our desire to investigate mosquito-resting behavior could be greatly enhanced by expanding the scope of future studies to include sugar-feeding behavior as in attractive toxic sugar baits (ATSBs). The addition of an attractive component in the laboratory studies may encourage exposure of the mosquitoes to the solution presented on the plants. Future evaluations directly comparing exposure of mosquitoes to TSBs versus ATSBs in the natural environment are needed.

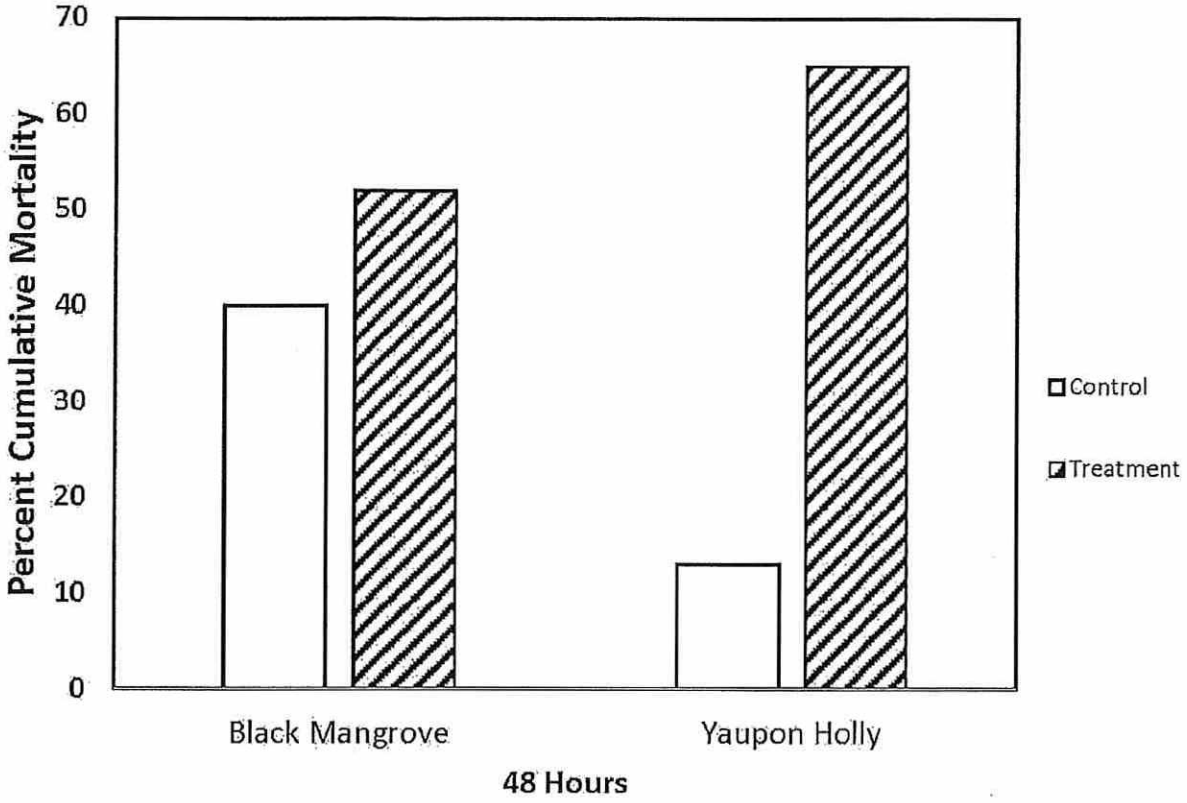

Fig. 1. Percent mortality of *Aedes taeniorhynchus* at 48 h after exposure to 1% boric acid sugar baits sprayed on black mangrove and yaupon holly cuttings in the laboratory.

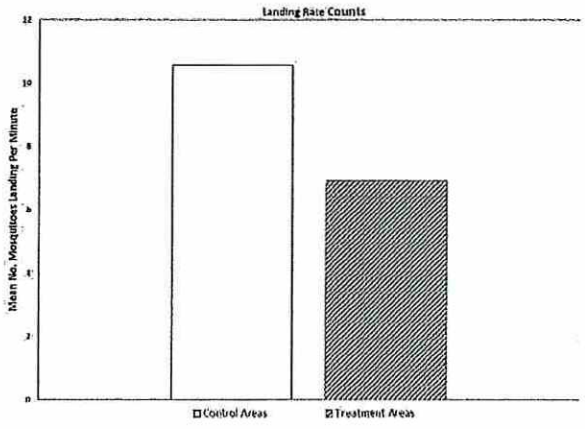

Fig. 2. Mean landing rate counts/minute by adult mosquitoes on human subjects after 1% boric acid sugar baits was sprayed on plants at Fish Island, St. Augustine, Florida.

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SUMMARY

A Toxic Sugar Bait (TSB, active ingredient 1% boric acid) was evaluated against *Aedes taeniorhynchus* (Diptera: Culicidae) in the laboratory and the field at St. Augustine, Florida. The laboratory component was comprised of plants located in known *Ae. taeniorhynchus* resting areas, i.e., black mangrove (*Avecennia germinans* L.) and yaupon holly (*Ilex vomitoria* Ait.). The results indicated that TSB sprayed on black mangrove and yaupon holly cuttings at 48 h resulted in significant mortality of resting *Ae. taeniorhynchus*, compared with the mortality of mosquitoes in the control group under the laboratory conditions. Also, the field studies indicated a significant reduction in mosquito populations after TSB was applied on plants.

Key Words: *Aedes taeniorhynchus*, attractive toxic sugar bait, boric acid

RESUMEN

Un Cebo Tóxico de Azúcar (CTA, ingrediente activo el ácido bórico al 1%) fue evaluado contra *Aedes taeniorhynchus* (Diptera: Culicidae) en el laboratorio y el campo en St. Augustine, Florida. El componente de laboratorio consistía de plantas

ubicadas en áreas conocidas donde *Ae. taeniorhynchus* reposa, como en mangle negro (*Avecennia germinans* L.) y acebo yaupon (*Ilex vomitoria* Ait.). Los resultados indicaron que el CTA rociado sobre recortes de mangle negro y el acebo yaupon a las 48 horas resultó en una mortalidad significativa de *Ae. Taeniorhynchus* en descanso, en comparación con la mortalidad de los mosquitos en el grupo control en las condiciones de laboratorio. Además, los estudios de campo indicaron una reducción significativa de las poblaciones de mosquitos después de la aplicacióon de CTA sobre las plantas.

Palabras Clave: *Aedes taeniorhynchus*, atractivo cebo tóxico de azúcar, ácido bórico

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MALARIA JOURNAL

RESEARCH Open Access

Quantifying the mosquito's sweet tooth: modelling the effectiveness of attractive toxic sugar baits (ATSB) for malaria vector control

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Abstract

Background: Current vector control strategies focus largely on indoor measures, such as long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS); however mosquitoes frequently feed on sugar sources outdoors, inviting the possibility of novel control strategies. Attractive toxic sugar baits (ATSB), either sprayed on vegetation or provided in outdoor bait stations, have been shown to significantly reduce mosquito densities in these settings.

Methods: Simple models of mosquito sugar-feeding behaviour were fitted to data from an ATSB field trial in Mali and used to estimate sugar-feeding rates and the potential of ATSB to control mosquito populations. The model and fitted parameters were then incorporated into a larger integrated vector management (IVM) model to assess the potential contribution of ATSB to future IVM programmes.

Results: In the Mali experimental setting, the model suggests that about half of female mosquitoes fed on ATSB solution per day, dying within several hours of ingesting the toxin. Using a model incorporating the number of gonotrophic cycles completed by female mosquitoes, a higher sugar-feeding rate was estimated for younger mosquitoes than for older mosquitoes. Extending this model to incorporate other vector control interventions suggests that an IVM programme based on both ATSB and LLINs may substantially reduce mosquito density and survival rates in this setting, thereby substantially reducing parasite transmission. This is predicted to exceed the impact of LLINs in combination with IRS provided ATSB feeding rates are 50% or more of Mali experimental levels. In addition, ATSB is predicted to be particularly effective against *Anopheles arabiensis*, which is relatively exophilic and therefore less affected by IRS and LLINs.

Conclusions: These results suggest that high coverage with a combination of LLINs and ATSB could result in substantial reductions in malaria transmission in this setting. Further field studies of ATSB in other settings are needed to assess the potential of ATSB as a component in future IVM malaria control strategies.

Background

In the last decade, declines in the incidence of *Plasmodium falciparum* malaria have been reported throughout sub-Saharan Africa, occurring concomitantly with the extensive scale-up of insecticide-based vector control and the switch to artemisinin-based combination therapy (ACT) as first-line treatment [1-3]. Vector control strategies have largely focused on interventions which attack the vector indoors, in particular the use of long-lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) with insecticides [4,5]. These are sometimes accompanied by efforts to control vector breeding sites through either source reduction or the application of larvicides [6]. This has resulted in substantial reductions in transmission and disease in many areas; however, in other areas, the reductions have been more modest [7]. This is partly due to the geographical variation in transmission potential which makes widespread elimination of the parasite difficult; however, there is also evidence that a residual population of outdoor-biting vectors, not targeted by indoor control measures, are able to sustain the parasite [8,9]. Thus it is clear that

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new vector control tools will be needed to maintain the recent gains made. Furthermore, these tools are essential in the face of evolving drug-resistance among parasites and insecticide-resistance among vectors [10].

Toxic sugar baits have been proposed as a novel vector control strategy that complements existing tools such as LLINs and IRS [11,12]. The strategy works by an "attract and kill" principle whereby mosquitoes are attracted to the fruity or flowery scent of the bait, and are then provided with a combination of sugar and an oral toxin such as boric acid, which is highly toxic to *Anopheles gambiae*, the primary African malaria vector [13,14]. The strategy has been extensively tested in Israel to suppress populations of the mosquito species *Anopheles sergentii*, *Anopheles claviger*, *Aedes caspius* and *Culex pipiens* [15-18] and has recently been tested in Bandiagara, a semi-arid area of Mali, to decimate populations of the malaria vector *An. gambiae s.l.* [14]. In Mali, ATSB solution sprayed onto vegetation near breeding sites was successful in reducing local vector densities by 90%, with the majority of remaining female mosquitoes being too young to transmit malaria. The strategy is, therefore, highly promising for malaria control in semi-arid areas of Africa, with further testing planned to determine its wider applicability.

A major benefit of ATSB is that, unlike LLINs and IRS, it targets female and male mosquitoes while they are outdoors. Larviciding is another important outdoor intervention, but is of limited use in rural areas where it is difficult to identify and treat all potential breeding sites [6,19,20]. Outdoor transmission is of growing importance as evidence suggests that intensive indoor control measures are causing transmission to shift from the mostly indoor-biting *An. gambiae* to the outdoor-adapted *An. arabiensis* [5,8,21,22]. Furthermore, *An. gambiae* appears to be becoming increasingly adapted to outdoor biting in some areas [9]. ATSB is also cheap and environmentally friendly, and oral toxins are not affected by the problem of insecticide-resistance [23]. That said, it is advisable that multiple toxins be used in an operational ATSB formula [14]. Effort will be required to ensure adequate vegetation coverage, particularly in less arid locations; however, ATSB benefits from the fact that sugar-feeding is a frequent behaviour for both male and female mosquitoes, and the sole food source for males [24,25].

This paper provides a quantitative basis for understanding the potential utility of ATSB as part of an integrated vector management (IVM) programme in Africa. Using results from the Mali field trial described earlier [14], mathematical models of sugar-feeding behaviour are fitted to the data to estimate parameters underlying the effectiveness of ATSB as a vector control strategy, including the rate of feeding on ATSB-sprayed plants and the expected lifetime of mosquitoes in the field following ingestion of the toxin. These parameters and an ecological model of *An. gambiae* and *An. arabiensis* dynamics are then used to investigate the impact of ATSB, as part of an IVM programme, on vector abundance and malaria transmission. The impact of a variety of vector control strategies on malaria transmission has been widely studied using mathematical models [26-30]; however, this study represents the first mathematical evaluation of the performance of ATSB, a highly promising, novel vector control strategy.

Methods

Trial data

Data were analysed from the above-mentioned ATSB field trial conducted near Bandiagara, Mali [14]. Two sites were monitored in this trial – an experimental site where ATSB was administered, and a control site where attractive (non-toxic) sugar bait (ASB) was used. Male and female catch numbers were recorded for six light traps at each site over a one-week pretreatment period and for 30 days post-treatment. The proportion of marked mosquitoes was also recorded, as in these experiments a coloured food dye that can be detected for several days after feeding was added to both ATSB and ASB solutions. To estimate age distribution among female mosquitoes, the number of gonotrophic cycles completed was recorded for a sample of 200 mosquitoes before and after the intervention, for both the experimental and control sites.

Basic model selection

A range of simple, deterministic models were fitted to the Mali data. These included models with and without decay of dye, models in which sugar-feeding rates were the same or different in the control and experimental settings, and models in which mosquito emergence was assumed to be constant or proportional to the population size (Additional file 1). For each model, posterior parameter distributions were estimated using a Markov Chain Monte Carlo (MCMC) sampling procedure, and the deviance information criterion (DIC) was calculated as a measure for model selection (Additional file 2: Table S1). The best model was characterized by different sugar-feeding rates in the two settings, no decay of dye and a constant rate of mosquito emergence. For this model, the equations for female mosquitoes in the experimental setting are,

$$\frac{dU_E}{dt} = bN_E - s_E U_E - \mu U_E \quad (1)$$

$$\frac{dM_E}{dt} = s_E U_E - \mu_{ATSB} M_E \quad (2)$$

Here, U and M represent the density of unmarked and marked female mosquitoes and the subscript

E represents the experimental setting throughout (equivalently, the subscript C represents the control setting throughout). The adult emergence rate, b, is chosen to match the death rate, μ, so that the population is at equilibrium in the absence of ATSB. The equilibrium population size, as measured by mosquito catch numbers, is N. For the control setting, identical equations apply with the exception that marked mosquitoes are not exposed to the toxin and so also die at the rate μ. Equivalent equations hold for males. The equations for this model can be solved, for the experimental setting, to give,

$$U_E(t) = N_E \frac{1}{\mu + s_E}\left(\mu + s_E e^{-(\mu+s_E)t}\right) \tag{3}$$

$$M_E(t) = N_E \frac{s_E}{\mu_{ASTSB}(\mu_{ATSB}-\mu-s_E)(\mu+s_E)} \times \left(\begin{array}{l} \mu_{ATSB}s_E e^{-(\mu+s_E)t} - \mu(\mu-\mu_{ATSB}+s_E) \\ +(\mu-\mu_{ATSB})(\mu+s_E)e^{-\mu_{ATSB}t} \end{array}\right) \tag{4}$$

Similarly, for the control setting, the equations are,

$$U_C(t) = N_C \frac{1}{\mu + s_C}\left(\mu + s_C e^{-(\mu+s_C)t}\right) \tag{5}$$

$$M_C(t) = N_C \frac{s_C}{\mu + s_C}\left(1 - e^{-(\mu+s_C)t}\right) \tag{6}$$

For a given set of parameter values, an expression for the model likelihood can be derived by assuming the observed mosquito catch numbers are sampled from a negative binomial distribution with mean equal to the model-predicted mosquito density and variance to be estimated. A normal prior was used for daily mosquito mortality μ, with a mean of 0.1 per day and a standard deviation of 0.01 per day [31]. Uninformative uniform priors were used for all other model parameters. Posterior parameter distributions were estimated using an MCMC sampling procedure (Additional file 1).

Model incorporating gonotrophic cycles

To accommodate the gonotrophic cycle data, the basic model (Equations 1, 2) was partitioned into unmarked females in the experimental setting, $U_{i,E}$, and marked females in the experimental setting, $M_{i,E}$, having completed i gonotrophic cycles, where $i \in \{0,1,\ldots,8\}$ (mosquitoes having completed eight or more cycles were grouped into the same category). Four models were then postulated to describe how the sugar-feeding rate may vary with cycle number: (i) feeding rate remains constant; (ii) feeding rate changes by a constant amount per cycle; (iii) feeding rate changes by a constant fraction per cycle; and, (iv) a step model in which feeding rate differs for mosquitoes having completed zero to two or three or more cycles (for more information, see Additional file 1). Once again, the DIC was used as a measure for model selection (Additional file 3: Table S2). The step model provided the best fit to the data and, for this model, the sugar-feeding rates vary with cycle number as,

$$s_i = \begin{cases} s_0, i \in \{0,1,2\} \\ m s_0, i \geq 3 \end{cases} \tag{7}$$

Here, s_i is the sugar-feeding rate for a female mosquito having completed i gonotrophic cycles, and m is the fractional change in sugar-feeding rate for mosquitoes having completed three or more cycles (as compared to those having completed 0–2 cycles). Age-dependency of the mosquito death rate was considered, however a constant death rate was chosen because: (a) the pre-intervention gonotrophic cycle data is consistent with a constant death rate; (b) experimental data suggesting a higher death rate following initial emergence has not been confirmed under field conditions [32,33]; and, (c) a constant death rate leads to conservative predictions of disease transmission since an elevated death rate following emergence shifts the age distribution towards younger mosquitoes unable to transmit disease [34]. In the experimental setting, the model equations are given by,

$$\frac{dU_{0,E}}{dt} = bN_E - (s_{0,E} + \mu + \delta)U_{0,E} \tag{8}$$

$$\frac{dM_{0,E}}{dt} = s_{0,E}U_{0,E} - (\mu_{ATSB} + \delta)M_{0,E} \tag{9}$$

$$\frac{dU_{i,E}}{dt} = \delta U_{i-1,E} - (s_{i,E} + \mu + \delta)U_{i,E}, i \in \{1,\ldots,7\} \tag{10}$$

$$\frac{dM_{i,E}}{dt} = \delta M_{i-1,E} + s_{i,E}U_{i,E} - (\mu_{ATSB} + \delta)M_{i,E}, i \in \{1,\ldots,7\} \tag{11}$$

$$\frac{dU_{8,E}}{dt} = \delta U_{7,E} - (s_{8,E} + \mu)U_{8,E} \tag{12}$$

$$\frac{dM_{8,E}}{dt} = \delta M_{7,E} + s_{8,E}U_{8,E} - \mu_{ATSB}M_{8,E} \tag{13}$$

Here, δ represents the reciprocal of the gonotrophic cycle length. The schematic for this model is shown in Additional file 4: Figure S1. Analogous equations apply in the control setting, replacing the subscript E with the subscript C. Analytic solutions to these equations are not feasible and so the differential equations must be solved numerically in order to compare the model predictions to the data.

Once again, an MCMC sampling procedure was used to estimate the posterior distributions of each of the model parameters. The likelihood function used was the same as for the basic models, multiplied by a term accounting for the comparison between the model-predicted and observed distribution of gonotrophic cycle number (Additional file 1). A normal prior was used for the parameter δ, with a mean of 0.33 per day and a standard deviation of 0.03 per day [34], and uninformative uniform priors were used for all other parameters.

Model of integrated vector management

The IVM model divides the mosquito life cycle into larval, pupal and adult stages, thus allowing stage-specific interventions to be modelled [35]. Density-dependence is modelled at the larval stage, based on a study in Tanzania suggesting a linear relationship between larval density and mortality [36]. Parameters were estimated from the entomological literature and the Garki Project, undertaken in the 1970s in the Garki District of Nigeria (Additional file 5: Table S3). With this framework in place, a variety of interventions were simulated in isolation and synchrony to calculate their expected effects on *An. gambiae* and *An. arabiensis* densities.

The EIR for a particular setting was derived by multiplying the human biting rate (the number of bites per person per year) by the sporozoite rate, S (the proportion of the vector population that is infectious for malaria). The sporozoite rate was calculated by averaging over the gonotrophic cycle number, i.e.,

$$S = \sum_i f_i S_i \tag{14}$$

Here, f_i represents the fraction of the female vector population having completed i gonotrophic cycles, and S_i represents the sporozoite rate of a female having completed i cycles. The sporozoite rate was calculated as a linearly-increasing function of cycle number accounting for the minimum number of cycles, σ, required for ingested parasites to become infectious in a mosquito [34],

$$S_i = \begin{cases} 0, i \le \sigma \\ \kappa Q_0 (i-\sigma), i > \sigma \end{cases} \tag{15}$$

Here, κ represents the probability that a vector becomes infectious per human bite, assuming it survives long enough, and Q_0 represents the proportion of blood-meals taken on humans in the absence of LLINs and IRS. Three transmission settings were considered with preintervention EIRs of 100 (very high transmission), 50 (high transmission) and 10 (moderate transmission). The human biting rate was varied according to the setting, and was consistent with estimates from Nigeria and Tanzania for the very high transmission setting [37-39]. Parameter estimates and their sources are included in Additional file 5: Table S3 and Additional file 6: Table S4.

Results

Estimates of exposure to ATSB and its impact on mortality using simple models

The best-fitting sugar-feeding model was one in which there are two classes of mosquitoes – marked and unmarked. In this model, after emergence from pupae, female and male mosquitoes are unmarked and become marked when feeding on ASB or ATSB-sprayed vegetation. In the control setting, marked and unmarked mosquitoes die at the same rate, while in the experimental setting, marked mosquitoes die at a faster rate due to the effect of the toxin. A schematic for this model is shown in Figure 1.

Figure 2 depicts model fits for both male and female mosquito catches in the experimental and control settings with associated parameter estimates summarized in Table 1. Visually, the model provides a good fit to the data; although the estimated variation in mosquito catch data is somewhat large. Of most interest are the estimates of sugar-feeding rates and death rates upon ingesting the toxin. These are summarized in Table 1 along with 95% credible intervals (CrIs).

The feeding rate of most relevance is that of females in the experimental setting, since only female mosquitoes bite and transmit malaria parasites. An ATSB feeding rate of 0.5 per female per day (95% CrI: 0.27-0.97) was estimated for the Mali experiment. Estimates of feeding rates differ significantly between the experimental and control settings (0.50 per day for the experimental setting versus 0.15 per day for the control setting) which could be due to differences in the relative abundance of sugar bait in the two settings (either in terms

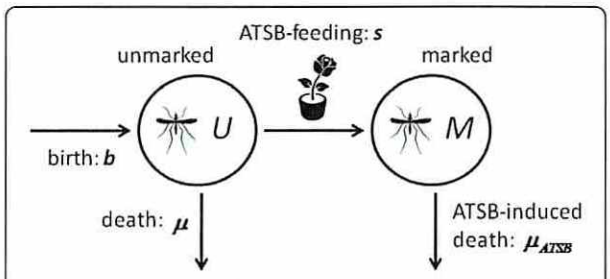

Figure 1 Schematic of the basic ATSB sugar-feeding model in the experimental setting. Female and male mosquitoes emerge at rate b into the unmarked class, U, and become marked, M, when feeding on ATSB-sprayed vegetation at rate s. In the control setting, marked and unmarked mosquitoes die at the same rate, μ, while in the experimental setting, marked mosquitoes die at a faster rate due to the effects of the toxin, μ_{ATSB}.

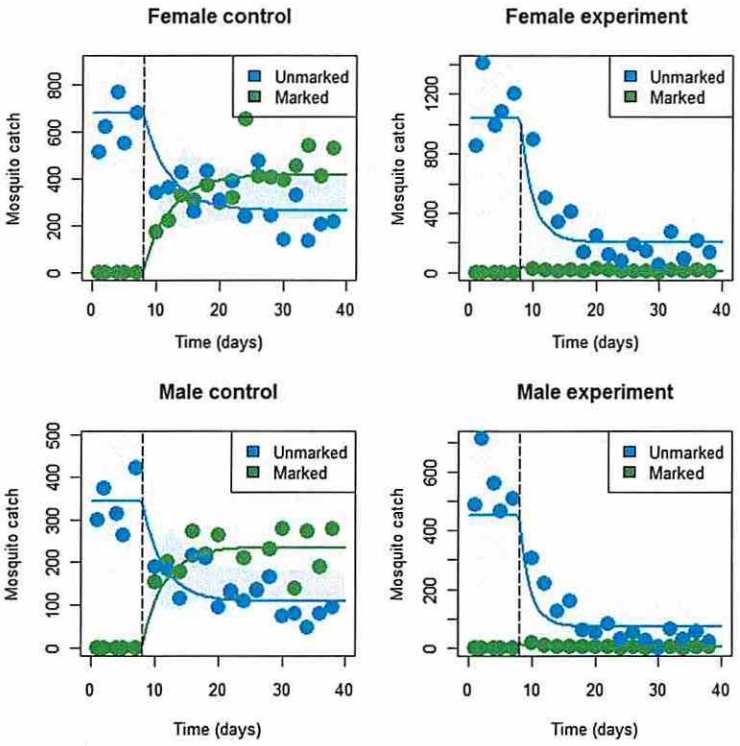

Figure 2 Basic model fits for both male and female mosquito catch data in the experimental and control settings. Dots represent mosquito catches, solid lines represent model predictions and shaded regions represent 95% of the model predicted variation in mosquito catch numbers.

of application level or the availability of natural sugar sources), or due to dye decay causing the ASB-feeding rates to be underestimated (in the experimental setting, dye decay can be ignored since toxin-induced death occurs at a faster rate). Given that mosquitoes also feed on natural sugar sources, the total sugar-feeding rate will be higher than both of these estimates.

The death rates following ingestion of ATSB are important indicators of the effectiveness of ATSB at reducing mosquito density. For females, an estimated death rate of 11.7 per day corresponds to a mean lifetime of 2.1 hours following ATSB consumption (95% CrI: 1.1-3.8 hours). This estimate is consistent with laboratory experiments showing 100% lethality within 12 hours [14]. It should be noted that, while relevant to mosquito density, this parameter is less relevant to malaria control since mosquitoes tend not to seek blood meals after feeding on ATSB [13].

Incorporating gonotrophic cycles

Female mosquitoes blood-feed to fuel the production of eggs. The number of blood-feeding and egg-laying

Table 1 Parameter estimates for basic sugar-feeding model

Parameter:	Prior distribution (per day):	Posterior estimate with 95% credible interval (per day):
Female ASB-feeding rate (control): $s_{f,C}$	Uniform (0,10)	0.15 (0.12 - 0.19)
Female ATSB-feeding rate (experiment): $s_{f,E}$	Uniform (0,10)	0.50 (0.27 - 0.97)
Male ASB-feeding rate (control): $s_{m,C}$	Uniform (0,10)	0.15 (0.12 – 0.19)
Male ATSB-feeding rate (experiment): $s_{m,E}$	Uniform (0,10)	0.46 (0.27 – 0.84)
Female death rate, μ_f	Normal (0.1,0.01)	0.094 (0.075-0.115)
Male death rate, μ_m	Normal (0.1,0.01)	0.094 (0.076-0.113)
Female ATSB death rate: $\mu_{f,ATSB}$	Uniform (0,100)	11.7 (6.3 - 22.6)
Male ATSB death rate: $\mu_{m,ATSB}$	Uniform (0,100)	11.0 (6.1 - 20.3)

(gonotrophic) cycles they have completed provides a measure of their age – each cycle takes approximately three days to complete [34] – and their ability to transmit pathogens. At the earliest, mosquitoes can become infected with malaria on their first gonotrophic cycle, and it takes at least another two cycles for the parasites to incubate within the mosquito [34]. This means that only female mosquitoes that have completed three or more gonotrophic cycles can be infectious to humans. Gonotrophic cycle numbers as high as eight were recorded in the Mali field trial [14] and these provide an opportunity to investigate trends in sugar-feeding with age.

Figure 3 shows model fits for the proportion of female mosquitoes having completed 0–2 or more than two gonotrophic cycles in the experimental setting. The intervention was at day seven and the first post-intervention data regarding gonotrophic cycles was collected at day 24, hence there is limited power to predict changes in the breakdown of gonotrophic cycle numbers between these time points. However, it is clear that three weeks after the intervention, very few female mosquitoes remained that had completed more than two gonotrophic cycles. In the control site, the gonotrophic cycle number distribution remained constant over time.

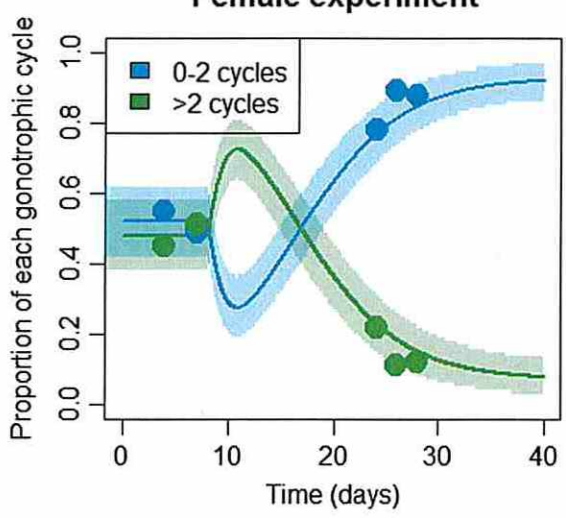

Figure 3 Proportions of female mosquitoes having completed zero to two or more than two gonotrophic cycles in the experimental setting. Dots represent observed results, solid lines represent model predictions and shaded regions represent 95% of the model-predicted variation in mosquito catch numbers. Although mosquito numbers in all categories decline after the addition of ATSB, females having completed zero to two gonotrophic cycles decline fastest initially because they have the highest sugar-feeding rate; however, the age distribution quickly shifts towards younger mosquitoes.

Table 2 shows revised estimates of sugar-feeding rates and ATSB-induced death rates for a model in which mosquitoes having completed zero to two and three or more gonotrophic cycles have distinct sugar-feeding rates. This was the best-fitting of four models in which sugar-feeding rates were allowed to vary with age (Additional file 3: Table S2). Interestingly, for all four models, a significant reduction in sugar-feeding rate with age was observed. The model suggested a sugar-feeding rate for females having completed zero to two gonotrophic cycles that was almost double the mean sugar-feeding rate estimated from the simpler, non-age-structured model. Balancing this, the estimated sugar-feeding rate for females having completed three or more gonotrophic cycles was about a quarter the mean sugar-feeding rate. The feeding rate on ATSB-sprayed vegetation in the experimental setting of 0.84 per day is consistent with empirical evidence that young mosquitoes sugar-feed more than once per day [14], since the actual sugar-feeding rate is higher than that solely on ATSB-sprayed sources.

The potential impact of ATSB as part of integrated vector management (IVM)

Current vector control strategies focus largely on LLINs and IRS; however both these interventions target adult mosquitoes while they are indoors. The addition of ATSB holds promise because it targets adult mosquitoes outdoors and also complements larviciding, which targets the aquatic stage of the mosquito life cycle (Figure 4A). Data on the pattern of mosquito activity (Figure 4B) also suggest that mosquitoes sugar-feed at different times to seeking a blood-meal – specifically, at dusk before blood-feeding, and, to a lesser extent, just before sunrise. This further highlights the potential synergy between ATSB and other vector control strategies.

To assess the potential contribution of ATSB to IVM strategies, the models and parameters described above were used in conjunction with an existing ecological model of *Anopheles* population dynamics [34] and an existing model [40] of the effects of LLINs and IRS on mosquito densities, modified slightly as in Griffin *et al.* [41]. For larviciding, the case of *Bacillus thuringiensis var. israelensis* (BTI) applied to larval breeding sites was considered [42]. BTI was found to reduce larval density by 88% where applied [42] and to increase larval and pupal death rates by a constant factor. Coverage levels for current vector interventions were assumed to be either 80% or 50% (Additional file 1), and ATSB was assumed to be implemented at levels leading to an exposure rate analogous to that in the Mali experimental setting [14] or at levels such that the exposure rate would be half that of the Mali setting. The combined model is described in Additional file 1.

At 80% coverage, LLINs and IRS are expected to significantly reduce *An. gambiae* density (Figure 5).

Table 2 Parameter estimates for sugar-feeding model incorporating gonotrophic cycles

Parameter:	Prior distribution (per day):	Posterior estimate with 95% credible interval (per day):
ASB-feeding rate (0–2 gonotrophic cycles, control): $s_{A,C}$	Uniform (0,10)	0.25 (0.18 - 0.31)
ASB-feeding rate (3 or more gonotrophic cycles, control): $s_{B,C}$	Uniform (0,10)	0.035 (0.009 – 0.076)
ATSB-feeding rate (0–2 gonotrophic cycles, experiment): $s_{A,E}$	Uniform (0,10)	0.84 (0.53 – 1.21)
ATSB-feeding rate (3 or more gonotrophic cycles, experiment): $s_{B,E}$	Uniform (0,10)	0.12 (0.03 – 0.27)
Female death rate, μ_f	Normal (0.1,0.01)	0.094 (0.081-0.110)
Female ATSB death rate: $\mu_{f,ATSB}$	Uniform (0,100)	12.2 (7.5 – 23.9)
Reciprocal of gonotrophic cycle length: δ	Normal (0.33,0.03)	0.34 (0.30-0.39)

However, LLINs are expected to have less effect on the more exophilic *An. arabiensis*. Interestingly, at Mali exposure rates, ATSB is expected to have a greater population suppressing effect than either LLINs or IRS – a trend also seen if ATSB exposure rates are halved. All three of these interventions result in an age distribution heavily skewed towards females having completed two or less gonotrophic cycles, which is encouraging for malaria control. ATSB and larviciding perform similarly well at reducing adult mosquito densities; however, since larvicides act before the adult life stage, they don't cause any changes in the adult age structure. If ATSB coverage is such that exposure rates are half those of the Mali experimental setting, larviciding has a bigger effect on the total mosquito density but a smaller effect on mosquitoes having completed three or more gonotrophic cycles. ATSB is therefore more efficient at reducing the number of mosquitoes that could potentially transmit malaria. If coverage with the other interventions is reduced to 50%, ATSB is predicted to outperform all of them even at 50% Mali exposure rates (Additional file 7: Figure S2).

Figure 6 and Additional file 8: Figure S3 show the expected impact of different combinations of interventions on *An. gambiae* and *An. arabiensis* densities assuming a pre-intervention density of 1,000 for both species, 369 of which have completed three or more gonotrophic cycles. A combination of LLINs and ATSB is expected to be extremely efficient at reducing population densities of both species and, in particular, the density of female mosquitoes having completed three or more gonotrophic cycles (reduced to ~2 for *An. gambiae* and ~5 for *An. arabiensis*). The LLIN/ATSB combination compares favourably against a combination of LLINs and IRS or LLINs and larviciding, even when ATSB exposure rates are halved (in which case, the density of *An. gambiae* having completed three or more gonotrophic cycles is reduced to ~7, and to ~23 for *An. arabiensis*). For the LLIN/larviciding combination, mosquito densities are reduced; however there is still a residual *An. arabiensis* population with a density of ~39 having completed three or more gonotrophic cycles. Addition of IRS to the LLIN/ATSB combination provides little benefit due to the efficiency of the LLIN/ATSB combination on its own.

Impact of IVM strategies including ATSB on EIR

Reductions in vector density give a clear comparison of the relative impact of IVM strategies; however a more

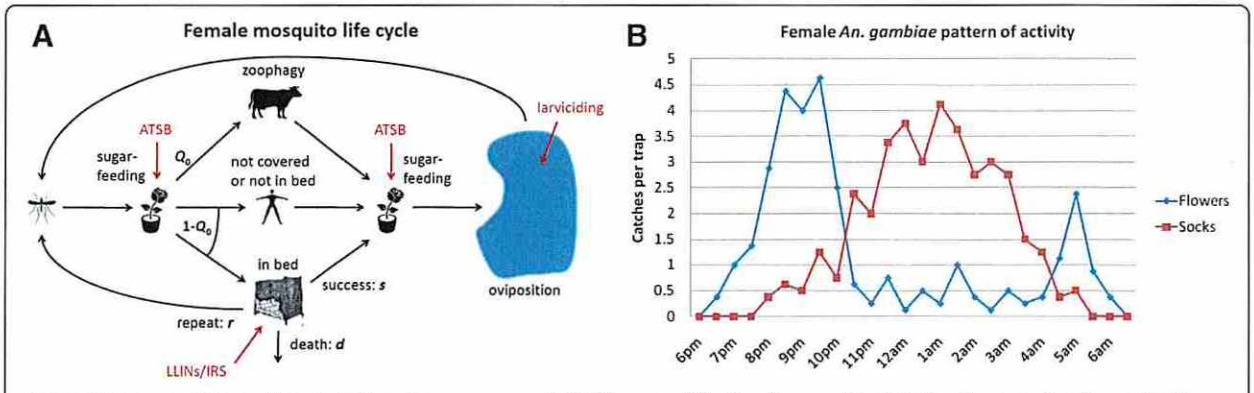

Figure 4 Sugar-feeding and integrated vector management. A) Life cycle of the female mosquito depicting the centrality of sugar-feeding and opportunities for vector control in red. **B)** Data on the pattern of activity of female *An. gambiae* mosquitoes. Mosquito catches on flowers peaked at 9 pm and 5 am, while catches on socks peaked around 1 am.

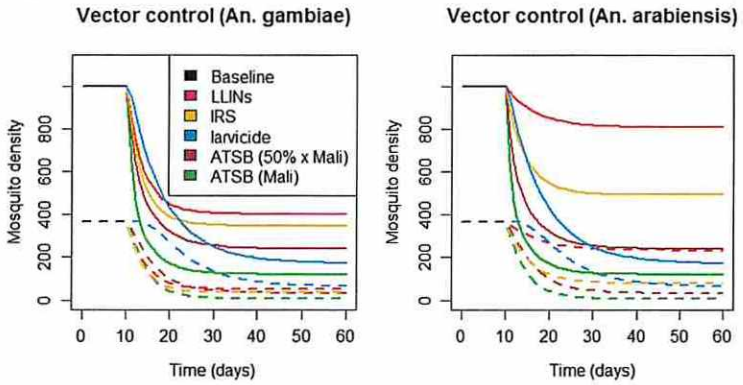

Figure 5 Time-series depicting the effects of vector control strategies on vector density in isolation. Solid lines represent total female mosquito density, dashed lines represent females having completed three or more gonotrophic cycles. Coverage levels are assumed to be 80% for all interventions (i.e. 80% of people sleeping under nets, 80% of houses sprayed with insecticide, and 80% of breeding sites treated with BTI). ATSB is assumed to be implemented at analogous levels to that in the Mali experimental setting, and at levels such that the exposure rate would be half that of the Mali experimental setting.

direct measure of human exposure to malaria is the entomological inoculation rate (EIR), defined as the average number of infective bites per person per year [39]. The EIR is more sensitive to the age breakdown of the vector population, since older mosquitoes are more likely to be infectious to humans.

Figure 7 shows the expected impact of the same interventions as shown in Figure 6 on EIR for three transmission settings, and Additional file 9: Figure S4 shows these for 50% coverage levels with current interventions. For simplicity of comparison, populations are assumed to be entirely either *An. gambiae* or *An. arabiensis*. The relative impact of the different combinations of interventions is the same in each setting, although the magnitude of the post-intervention EIR differs. For 80% coverage levels in settings with baseline EIRs of 50 and 100, only the LLIN/ATSB combination (with Mali ATSB exposure rates) is expected to reduce EIRs to less than one infective bite per person per year for both species, which is the value thought necessary to achieve local elimination [43-45]. For 50% coverage levels and ATSB exposure rates at 50% of those in Mali, only the LLIN/ATSB combination is expected to reduce EIRs to less than one in the moderate transmission setting and to less than ten in the high and very high transmission settings. These results should not be interpreted as predictive; but they do suggest that ATSB could potentially play an important role in vector control in a range of transmission settings.

A question of great relevance to IVM planning is the ATSB exposure rate at which a combination of

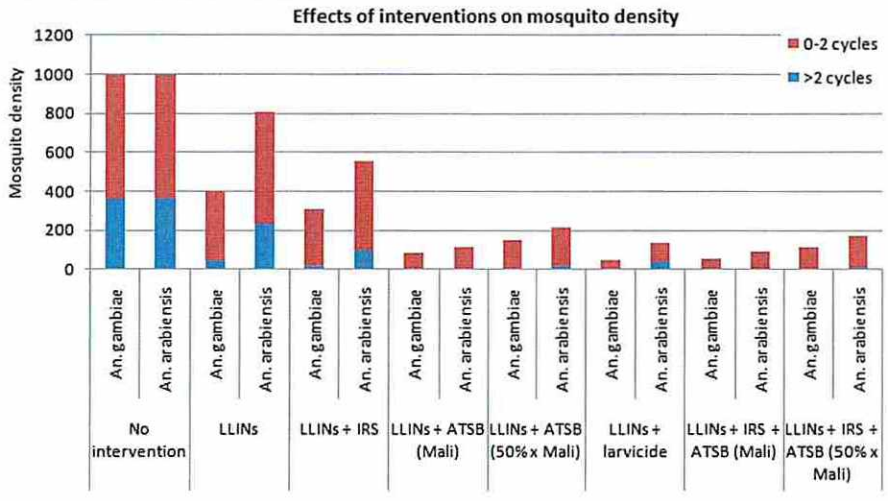

Figure 6 Expected impact of IVM strategies on mosquito density.

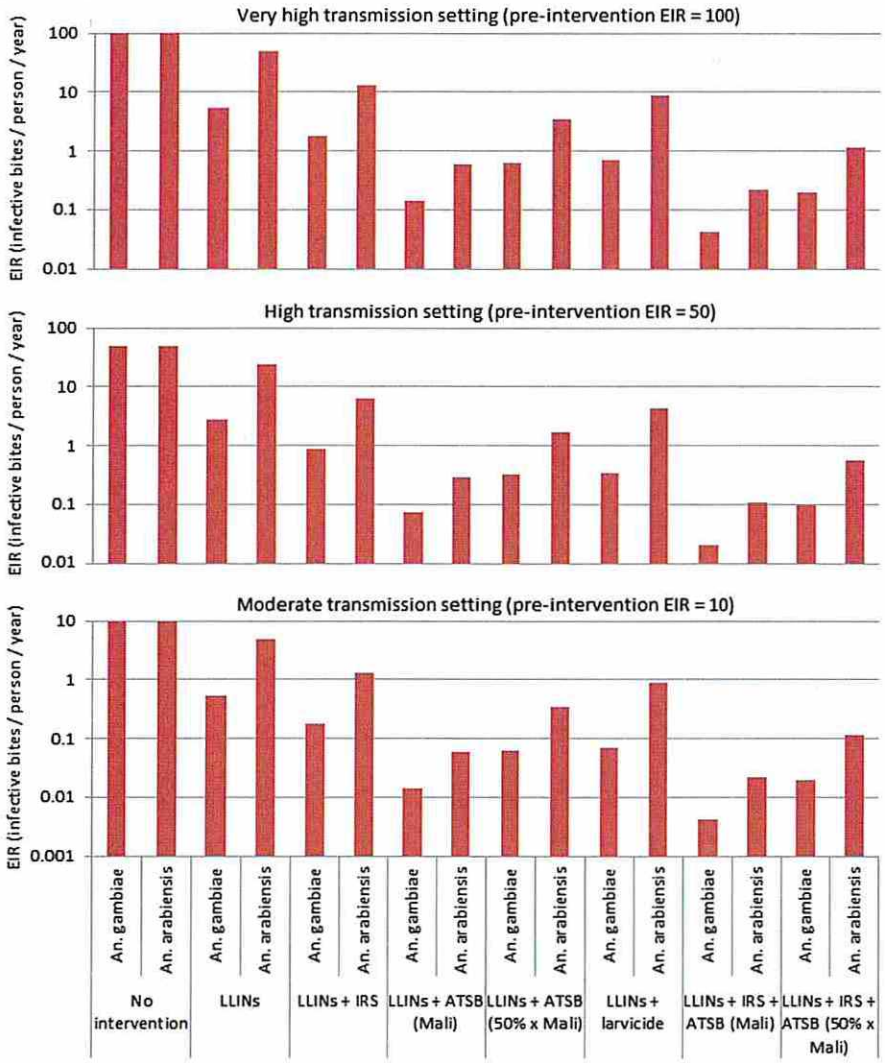

Figure 7 Expected impact of IVM strategies on EIR. Model predictions are shown for three transmission settings with pre-intervention EIRs of 100 (very high transmission), 50 (high transmission) and 10 (moderate transmission).

LLINs and ATSB is expected to cause a larger reduction in malaria transmission (as measured by EIR) than a combination of LLINs and IRS. LLINs are now widely distributed in many malaria-endemic countries [4,7], and hence 80% LLIN coverage levels are assumed for these calculations. IRS is less widespread and more likely to be replaced by alternative interventions; however, before being replaced, the new intervention should be expected to be more effective at reducing malaria transmission than IRS when used in combination with the already-present LLINs. Figure 8 shows the ATSB exposure rates (measured as a fraction of Mali exposure rates) required to achieve the same reduction in EIR as IRS at a range of coverage levels between 0 and 100%. To compensate for IRS at an optimistic coverage level of 80%, modelling suggests that ATSB exposure rates of ~36% of Mali levels (34% for *An. arabiensis* and 38% for *An. gambiae*) would be required to achieve the same reduction in malaria transmission (these results are independent of the baseline EIR for this model). The lower requirement for *An. arabiensis* is due to it being relatively exophilic and hence more susceptible to outdoor control measures. The predicted effectiveness of ATSB coverage levels less than in the Mali experimental setting is encouraging; however, further experiments will be required to determine the relationship between coverage level and exposure rate in a range of environmental settings, including in lush settings with an abundance of natural sugar sources.

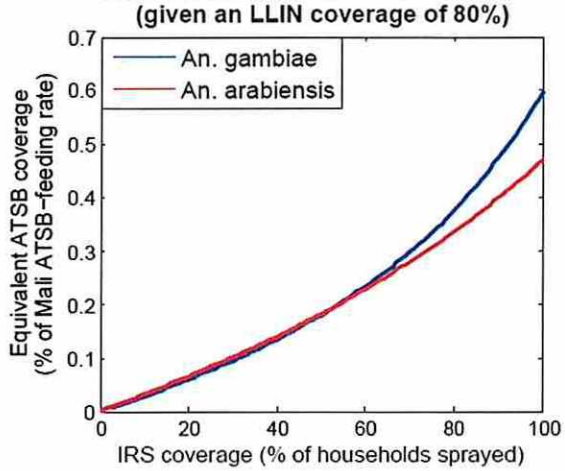

Figure 8 ATSB coverage required to achieve the same reduction in transmission as IRS (given an LLIN coverage of 80%).

Discussion

The promise of ATSB described here directly follows from extending the results of a successful field trial in Bandiagara, Mali [14] to a range of different transmission intensities, and modelling its impact in combination with a variety of other vector control strategies. The models suggest that high coverage with a combination of LLINs and ATSB at levels similar to those in the Mali field trial is expected to cause significant reductions in EIR, exceeding the predicted impact of LLINs in combination with other interventions such as IRS or larviciding. Furthermore, ATSB is expected to perform favourably even at half the exposure rates of the Mali field trial.

The benefit of ATSB is that it kills mosquitoes while they are outdoors, thus targeting a different stage of the mosquito gonotrophic cycle than LLINs and IRS [46]. Larviciding targets a different stage of the mosquito life cycle; however ATSB has the advantage that it skews the adult age distribution towards younger mosquitoes, which is beneficial for malaria control because only older mosquitoes have time to acquire, incubate and transmit the parasite. It is also cheap and environmentally friendly and, while not modelled here, it targets both male and female mosquitoes.

An interesting result from the model fits was a significant trend in declining sugar-feeding rate with age among female mosquitoes. Since older mosquitoes are more likely to transmit malaria, a strategy that targets these older mosquitoes is desirable; however, if mosquitoes are targeted when they are young, they will not reach the required age to transmit malaria, so both approaches are effective. This is evidenced by the scarcity of mosquitoes having completed more than two gonotrophic cycles within a few weeks of ATSB application in Mali (Figure 3). That said, it is not clear the extent to which this trend is influenced by the high rate of sugar-feeding following emergence [25]. Regardless, the results from this trial suggest a high death rate among young mosquitoes, which is predicted to reduce the number of adult mosquitoes capable of transmitting malaria similarly to strategies that target adult mosquitoes in an age-independent manner.

Also worthy of note is that the sugar-feeding rates estimated here are for ATSB and ASB-sprayed vegetation at the coverage levels of the Mali experiment. An estimate of the total sugar-feeding rate on all available vegetation would be of interest to understanding the maximum potential of ATSB at reducing mosquito density. One way to measure this would be to spray patches of vegetation with ASB containing different coloured dyes. Coloured and multicoloured mosquitoes could then be used to infer the total sugar-feeding rate in a similar manner to how total population size is inferred in a traditional mark-release-recapture experiment. Also of interest is the relationship between coverage level and ATSB-feeding rate. In the Mali trial, one square metre spots of vegetation were sprayed every three metres around breeding sites [14] leading to the ATSB-feeding rates estimated here. A relationship between these variables would assist in operational and cost-effectiveness analyses.

The performance of ATSB in different geographic and seasonal settings is of great interest. Field trials have thus far been conducted in Mali and Israel [14-18] and provide a proof of principle in semi-arid areas. In Israel, ATSB has been shown to outcompete natural sugar sources [47] and to reduce mosquito populations even in sugar-rich environments [48]. Modelling results presented here predict ATSB to be effective even if exposure rates are half those of the Mali experiment. However, the performance of ATSB remains to be tested in settings with a greater abundance of natural sugar sources. A further complication is that heavy rains can wash ATSB off of vegetation, making reapplication necessary during the rainy season. A complementary approach is the provision of covered bait stations, which have proven successful in Israel [15,16], and are currently undergoing field testing and product development in other settings. Another product enhancement being considered is combining ATSB with larvicides which mosquitoes may carry to breeding sites after sugar-feeding.

As for any modelling exercise, simplifications have been made and limitations exist that mean that the

results are indicative rather than predictive. The sugar-feeding model is parameterized by fitting to the available trial data from one semi-arid location; however, the parameter estimates include a high degree of uncertainty. Furthermore, the true underlying dynamics may be more complicated than suggested by the simple parsimonious models explored here. For instance, sugar-feeding rates are likely to decline more gradually with age than was possible to detect by fitting to the available data and dye decay was not incorporated but is known to occur in the wild [14]. However, the general trends inferred here capture important features of vector control with ATSB. Parameter estimates for other vector control strategies are collated from several different locations and neglect phenomena such as waning of efficiency with time. Whilst the LLIN model captures the effect size observed in randomized trials, both the IRS and larviciding models have not been validated against trial data. Furthermore, *An. gambiae* and *An. arabiensis* have been considered as separate entities here, while future studies could investigate potential shifts in species composition under a variety of IVM combinations using a species competition model [49]. Therefore, the IVM model predictions should be interpreted in this light as providing insight into the potential of ATSB to contribute to future integrated vector control programs rather than precise predictions.

Conclusions

In summary, the models presented suggest that ATSB, or modifications of this approach to target outdoor mosquitoes, could be important to consider in future IVM programmes, especially in combination with LLINs and in semi-arid areas. ATSB kills mosquitoes while they are outdoors and skews the adult age distribution towards younger mosquitoes, leading to substantial reductions in both sporozoite rate and EIR. Further field testing is needed to address operational issues (in particular the degree of overall coverage that can be obtained) and to determine its efficacy in a range of other settings. If the predictions of this modelling effort hold true, ATSB could be a useful additional tool for malaria control in permissive settings.

Additional files

Additional file 1: Further details on sugar-feeding and integrated vector management models, model fitting and parameter values.

Additional file 2: Table S1. Model comparison for basic models.

Additional file 3: Table S2. Model comparison for models incorporating gonotrophic cycle number.

Additional file 4: Figure S1. Schematic of sugar-feeding model incorporating gonotrophic cycles in the experimental setting. Umarked, U_i, and marked, M_i, females are partitioned into those having completed i gonotrophic cycles, where $i \epsilon \{0,1,\ldots,8\}$ (mosquitoes having completed eight or more cycles are grouped into the same category). Female mosquitoes emerge at rate b into unmarked class, U_0, and become marked, M_0, when feeding on ATSB-sprayed vegetation at rate s_F. In the control setting, marked and unmarked mosquitoes die at the same rate, μ, while in the experimental setting, marked mosquitoes die at a faster rate due to the effects of the toxin, μ_{ATSB}. Both marked and unmarked mosquitoes have a gonotrophic cycle length of $1/\delta$.

Additional file 5: Table S3. Parameter estimates for IVM model that are species-invariant.

Additional file 6: Table S4. Parameter estimates for IVM model that vary between species.

Additional file 7: Figure S2. Time-series depicting the effects of vector control strategies on vector density in isolation. Solid lines represent total female mosquito density, dashed lines represent females having completed three or more gonotrophic cycles. Coverage levels are assumed to be 50% for all interventions (i.e. 50% of people sleeping under nets, 50% of houses sprayed with insecticide, and 50% of breeding sites treated with BTI). ATSB is assumed to be implemented at analogous levels to that in the Mali experimental setting, and at levels such that the exposure rate would be half that of the Mali experimental setting.

Additional file 8: Figure S3. Expected impact of IVM strategies on mosquito density. Red bars represent females having completed less than three gonotrophic cycles and blue bars represent females having completed three or more gonotrophic cycles. Coverage levels are assumed to be 50% for all interventions (i.e. 50% of people sleeping under nets, 50% of houses sprayed with insecticide, and 50% of breeding sites treated with BTI). ATSB is assumed to be implemented at analogous levels to that in the Mali experimental setting, and at levels such that the exposure rate would be half that of the Mali experimental setting.

Additional file 9: Figure S4. Expected impact of IVM strategies on EIR. Coverage levels are assumed to be 50% for all interventions (i.e. 50% of people sleeping under nets, 50% of houses sprayed with insecticide, and 50% of breeding sites treated with BTI). ATSB is assumed to be implemented at analogous levels to that in the Mali experimental setting, and at levels such that the exposure rate would be half that of the Mali experimental setting. Model predictions are shown for three transmission settings with pre-intervention EIRs of 100 (very high transmission), 50 (high transmission) and 10 (moderate transmission).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JMM, MTW, GCM and JCM devised the study and objectives. GCM provided the data. JMM and MTW developed the model and analysed the data. JMM wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Effect of CO_2 concentration on mosquito collection rate using odor-baited suction traps

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ABSTRACT: Carbon dioxide (CO_2) has been used for decades to enhance capture of host-seeking mosquitoes when released in association with traps commonly used by mosquito and vector control agencies. However, there is little published work evaluating the effect of altering CO_2 release rates relative to the number of mosquitoes captured in these traps. This study investigated how varying CO_2 concentration altered the mosquito collection rate at a freshwater wetlands in southern California. Host-seeking mosquitoes were captured in CDC-style traps baited with one of six CO_2 release rates ranging from 0-1,495 ml/min from gas cylinders. Species captured were *Aedes vexans*, *Anopheles franciscanus*, *An. hermsi*, *Culex erythrothorax*, and *Cx. tarsalis*. A biting midge, *Culicoides sonorensis*, was also captured. For all species, increasing CO_2 release rates resulted in increasing numbers of individual females captured, with the relative magnitude of this increase associated to some extent with known feeding preferences of these species. We found that variation in CO_2 release rate can significantly alter mosquito capture rates, potentially leading to imprecise estimates of vector activity if the relationship of CO_2 release rate to mosquito capture rate is not considered. ***Journal of Vector Ecology* 42 (1): 44-50. 2017.**

Keyword Index: Mosquito trap, *Culex, Anopheles, Culicoides*, host-seeking, surveillance.

INTRODUCTION

Hematophagous insects rely on biochemical cues such as odors to locate and identify hosts on which to feed. Carbon dioxide is one of the most important olfactory stimuli involved in orientation toward hosts by mosquitoes and other blood feeding insects (Gillies 1980). Respiration involving CO_2 output is common to all vertebrates and therefore serves as a signal for their presence.

Surveillance and monitoring for changes in the abundance and activity of host-seeking mosquitoes is critical to assess the public health risk for mosquito-transmitted pathogens like West Nile virus (WNV). Surveillance should employ efficient collection methods that allow for definitive species identification while also providing an accurate representation of species host-seeking activity. Numerous studies have shown that the release of CO_2 will enhance the capture of host-seeking mosquitoes when released with traps commonly used by mosquito control agencies (e.g., CDC light trap (Sudia and Chamberlain 1962) or Encephalitis Virus Surveillance trap (Rohe and Fall 1979)). However, there is little published work analyzing the effect of altering CO_2 release rates relative to the number of mosquitoes captured in the field. Previous non-laboratory studies by Reeves (1953) and Reisen et al. (2000) focused primarily on *Culex tarsalis* Coquillett attraction to traps using measured CO_2 release rates simulating specific hosts (chicken, human, cow) and in relation to varying trap design with CO_2 presentation (e.g., pulsating vs consistent CO_2 release), respectively.

There are three common sources for CO_2 used in mosquito traps: dry ice, compressed gas, and hydrocarbon fuel combustion. Dry ice is most commonly used for vector surveillance by mosquito control and public health agencies due to lower cost and portability. However, the concentration of CO_2 released by dry ice is highly variable, as the quantity and surface area (solid block or pellet) of dry ice used with traps will often differ considerably among trap designs. Additionally, sublimation rates vary with the type of holding container and environmental conditions in the field.

Host CO_2 output varies considerably across a range of potential animal hosts. For example, Roberts (1972) found the CO_2 emissions of beef heifers ranged from 1,200 to 1,800 ml/min, while Gillies and Wilkes (1974) determined CO_2 output for chickens to be 50 ml/min. The CO_2 output of a standard CDC-style suction trap baited with dry ice held in an insulated container has been recorded to range from 1,000-1,500 ml/min (Mullens 1995) or 641-892 ml/min (Eshun et al. 2016), or roughly equivalent to a large mammal.

Several investigators have documented changes in the capture rate of biting flies when releasing variable CO_2 concentrations (release rate or flow rate) to attract these flies to a suction trap. Carestia and Savage (1967) used traps with CO_2 flow rates of 250, 500, 1,000, and 2,000 ml/min and reported that mosquito capture generally increased with increasing CO_2 release rate for a number of species (*Anopheles quadrimaculatus* Say, *An. walkeri* Theobald, *An. punctipennis* Say, *Aedes vexans* (Meigen), *Culex pipiens* L., and *Cx. salinarius* Coquillett), but traps in their study were not run concurrently and thus could not be directly compared for differences among the CO_2 release rates. Additionally, the relationship of CO_2 release rate and insect capture rate may differ among species, as later studies showed that increasing the CO_2 concentration released was not always associated with an increased capture of host-seeking insects. For example, Pfuntner et al. (1988) found that while capture of *Cx. tarsalis* increased significantly when CO_2 release was increased from 250 to 1,000 ml/min, the capture of *Cx. quinquefasciatus* Say and *Cx. sitgmatosoma* Dyar was not altered by the increased CO_2 release. In contrast, Mullens and Gerry (1998) observed that capture of *Cx. quinquefasciatus* actually decreased significantly when the CO_2 release rate was increased from 300 to 1,000 ml/min, while simultaneous capture of the highly mammalophilic biting midge *Culicoides sonorensis* Wirth & Jones increased with the higher CO_2 release rate. For black flies identified as *Cnephia (Stegopterna) mutata* (Malloch)

and *Prosimulium hirtipes* (Fries), it was determined that CO_2 release rates exceeding 500 ml/min did not further increase their collection (Frommer et al. 1976). Both *C. (Stegopterna) mutata* and *P. hirtipes* are known to bite humans (Mokrey 1978, Mason and Schmanchuck 1990) though *C. mutate* is also recorded to be autogenous and thus does not require an initial blood meal (Mokrey 1978).

The objectives for this study were (1) to investigate how varying CO_2 concentration alters capture of mosquitoes at a southern California wetlands, and (2) to determine if changes in the mosquito capture rate were associated with increasing CO_2 concentration similarly for available host-seeking species at the wetlands site.

MATERIALS AND METHODS

Host-seeking mosquitoes, and other hematophagous Diptera, were collected from 10 July 2013 through 28 August, 2013 at the San Jacinto Wildlife Area (SJWA; 33°52′14.19″N, 117°7′6.86″W). SJWA is a managed freshwater wetlands located in the inland southern California desert of Riverside County. This area is known to have high mosquito activity and to contain diverse avian and mammalian fauna (Lura et al. 2012, CDFW 2016). Eight CDC style suction traps (Model 512, JW Hock Inc., Gainesville, FL) were used to collect host-seeking Diptera. Six of the traps were augmented with CO_2 from a 567 g cylinder tank equipped with a two-staged regulator, a flow restrictor to maintain a steady flow of CO_2, and vinyl plastic tubing placed so that CO_2 was released just above the trap entrance. Traps were placed 30 m apart in a circular trapline in an open field adjacent to mosquito production and resting sites (Figure 1). This arrangement was used to ensure trap independence (Brown et al. 2008), unobstructed CO_2 plume structure (Murlis and Jones 1981), and to minimize varying effects of vegetation on mosquito collections. CO_2 concentrations (flow rates) evaluated were 15, 47, 149, 473, and 1,495 ml of CO_2 per min; these flow rates were half-log increases starting at 15 ml/min, which was the lowest flow rate that could be maintained consistently by the regulators used in this study.

Trapping was conducted two nights per week during six weeks, with trap nights separated by 2-3 days within each week. Trapping began 30 min after sunset and ended two h later to cover the peak mosquito activity period at this site based upon preliminary studies. The 2-h trapping period also assured that CO_2 flow rates remained consistent over the entirety of the trapping period. Low CO_2 flow rates (15 and 47 ml/min) were tested on the first night each week (low CO_2 nights), while high CO_2 flow rates (473 ml/min and 1,495 ml/min) were tested on the second night each week (high CO_2 nights). High and low flow rates were separated by night to avoid masking or interference of traps with low CO_2 flow rates by those with high CO_2 flow rates. An intermediate or mid-range flow rate (149 ml/min) and a negative control (0 ml/min) were included on all nights in order to make comparisons across collection nights. During each trap night, two traps were set with each of the four flow rates to be tested on that night, for a total of eight traps utilized per night. One trap with each flow rate was randomly assigned to the first four trap positions of the circular trapline (Figure 1), and the second trap with the same flow rate was then placed at the opposing trap position in the circular trapline. Flow rates were checked at the beginning and end of each trapping period in order to validate a constant CO_2 flow rate throughout the trapping period. Weather conditions were hot and dry. Temperatures recorded at the CIMIS (California Irrigation Management Information System) weather station near Lake Perris averaged 35 (max) to 16 (min)° C during the sampling periods (Figure 2). The prevailing wind direction was from the west and northwest with average daily wind speed of 8 kph.

Figure 1. Trap arrangement at the wetlands of the San Jacinto National Wildlife Area in southern California.

For each collection night, insects captured in the two traps with the same CO_2 flow rate were combined and sorted to species and sex, and enumerated, with the total number of females captured divided by two to give a mean per trap night count for females of each species collected at each CO_2 flow rate tested. Mean counts were subsequently transformed to $\log_{10}$ (n+1) and analyzed separately for low and high CO_2 nights by ANOVA (Proc GLM in SAS; Cary, North Carolina, U.S.A.) with week and CO_2 flow rate as independent variables. Means for significant variables were separated using the post-hoc least significant difference (LSD) test. The use of traps with the mid-range CO_2 flow rate (149 ml/min) on all nights allows for further comparison of mean insect captures across all CO_2 release rates.

To determine whether using traps with either low or high CO_2 flow rates in the trap line differentially impacted mid-range flow rate trap captures, mean counts for mid-range flow rate traps were paired by week (one low and one high CO_2 night per week) due to significant differences in mean counts among weeks for some species, and evaluated using a paired t-test (SAS) for differences among high and low CO_2 nights. For species with mid-range flow rate trap counts that did not differ between low and high CO_2 nights (all but *An. hermsi* Barr & Guptavanij which was captured in very low numbers), the relationship of female capture to CO_2 concentration was subsequently determined across all CO_2 release rates tested. To adjust for the natural variation in host-seeking activity among weeks, it was necessary to transform each mean trap count into a relative count that was a proportion of the species-specific mean capture for the mid-range flow rate on the same trap night.

To evaluate the effect of increasing CO_2 concentration on female captures across both low and high CO_2 concentrations

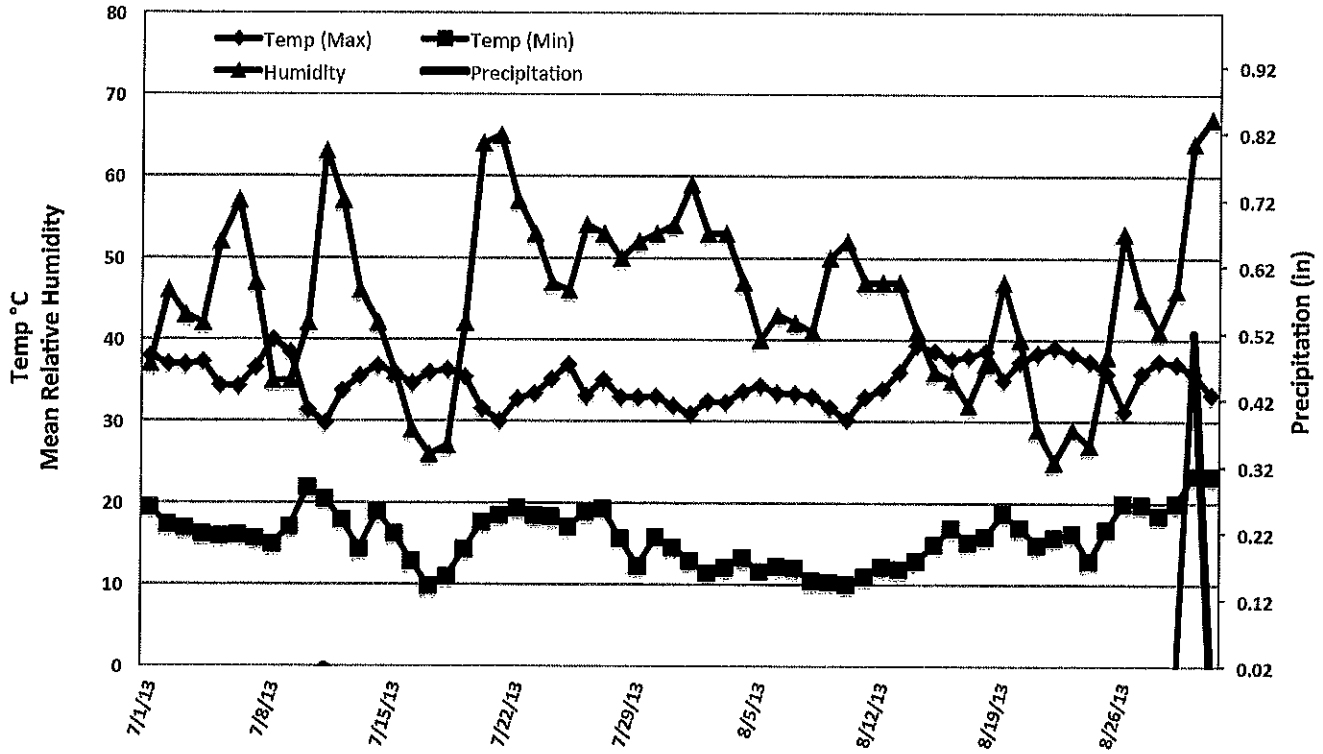


Figure 2. Meteorological data (Mean temperature, humidity and precipitation) taken from the CIMIS (California Irrigation Management Information) weather station located near lake Perris.

tested, we corrected for the natural variation in host-seeking insect activity among nights by dividing the species-specific mean capture for each CO_2 flow rate on each trap night by the species-specific mean capture for the mid-range flow rate on the same trap night to give a proportional mean per trap capture value for each CO_2 flow rate tested. Data was then analyzed by simple linear regression (R statistical package) against CO_2 concentration.

RESULTS

A total of 34,196 female mosquitoes representing five species within three mosquito genera (*Aedes, Anopheles, Culex*) as well as 2,169 biting midges (*C. sonorensis*) were collected (Table 1). The most abundant mosquitoes were *Cx. tarsalis* and *Cx. erythrothorax* Dyar (57% and 40% of total mosquitoes collected, respectively). Other mosquito species collected in the order of their abundance were *Ae. vexans, An. hermsi,* and *An. franciscanus* McCracken.

Table 1. Total number of biting flies captured in all traps.

Species	Females	Males	Blood fed
Aedes vexans	816	0	1
Anopheles franciscanus	87	0	0
Anopheles hermsi	161	0	1
Culex erythrothorax	13,796	68	6
Culex tarsalis	19,336	415	3
Culicoides sonorensis	2,169	0	0
Total	36,365	483	11

For all species, the number of females captured increased significantly with increasing CO_2 concentration on both high CO_2 nights [*Cx. erythrothorax* (F=230.64; df = 3,15; P<0 .0001), *Cx. tarsalis* (F=401.38; df=3,15; P<0.0001), *Ae. vexans* (F=17.99; df=3,15; P<0.0001), *An. hermsi* (F=12.16; df=3,15; P=0.0003), *An. franciscanus* (F=14.42; df=3,15; P<0.0001), and *C. sonorensis* (F=167.32; df=3,12; P<0.0001)] and low CO_2 nights [*Cx. erythrothorax* (F=382.57; df=3,15; P<0 .0001), *Cx. tarsalis* (F=195.83; df=3,15; P<0.0001), *Ae. vexans* (F=7.81; df=3,15; P=0.0023), *An. hermsi* (F=9.37; df=3,15; P=0.001), *An. franciscanus* (F=6.47; df=3,15; P=0.005), and *C. sonorensis* (F=11.59; df=3,12; P=0.0003)], although the increase in trap counts for *Ae. vexans* on low CO_2 nights was only in comparison to the negative control (no CO_2) traps (Figure 3). While there was a trend for reduced mean counts in the mid-range CO_2 flow rate traps on high CO_2 nights relative to low CO_2 nights, this difference was not significant except for *An. hermsi* (t=3.29; df=1,5; P<0.05), which was captured only in very low numbers.

There was a statistically significant linear relationship between the CO_2 concentration and the standardized proportional capture for *Cx. tarsalis* ($R^2 = 0.93$, $p<0.001$), *Cx. erythrothorax* ($R^2 = 0.88$, $p < 0.001$), and *C. sonorensis* ($R^2 = 0.87$, $p < 0.001$) with proportional captures for species collected in lower numbers still predicted primarily (R^2=0.44-0.51) by the CO_2 flow rate (Figure 4).

Simple linear regression of species-specific mean trap counts against CO_2 flow rate showed that mosquitoes were captured at a similarly increasing rate over the range of CO_2 concentrations tested (slope=0.0015-0.0031), while the more mammalophilic

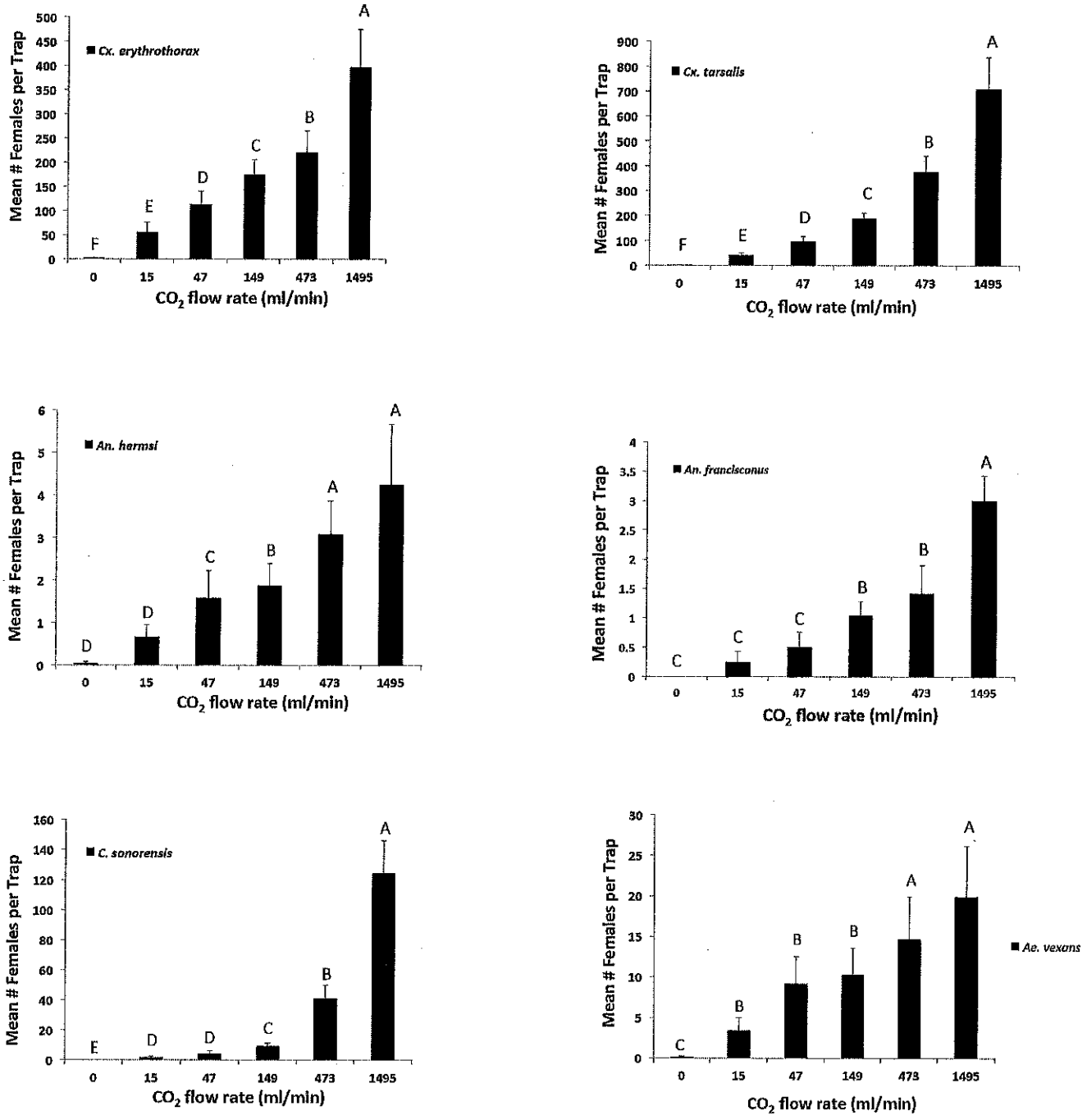
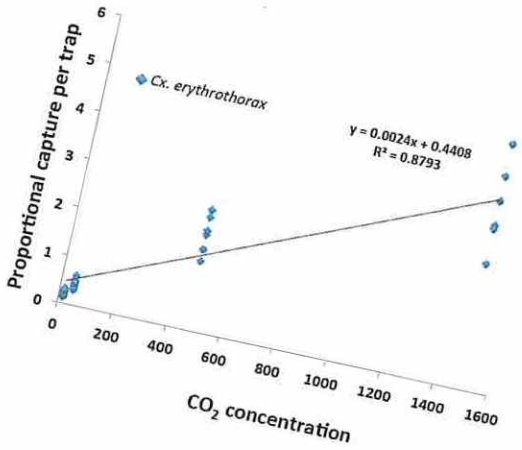
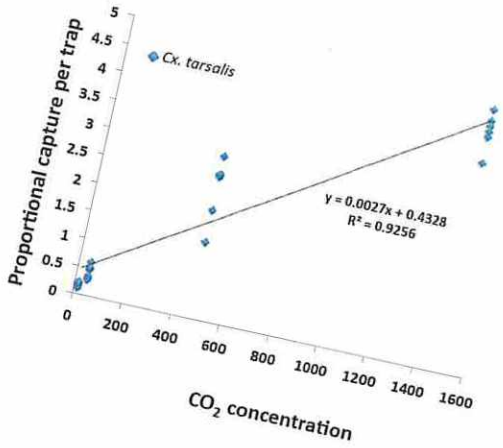
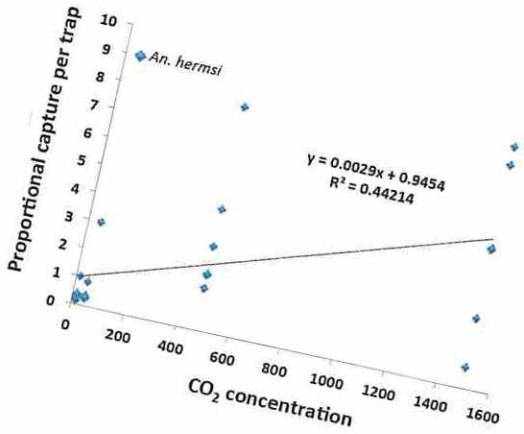
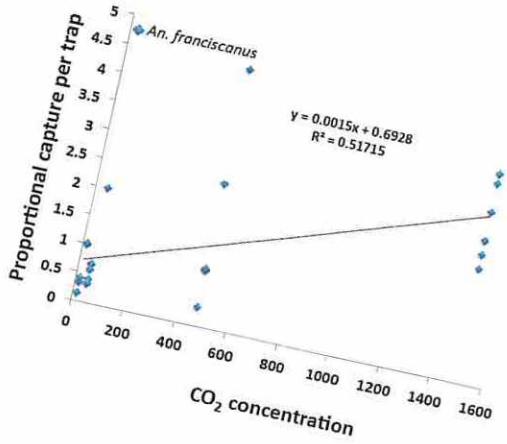


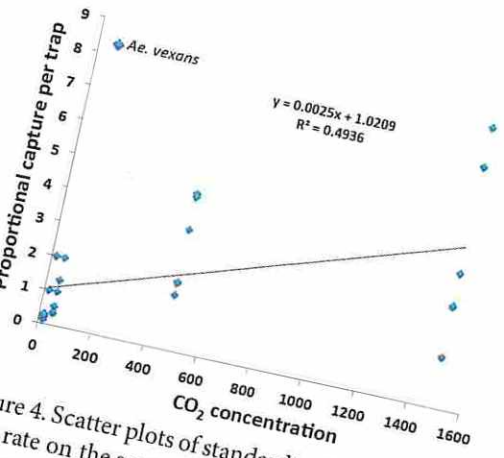
Figure 3. Mean (SE) per trap capture of female mosquitoes and biting midges at CO_2 flow rates tested. Bars with the same letter are not significantly different (LSD test (P>0.05)).

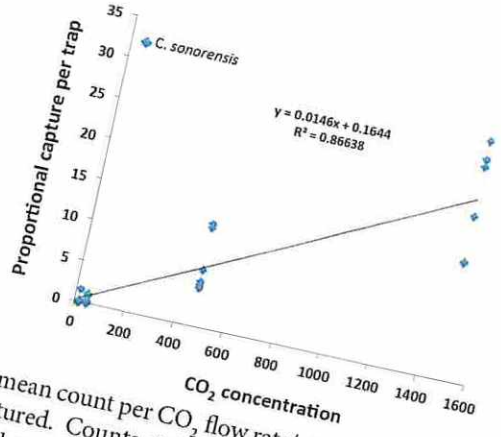












ure 4. Scatter plots of standardized proportional counts per trap night (mean count per CO_2 flow rate/mean count of the midrange CO_2 rate on the same night) for female mosquitoes and biting midges captured. Counts at the midrange CO_2 flow rate (149 ml/min) are sarily excluded from analysis as this counts were used to standardized counts for all other CO_2 flow rates.

biting midge (*C. sonorensis*) was captured at a substantially greater rate with increasing CO_2 concentration (slope=0.015). From the mid-range CO_2 flow rate (149 ml/min) to the highest flow rate tested (1,495 ml/min), there was a four-fold increase in capture for all mosquito species, except *An. hermsi* which increased two to three-fold over this CO_2 range, in contrast to a 25-fold increase in the capture of *C. sonorensis* (Figure 4).

A total of 483 males of two mosquito species, *Cx. tarsalis* (85%) and *Cx. erythrothrorax* (15%), was collected. Males of both species were collected in similar numbers across all CO_2 flow rates, indicating males exhibit no increased or decreased orientation toward traps based upon the presence or concentration of CO_2 or upon the differences in numbers of females collected during the trapping period by traps with varying CO_2 concentration (Figure 5).

DISCUSSION

Overall, CO_2 concentration influenced the capture of all host-seeking Diptera sampled in this study, with increases in CO_2 concentration resulting in increasing capture of host-seeking insects across all flow rates examined (15-1,495 ml/min). For all mosquito species, the number of females captured increased at an approximately similar rate with increasing CO_2 concentration, perhaps as a result of the similar opportunistic feeding behavior of these species. In contrast, captures of the highly mammalophilic biting midge (*C. sonorensis*) increased at a much faster rate, particularly at the higher CO_2 concentrations, probably reflecting their preference for biting large mammals like cattle, sheep, deer, or horses as compared to the broader host range of the mosquito species captured.

This study is generally in agreement with earlier studies that showed a positive relationship between numbers of insects captured and CO_2 concentration for blood feeding insect species that demonstrate a willingness to feed on a wide range of avian and mammalian hosts. Using traps baited with CO_2 (0, 250, 400, 500, and 1,000 ml/min) Carestia and Savage (1967) found that increases in CO_2 concentrations resulted in increasing capture of *An. punctipennis*, *Ae. vexans*, and *Cx. salinarius*. McIver and

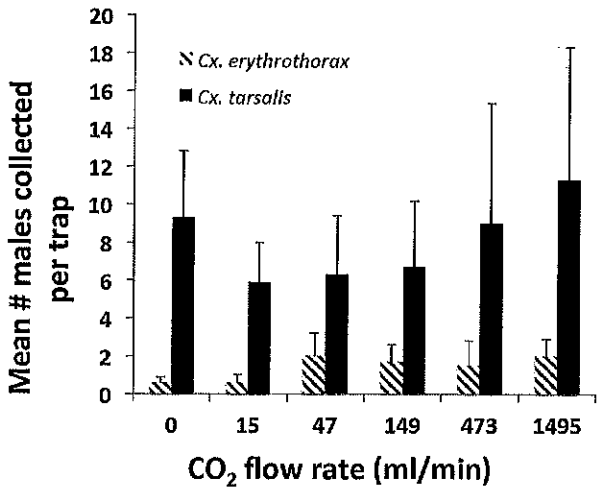


Figure 5. Mean [SE] per trap capture of male mosquitoes by CO_2 release rate.

McElligott (1989) reported a significant increase (six-fold) in *Ae. vexans* capture using ramp traps baited with 4,000 ml/min in comparison with those baited with 1,000 ml/min. Our observations for *Cx. tarsalis* are consistent with studies by Reeves (1953) and Pfuntner et al. (1988) which reported significant increases in capture of *Cx. tarsalis* when CO_2 release rates were increased from 250 to 1,000 ml/min, and with a study by Reisen et al. (2000) which reported increased capture of *Cx. tarsalis* as CO_2 release rates increased from 500 to 1,500 ml/min.

It is important to note that the mosquito species collected in the current study are all generalist feeders. Therefore, it is perhaps not surprising to see that all the species sampled in this study responded to increasing CO_2 concentrations with a similar rate of increasing capture. We had expected to capture *Cx. quinquefasciatus* at this site, given the collection of this species in an earlier study at this same location (Lura et al. 2012). It was anticipated that we might see a negative relationship between CO_2 concentration and trap captures for this species given the earlier report by Reeves (1953) and by Mullens and Gerry (1998), and the reduced feeding by this species on large mammals (horses) relative to other mosquito species captured in this region of California (Gerry et al. 2008).

While it would be expected perhaps that a species like *C. sonorensis*, which has a feeding preference for large mammals, would also have a strong positive relationship between number of insects captured and CO_2 concentration, it was nevertheless surprising how rapidly trap captures increased for *C. sonorensis* when CO_2 concentration increased from the mid-range flow rate of 149 ml/min to the highest flow rate of 1,495 ml/min relative to the more generalist feeding mosquitoes. The very large increase in trap capture of *C. sonorensis* only at higher CO_2 rates is consistent with previous studies by Mullens (1995) and Mullens and Gerry (1998), and contrasts with the slower rate of increase at the highest CO_2 flow rates for all the mosquito species captured in the current study. In this study, the increase in captures of *C. sonorensis* from the mid-range to highest CO_2 rate was 25-fold relative to the increase in mosquito captures which were three-fold increases over this same CO_2 range.

We hypothesize that higher CO_2 release rates resulted in an expanded odor plume covering a greater area compared to lower release rates. This would increase the opportunity for more distant host-seeking mosquitoes to detect and initiate orientation behaviors toward the CO_2 source. More mammalophilic or generalist species, like those captured in this study, might be expected to continue to orient toward the odor source even at the higher CO_2 concentrations that would be encountered near the trap. In contrast, ornithophilic species may be better adapted to responding to lower CO_2 release rates, more typical of small animals and birds. These ornithophilic species might be expected to be repelled by high concentrations of CO_2 or just simply fail to find the trap (CO_2 source) under the high CO_2 concentration conditions near the trap. It would be interesting to repeat this study at a site where mosquito species collected were not generalist host feeders but had a more narrow feeding preference for either birds or large mammals.

This study highlights the importance of standardizing CO_2 release rates used with traps when conducting vector surveillance. Medically important Diptera collected during this study showed

significant differences in capture rate with increasing CO_2 flow rate. Variation in CO_2 release rate might therefore impact mosquito surveillance interpretations regarding vector abundance and activity. For example, the mean trap count for *Cx. tarsalis* (an important vector of WNV) would be expected to vary by 20% or more across a range of probable CO_2 flow rates when using dry ice as the CO_2 source (1,000 to 1,495 ml/min, according to Mullens 1995). At our study site, this variation in CO_2 flow rate could have resulted in an increase of several hundred *Cx. tarsalis* captured per trap night between the low and high CO_2 flow rate within this range., The resulting increase in mosquito capture, due solely to variation in CO_2 rate, might be incorrectly interpreted as a worrisome increase in mosquito activity and pathogen transmission risk from one surveillance period to the next, when the increase in mosquito capture might in fact be due solely to variable CO_2 release among trap nights.

While mosquito capture may vary with CO_2 source (dry ice or compressed gas) (Reisen et al. 2000), we can reasonably expect that the capture rate for the mosquito and biting midge species captured in this study would similarly increase with increasing CO_2 flow rates, even if the actual rate of increase might differ somewhat among the CO_2 sources. Additional field studies comparing mosquito collections from dry ice baited traps with traps using CO_2 released from pressurized gas tanks are warranted to measure differences between these two methods of CO_2 presentation.

The typical CO_2 output of a dry ice baited suction trap falls within the range of flow rates tested in this study (Mullens 1995, Eshun et al. 2016). If dry ice is utilized with the trap, the CO_2 release rate could be estimated by the loss in dry ice weight due to sublimation of the gas during the trapping period. Even if CO_2 rates vary somewhat by trap night, collections could be adjusted based upon the direct relationship between CO_2 concentrations and mosquito capture rates. Our study suggests that if standard trap designs and suitable collection sites are utilized to minimize design and site effects (Resien et. al. 2000), the host seeking activity of species captured in this study could be effectively modeled, even with variation in CO_2 release, so that surveillance data could be more reliably used.

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MALARIA JOURNAL

RESEARCH Open Access

Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the *Anopheles gambiae* complex in Mali, West Africa

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Abstract

Background: Based on highly successful demonstrations in Israel that attractive toxic sugar bait (ATSB) methods can decimate local populations of mosquitoes, this study determined the effectiveness of ATSB methods for malaria vector control in the semi-arid Bandiagara District of Mali, West Africa.

Methods: Control and treatment sites, selected along a road that connects villages, contained man-made ponds that were the primary larval habitats of *Anopheles gambiae* and *Anopheles arabiensis*. Guava and honey melons, two local fruits shown to be attractive to *An. gambiae* s.l., were used to prepare solutions of Attractive Sugar Bait (ASB) and ATSB that additionally contained boric acid as an oral insecticide. Both included a color dye marker to facilitate determination of mosquitoes feeding on the solutions. The trial was conducted over a 38-day period, using CDC light traps to monitor mosquito populations. On day 8, ASB solution in the control site and ATSB solution in the treatment site were sprayed using a hand-pump on patches of vegetation. Samples of female mosquitoes were age-graded to determine the impact of ATSB treatment on vector longevity.

Results: Immediately after spraying ATSB in the treatment site, the relative abundance of female and male *An. gambiae* s.l. declined about 90% from pre-treatment levels and remained low. In the treatment site, most females remaining after ATSB treatment had not completed a single gonotrophic cycle, and only 6% had completed three or more gonotrophic cycles compared with 37% pre-treatment. In the control site sprayed with ASB (without toxin), the proportion of females completing three or more gonotrophic cycles increased from 28.5% pre-treatment to 47.5% post-treatment. In the control site, detection of dye marker in over half of the females and males provided direct evidence that the mosquitoes were feeding on the sprayed solutions.

Conclusion: This study in Mali shows that even a single application of ATSB can substantially decrease malaria vector population densities and longevity. It is likely that ATSB methods can be used as a new powerful tool for the control of malaria vectors, particularly since this approach is highly effective for mosquito control, technologically simple, inexpensive, and environmentally safe.

Background

One of the key challenges for successful malaria control and eventual malaria elimination in African countries is to implement highly efficient malaria vector control to reduce annual entomological inoculation rates (EIRs) to below one infective bite per person per year [1]. Such reductions are required to drive down levels of malaria prevalence to achieve local malaria elimination [2]. Currently, there are no documented examples anywhere in Africa where annual EIRs have been reduced and sustained to levels < 1 using available vector control tools [3].

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Current options for vector control tools to tackle the malaria problems in African countries are limited. Most national malaria control programmes use long-lasting insecticide-treated nets (LLINs) and/or indoor residual spraying (IRS) [4,5], and there is a growing interest in environmental management and larval control [6-8]. These proven methods can reduce malaria parasite transmission by > 90%, and correspondingly reduce the incidence of new infections and malaria-related mortality. However, they do not consistently reduce malaria prevalence because even barely detectable low numbers of infective bites per person per year can be associated with malaria prevalence rates over 20% [1]. The lack of viable new methods for vector control is one reason why integrated vector management (IVM) strategies [9,10] have not been fully embraced and implemented [11]. Clearly, new vector control tools that can be used in conjunction with current methods are required as successful malaria control programmes transition their goals to country-wide malaria elimination [5].

This paper addresses whether newly developed ATSB methods may be suitable for malaria vector control in Africa. The ATSB methods, developed and tested extensively in Israel [12-16] represent a new form of mosquito control based on an "attract and kill" principle. The ATSB approach uses fruit or flower scent as an attractant, sugar solution as a feeding stimulant, and oral toxin to kill the mosquitoes. The ATSB solutions are either sprayed on vegetation or suspended in simple bait stations, and the mosquitoes ingesting the toxic solutions are killed. As such, this method targets sugar-feeding female and male mosquitoes outdoors. Plant sugars or "sugar meals" represent an important source of energy for female mosquitoes and are the only food source for males [17,18]. Data on mosquito orientation to plant volatiles and their attraction to plant odors were recently reviewed [19]. Over a range of arid environments in Israel, field trials of ATSB methods have proven highly effective in decimating local populations of diverse mosquito species. In addition to being highly effective, technologically simple, and low-cost, the ATSB methods are based on the use of oral toxins as opposed to contact insecticides used in LLINs or IRS. As such, this new approach circumvents many of the traditional problems relating excito-repellency and the development of insecticide resistance in mosquitoes [20,21].

The objective of this study was to conduct a controlled field trial of ATSB plant-spraying methods to determine impact on malaria vector densities and longevity in a semi-arid malaria endemic area of Mali. This study represents the first evaluation of ATSB methods for malaria vector control in Africa.

Methods

Study sites

The study was conducted at the margins of the inland delta of the river Niger in Bandiagara District, approximately 650 km northeast of Bamako, Mali. In this semi-arid area, the rainy season is between July and September with a peak of malaria transmission in October. Malaria vectors include 99.8% *Anopheles gambiae* s.l., of which 86% are *An. gambiae* s.s and 14% are *Anopheles arabiensis* and *Anopheles funestus* [22]. Malaria transmission is seasonal with virtually undetectable transmission during the dry season and up to 25 infective bites per person per month during peak periods of transmission. The prevalence of *Plasmodium falciparun* infection varies from 45% during the dry season to > 65% at the end of the rainy season [23].

An area along the main road, connecting Bamako and Gao, about 50 km north of Sevare provided ideal testing conditions for an ATSB field trial, in terms of both representative local environmental conditions and relatively isolated ecological "island" settings with abundant larval habitat containing high densities of *An. gambiae* s.l. The area contains numerous clusters of three to five ponds for collecting rainwater with the ponds varying in size from 3,000 to > 10,000 square meters. They were artificially created to assist the semi-nomadic population during the dry season and are used as a water supply for local livestock and the shallow areas for rice paddies. The clusters of ponds are separated from each other by 0.5 to 3 km and are surrounded by arid vegetation. Larval surveys conducted in seven clusters of ponds along a road that interconnects local villages showed that most of the ponds contained *An. gambiae* s.l. larvae. From the seven clusters of ponds, two clusters of ponds with high densities of *An. gambiae* larvae were selected as study sites for the ATSB field trial. Each of these sites included a group of man-made reservoir ponds surrounded by partially flooded rice paddies. The experimental treatment site included six ponds which covered an area of ~3.8 ha and the distance between this group and the closest cluster of ponds was ~2.0 km. The control site included a group of four ponds covering ~1.4 ha which were at least ~0.5 km away from other groups of ponds and 15 to 20 km from the selected treatment site.

Preparation of ASB and ATSB solutions

The ASB solution included juice of ripe/overripe fruits, 30% Guava juice, 30% Honey Melon juice, 25% water, 12% brown Sugar W/V, 2% local millet beer, and 1% (W/V) BaitStab™ concentrate (Westham, Israel) for preservation and stabilization of the bait. Guava and honey melons were selected for the ASB based on their local availability and their high level of attractiveness for *An. gambiae* s.l.

based on comparative field tests in Mali using 26 different types of local fruits (unpublished data). Locally available millet beer was used to start the fermentation process. BaitStab™ is a blend of preservatives and slow-release substances used to preserve food-grade material, and was brought as the only ingredient from Israel [15]. Crushed fruits and the other components were left for two days to ferment in covered plastic buckets in the sun. The liquid, sifted by sieve and then by cloth, was stored at ambient temperature. The pulp was used for goat and chicken feed. ATSB was made by adding the toxin boric acid [24] 1% (W/V) to ASB liquid. Food dye markers of 0.5% W/V Food blue No. 1 or E122, Azorubine, (red) (Stern, Natanya, Israel) were added to ASB and ATSB. A laboratory experiment at University of Bamako confirmed that colonized *An. gambiae* females and males readily fed on the ATSB solution containing food dye marker, most within two hours, and that mortality rates of fed mosquitoes at 12 h were 99.6% (n = 259) for females (2 replicates) and 100% (n = 309) for males (2 replicates) compared to < 1% for control cages of females and males provided only ASB solution.

Field application of ASB and ATSB solutions

The ASB and ATSB solutions were sprayed with a 16-liter back-pack sprayer (Killaspray, Model 4526, Hozelock, Birmingham UK) in aliquots of ~80 ml on 1 m^2 spots at distances of ~3 m on the vegetation around the ponds and rice paddies. Predominant types of plants sprayed at the two sites included rice, sedges, grasses, and non-flowering herbaceous plants. One sprayer completed the applications in less than two hours per site.

Study design and methods for the ATSB field trial

The field trial was conducted over a period of 38 days, at the end of the malaria transmission season, beginning in mid-November 2008. During this period, adult mosquitoes were sampled a total of 20 times at each site using 6 CDC light traps (Model 512, John W. Hock, Gainesville, FL) without attractants in fixed positions between the ponds. Bait solutions were sprayed on day 8 of the experiment, ASB at the control site and ATSB at the experimental site. The designation of control and experimental treatment sites was done just prior to day 8 based on CDC light trap data showing higher densities of *An. gambiae* s.l. at the experimental treatment site. Collected mosquitoes were sexed and checked for food dye marker using a dissection microscope [13]. They were then preserved in 70% ethanol for species identification by classical taxonomic methods [25] and by PCR to identify species in the *An. gambiae* complex [26]. The physiological age of female mosquitoes was determined by dissecting ovaries and counting the number of dilatations [27]. From the control and treatment sites, live female *An. gambiae* s.l. were randomly selected for age-grading from collections on days 4 and 6 pre-treatment and days 24, 26 and 28 post-treatment.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 4.0 (GraphPad Software Inc., Calif). Comparisons between light trap catches of female and male *An. gambiae* s.l. at the control and treatment sites were performed using the two-tailed Student's t test. The Z-test was used to compare proportions of female *An. gambiae* s.l. at control and treatment sites that completed more than three gonotrophic cycles. This assessment of "older" females is relevant because gonotrophic cycles of *An. gambiae* are generally three days and the first sporozoites of *P. falciparum* are normally observed in females developing their fourth batch of eggs [28,29].

Results

PCR analysis showed that two species of the *An. gambiae* complex inhabited the study sites, *An. gambiae* and *An. arabiensis*. *Anopheles gambiae* predominated, and just prior to spraying ASB and ATSB solutions comprised 76.6% (n = 47) and 81.5% (n = 52) of the population in the control and treatment sites, respectively. Post-treatment, the proportion of *An. gambiae* was 81.5% (n = 52) and 100% (n = 54) in the control and treatment sites, respectively.

ATSB treatment reduced densities of female and male *An. gambiae* s.l. by about 90%. After spraying ATSB in the treatment site, population densities of female and male *An. gambiae* s.l. declined rapidly over a week and then stabilized at low levels (Figure 1). The pre-treatment of catch 184.6 ± 15.7 females and 55.9 ± 4.7 males per trap decreased to 26.4 ± 3.80 females and 7.35 ± 1.23 males in the last 22 days of the experiment. The control site population was relatively stable yielding pre-treatment levels of 101.6 ± 9.3 females and 55.9 ± 4.66 males per trap, and 118.83 ± 5.82 females and 54.0 ± 3.24 males in the last 22 day of the experiment. A decrease in numbers of mosquitoes collected post-treatment at the ATSB treatment site, when compared with the control site, was highly significant for both females ($t = 8.747$, $df = 13$; $p < 0.0001$) and males ($t = 11.91$, $df = 13$; $p < 0.0001$).

ATSB treatment correspondingly affected the longevity of female *An. gambiae* s.l. as shown in table 1, which summarizes results from the classification of females by age-grading. At the experimental site, most females remaining after ATSB treatment had not completed a single gonotrophic cycle, and only 6% had completed more than three gonotrophic cycles compared with 37% pre-treatment. During the same period at the control site sprayed with ASB (without toxin), the proportion of females completing more than three gonotrophic cycles increased from 28.5% pre-treatment to 47.5% post-treatment.

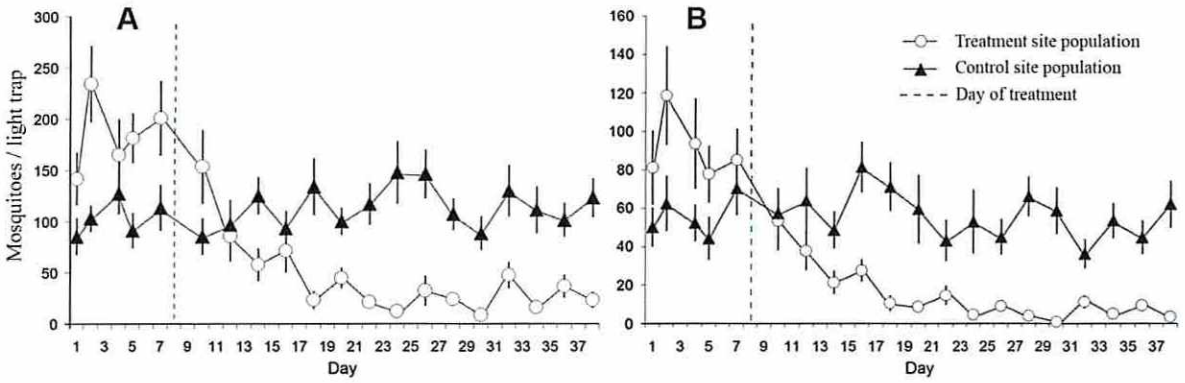

Figure 1 Relative abundance of *Anopheles gambiae* s. l. females (A) and males (B) in the ATSB-sprayed experimental treatment site and the ASB-sprayed control site, determined by CDC light trap sampling during the 38-day field trial in Bandiagara District, Mali.

At the control site, a high proportion of the *An. gambiae* s.l. females and males fed on the ASB solution sprayed on plants. The proportion of mosquitoes showing evidence of the coloured food dye included in the ASB solution included 56.4% of the females (n = 10,250) and 62.2% of the males (n = 5,071). Even on the last day of collection, 71.1% of the females (n = 741) and 75.1 of the males (n = 373) were marked. A much lower proportion of the mosquitoes contained dye marker at the ATSB treatment site, 3.9% of the females (n = 3,952) and 3.2% of the males (n = 1,325).

Discussion

The results of this field trial in Mali show that under local conditions a single application of ATSB solution by plant-spraying markedly reduced the relative abundance of *An. gambiae* s.l. populations and their longevity. Within a week after spraying, densities of adult females and males at the treatment site were reduced by around 90% and remained low throughout the remainder of the monitoring period. Clearly, the ATSB treatment was highly effective in killing the "older" more dangerous females as shown in table 1. Reducing the proportion of "older" females is a key factor in reducing malaria transmission [30]. The pronounced impact of the ATSB is comparable to that demonstrated in ATSB field trials in Israel [12-16] and establishes that this method for mosquito control is also highly effective for targeting and killing major malaria vectors in semi-arid areas of Africa.

By using a dye marker in the ASB solution applied at the control site, as in previous studies in Israel [12-16], we demonstrated that a high proportion of the local *An. gambiae* s.l. populations were making contact with and feeding on the solution sprayed on local plants. The observed marking rates for females (56.4%) and males (62.2%), however, represent only minimal rates of contact

Table 1: Age-group classification of *Anopheles gambiae* s.l. females before and after a single application of ASB (control) or ATSB (experimental treatment) on local vegetation

Site and time	Females examined	% females by observed numbers of dilatations in dissections of ovaries								
		0	1	2	3	4	5	6	7	>8
Control pre-treatment	200	25.5	23.5	14.5	8	7	4.5	4	3.5	9.5
Control post-treatment	200	12.5	14	16	10	10.5	6.5	9	7	14.5
Experimental pre-treatment	200	23	16.5	12.5	11	8.5	5.5	6	5.5	11.5
Experimental post- treatment	200	52	24.5	9.5	8	3	1.5	0.5	0	1

The difference between the proportion of females with more than 3 gonotropic cycles in the experimental site before and after treatment was significant ($z = 7.185$; $p < 0.05$).

as the dye marker persists for only about two days due to digestion processes while the mosquitoes can sugar-feed throughout their lifespan. The finding of marked mosquitoes on the last day of collection highlights that the sprayed ASB solution was still present at the very end of the trial. The low percentage (< 5%) of mosquitoes caught with colored ATSB from the treatment site indicates that a high percentage died before again flying where they could be caught, with the possibility that some may have exhibited behavioral changes after feeding on the bait that would have altered their probability of capture [31].

The results demonstrate how ATSB is effective when applied to various types of vegetation located in the vicinity of local mosquito populations, including that which exists around natural larval habitats and is likely used by both newly-emerged and older mosquitoes as outdoor resting sites. This approach is similar to the most recent studies in Israel [16,32] but differs from initial studies of ATSB plant-spraying in Israel where ATSB was selectively sprayed on flowering plants known to be highly attractive to mosquitoes as sugar sources [12,13]. The two approaches are both highly effective and potentially complimentary but the method used in this study and a recent study in Israel [32] is technically simpler as it does not require *a priori* knowledge the most attractive plants. It only requires some basic skills in identifying larval habitats and general types of vegetation that may be used by mosquitoes as outdoor resting sites [32].

The preparation of ATSB solution is technically quite simple. Four of the key ingredients are readily available at the local community level: water, unrefined brown sugar, beer, and ripe/overripe fruit. While initial studies in Israel used nectarines [14,15] and plums in Florida [33], guava and honey melons were used instead based on local availability at the time of our studies and on our comparative tests of the attraction of *An. gambiae* s.l. to various local fruits and seed pods in Mali (unpublished). Even fruits that are close to rotting and are therefore unsuitable for trade and human consumption can be used, and leftover products can be used to feed domestic animals and fowl. As the chemical identity of the attractive ingredients in the fruits has not been determined, at this point it is not possible to substitute a synthesized chemical attractant. Two of the ingredients must be purchased, the BaitStab™ for preservation and stabilization, and the oral toxin, but both are very inexpensive. At the study area in Mali, the boric acid was purchased at the local market.

Rather than using Spinosad ("Tracer™"; Dow Agrosciences, Calgary, Canada) as the oral toxin as in the proof-of-concept studies in Israel [14,15], we instead used boric acid, which is highly lethal to mosquitoes [24,31]. Preliminary laboratory testing in Bamako confirmed high toxicity to *An. gambiae*. The advantage of using boric acid is that it is very inexpensive, readily available, is stable (in contrast to Spinosad which decays by UV), and has a mammalian toxicity level about as low as table salt [34]. The boric acid proved highly effective in our initial field trial. This is not surprising because boric acid and a number of different insecticides have been used for many years as oral toxin for the control of ants, cockroaches, fruit flies, and house flies. Studies by Allan [35] have recently shown that, when delivered orally, a wide variety of different insecticides are effective against mosquitoes, with apparently no repellency effects. The study concluded that baits with oral toxins for mosquitoes using a phagostimulant, such as sucrose, are effective in causing mortality [35]. Longer-term, operational strategies using ATSB solutions with mixtures of 2 or more different insecticides may help minimize the emergence of resistance in local populations of mosquitoes, which is of course already a concern for the insecticides associated with LLIN and IRS use for malaria vector control in Africa [36,37].

This first field trial of ATSB methods in Mali begins to explore some of the ultimate impacts of the ATSB approach for malaria vector control in Africa. In addition to the ATSB plant-spraying methods tested here, it also might be possible to deliver the same ATSB solution using very simple bait stations that have proven successful in Israel [14,15]. Ultimately, we expect that strategies will emerge for co-use of both plant-spraying and bait stations to achieve maximal killing of local vector populations. As the malaria vectors in Africa, *An. gambiae*, *An. funestus* and to a lesser degree *An. arabiensis*, show a pronounced tendency to rest inside houses where they feed on humans [38], it may also be possible to use ATSB methods directly outside or inside houses. Though there were indications of a differential impact on *An. arabiensis* in this trial (i.e., none remained after ATSB treatment), the numbers identified by PCR were too small to detail with certainty that the ATSB treatment had a more pronounced impact on this malaria vector which is well-known to be more exophilic than *An. gambiae*.

Beyond this initial field trial, the full impacts of ATSB need to be determined by field assessments on a larger scale and of longer duration at the village and/or district levels with designs that measure impact not only on vector densities and vector longevity, but also measure malaria parasite transmission (e.g., EIRs), and malaria burden in human populations (e.g., incidence and prevalence of malaria cases). It is also important to determine the additive effects of ATSB when used in combination with existing vector control methods including LLINs and IRS, as it is not likely that ATSB methods alone would be sufficient to meet programmatic goals for malaria vector control. It is also important to determine the full impact on all mosquito species, not just the malaria vectors. In Mali, for example, *Culex quinquefasciatus* is

locally abundant and serves as a major nuisance-biting mosquito and a vector of filariasis in some areas [39].

The range of environments in Africa where ATSB methods can be used effectively remains to be determined. They will likely work best in arid and semi-arid areas where natural flowering plants are limited, as the effectiveness of the ATSB methods depends on their ability to outcompete natural plant sources of sugar available to mosquito populations [40]. Though this first field trial was conducted in a semi-arid area of Mali, the actual study sites containing multiple ponds that were highly productive larval habitats surrounded by both natural vegetation and rice paddies were, in general, fairly typical of *An. gambiae* s.l. habitats over a range of environments found in malaria endemic areas of Africa. The ATSB methods may also work well in urban setting and in environmentally altered environments that lack biologically diverse groups of indigenous flowering plants that would naturally sustain mosquito populations. They may also be effective for use in large-scale irrigation areas where rice, for example, is cultivated (rice plants apparently do not provide a source of sucrose for mosquitoes).

There are three further considerations worth noting. First, to optimize performance of ATSB plant-spraying and bait station methods, there is a need to determine the coverage of plant spraying needed and also the density of bait stations needed to achieve effective control. In this first field trial in Mali, spraying just a series of 1 m^2 spots of vegetation every 3 m around breeding sites was apparently sufficient. Second, a logistical consideration is that heavy rains will wash off ATSB sprayed on plants and so re-applications during the rainy seasons may be needed. This is one reason why bait stations are equipped with covers [14,15]. During some periods of the year it may be feasible to use both plant-spraying and bait stations but this will depend on local circumstances. Third, ATSB approaches have only minimal risks to humans. Ongoing studies in Israel are determining potential impacts of ATSB on non-target insects. Strategies for spraying ATSB on non-flowering plants may be better than spraying the most attractive flowering plants, in terms of minimizing damage to non-target insects. Honey bees or any of the many species of pollinating bees may be affected and so in Israel suitable metal grids for bait stations have been developed that allow mosquitoes to pass but keep honey bees out (G. Müller and Y. Schlein, unpublished data). Overall, spraying non-flowering vegetation seems to be environmentally safe, except that non-biting midges (Diptera: Chironomidae) feed in similar proportions to the mosquitoes. The ATSB methods may pose only limited environmental risks for the following reasons: 1) whole classes of pollinating insects orient using optical targets rather than scents, 2) beneficial predatory insects will not be harmed because they do not feed on sugar, and 3) pollinating insects are typically absent from some of the prime target areas for ATSB treatment, such as rice fields and areas around mosquito larval habitats where there is minimal flowering vegetation.

In conclusion, this first field trial of ATSB methods in Mali provides a strong indication that such strategies will be very effective for malaria vector control in Africa. If tested further and found to be effective across a range of malaria endemic environments in Africa, it is likely that ATSB approaches could soon be added as a major component of IVM-based malaria vector control programmes. ATSB methods differ from and potentially complement LLIN and IRS methods, which focus on indoor-feeding and resting mosquitoes, because they have so far proven effective in outdoor habitats for killing all physiological states of females and at the same time also kill male mosquitoes. By targeting sugar-feeding mosquitoes in outdoor environments it is likely that their use will on the micro-scale overlap significantly in space with other key life history strategies of malaria vectors including mating and oviposition, both of which are temporally associated with sugar-feeding. Thus, in terms of malaria vector control in Africa, the ATSB methods when used operationally will likely reduce both total numbers of recently emerged female anophelines before they enter houses to feed on humans and the proportion of females exiting houses to oviposit and then returning to houses to re-feed on humans. This strategy to broaden the currently narrow segments of vector populations targeted for control (i.e., those females that feed and rest indoors) is certainly consistent with the broad-based IVM concepts being promoted and implemented throughout Africa.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GM, YS and JB conceived and planned the study, interpreted results, and wrote the paper. GM directed and performed the field experiments, and analyzed the data. ST and SD facilitated field experiments by selecting study sites and obtaining local clearance from community leaders, and along with MTo, MTr, assisted with the field and laboratory experiments, and data management. All authors read and approved the final manuscript.

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Field experiments of *Anopheles gambiae* attraction to local fruits/seedpods and flowering plants in Mali to optimize strategies for malaria vector control in Africa using attractive toxic sugar bait methods

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Abstract

Background: Based on recent studies in Israel demonstrating that attractive toxic sugar bait (ATSB) methods can be used to decimate local anopheline and culicine mosquito populations, an important consideration is whether the same methods can be adapted and improved to attract and kill malaria vectors in Africa. The ATSB approach uses fruit or flower scent as an attractant, sugar solution as a feeding stimulant, and an oral toxin. The ATSB solutions are either sprayed on vegetation or suspended in simple bait stations, and the mosquitoes ingesting the toxic solutions are killed. As such, this approach targets sugar-feeding female and male mosquitoes. This study examines the attractiveness of African malaria vectors to local fruits/seedpods and flowering plants, key biological elements of the ATSB approach for mosquito control.

Methods: Three field experiments were conducted at sites in Mali. The attraction of *Anopheles gambiae* s.l. to 26 different local fruits and seedpods was determined at a site in the semi-arid Bandiagara District of Mali. Wire mesh glue traps with fruits/seedpods suspended on skewers inside were set along a seasonal lagoon. Seven replicates of each fruit/seedpod species were tested, with a water-soaked sponge and a sugar-soaked sponge as controls. The attraction of *An. gambiae* s.l. to 26 different types of flowering plants was determined at a site near Mopti in Mali. The flowering plants held in a water-filled buried container were tested using the same glue traps, with controls including water only and sugar solution. Six replicates of each selected plant type were tested on transects between rice paddies. Additional studies using CDC light traps were done to determine the relative densities and periodicity of *An. gambiae* s.l. attraction to branches of the most highly attractive flowering plant, branches without flowers, human odor, and candescent light.

Results: Of the 26 fruits and seedpods tested, 6 were attractive to *An. gambiae* s.l. females and males, respectively. Guava (*Psidium guajava*) and honey melon (*Cucumis melo*) were the two most attractive fruits for both females and males. Of the 26 flowering plants tested, 9 were significantly attractive for females, and 8 were attractive for males. *Acacia macrostachya* was the most attractive flowering plant. Periodicity studies using this plant showed peaks of *An. gambiae* s.l. attraction between 1930 and 2200 h and 0400-0500 h, which differed considerably from the response to human odors, which expectedly peaked at around midnight.

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Conclusion: These field experiments in Mali highlight that female and male *An. gambiae* s.l. have pronounced differences in attraction for diverse types of indigenous fruits/seedpods and flowering plants. The identification of attractive fruits and seedpods shows that a variety of indigenous and locally abundant natural products could potentially be used as juices to make ATSB solution for mosquito control. As well, the simple methods used to identify the most attractive flowering plants provide valuable insights into the natural history of sugar feeding for *An. gambiae* s.l. These observations can be used to guide future strategies for employing ATSB methods for malaria vector control in Africa. They also provide a basis for subsequent chemical analysis and development of attractive baits for mosquito control.

Background

In Mali, the most important malaria vectors are *Anopheles gambiae sensu stricto* and *Anopheles arabiensis* [1], vectors that are important across Africa [2]. These vectors are currently being targeted for malaria vector control by long-lasting insecticide-treated bed nets (LLIN) disseminated throughout Mali and also indoor residual spraying (IRS) in select areas of the country. Investigators in Mali are also searching for additional vector control methods that could eventually be used in conjunction with LLINs and IRS as part of integrated vector management (IVM) [3-5].

Highly successful ATSB methods for mosquito control developed and extensively field-tested in Israel [6-10] are now being evaluated in Mali. An initial field trial in Mali showed that ATSB plant-spraying methods could reduce female and male *An. gambiae* s.l. populations by around 90% and reduce the proportion of older females by about 8-fold [11]. As part of the ATSB optimization process, it is necessary to identify the most attractive fruits/seedpods to make the ATSB solution for general application as bait stations [8,9] or spraying on plants surrounding breeding sites or human habitation [10-12]. It is also useful to identify the most attractive flowering plants that could be selectively sprayed to reduce mosquito populations, as has been detailed in Israel [7].

Under prevailing local conditions in Africa, there is a scarcity of information on the attraction of *An. gambiae* complex species to fruit/seedpods and flowering plants. There have been some elegant mosquito-plant relation studies in western Kenya [13-15]. However, similar studies or research that employs the field techniques for studying sugar-feeding pioneered in Israel [6-10,12] have not been conducted in Mali or anywhere in West Africa. The identification of the most attractive fruits/seedpods and flowering plants is needed to better understand the biology of mosquito-plant relations. This is highly critical for strategies to adapt, improve, and optimize recently developed ATSB strategies for malaria vector control in Africa.

The objectives of this study in Mali were to determine the fruits/seedpods and flowering plants that *An. gambiae* s.l. are attracted to and the nocturnal periodicity of sugar-feeding on flowering plants by the main malaria vectors. The approach and results provide valuable information to further guide the employment of ATSB methods for malaria vector control in Mali and elsewhere in Africa [11].

Methods

Study sites

The studies were conducted in the Inner Niger Delta, a large area of lakes and floodplains in Central Mali in the middle of the otherwise arid Sahel. During the wet season, the swamps and lakes are flooded naturally irrigating the nearby land. The rainy season usually begins in July and lasts until mid-October in the south, and from mid-July to mid-September in the north. The rainy season with annual precipitation of about 600 mm is between July and September with a peak of malaria transmission in October. Previous studies have shown that malaria vectors include 99.8% *Anopheles gambiae* s.l., of which 86% are *An. gambiae* s.s and 14% are *An. arabiensis*, and *Anopheles funestus* [16]. Malaria transmission is seasonal with virtually undetectable transmission during the dry season and up to 25 infective bites per person per month during peak periods of transmission. The prevalence of *Plasmodium falciparun* infection varies from 45% during the dry season to >65% at the end of the rainy season [17].

The study of *An. gambiae* s.l. attraction to fruits/seedpods was conducted in Bandiagara district, approximately 650 km northeast of Bamako, at the eastern outskirts of the delta. The experimental site was approximately 50 km north of Sevare near a seasonal lagoon. The lagoon was about 4 km long and surrounded by wooded grassland dominated by *Acacia* species.

The study of *An. gambiae* s.l. attraction to flowering plants and the periodicity studies were conducted near a small village 3 km north of Mopti at the confluence of the Niger and Bani River. The experimental site was on a small natural island linked by a dyke to the town. The western side of this peninsula is bordered by the Niger and the eastern-side rice fields extend several kilometers. Experiments for attraction to flowers were

conducted along a small road crossing the rice fields toward the east (in a 90 degree angle to the village) with the trap line starting about 600 m from the village. *An. gambiae* s.l. was breeding in large numbers in these rice fields. The studies of diel rhythm of activity were conducted nearby along the edge of the island near the rice fields at a distance of about 300 m and parallel to the village.

Attraction to fruit/seedpods

The attractiveness of 26 different types of fruits and seedpods, identified to genus and species (Faculty of Medicine, Pharmacy and Odontostomatology, University of Bamako, Bamako, Mali), was determined using a specially designed glue trap shown in Figure 1. It was constructed as follows: 1.5 L plastic bottles were cut in half, buried in the ground, and filled up with water. The soil around the bottle was compressed and watered to consolidate. Plastic nets, 70 × 70 cm, (0.8 cm square holes, 0.2 cm wide netting) were rolled to cylinders and fixed by plastic tie wraps, the top was closed with the same material. The net-cylinder was placed on the buried bottle and fixed with 20 cm long wooden stakes to the ground. In each cylinder we placed about 0.5 kg of ripe fruit cut to pieces and arranged on wooden stakes (40 cm in length) and then painted the cylinders with glue (Tangle Foot, Tel Aviv, Israel). The experiments were conducted over 10 consecutive days in November 2008. A total of 30 traps, 20 m apart, were placed along a lagoon. Every night two types of controls (two from each type: glue trap baited with a water-soaked sponge and glue trap baited with sugar-soaked sponge; 20 replicates of each type during the experiment) and 28 fruit-baited traps were employed (with two to three samples of the same fruit species per night totaling seven replicates). Mosquitoes caught on the glue traps were counted and samples were stored in 70% ethanol for species identification according to morphology [18] and by PCR [19]. On each test day the cylinders were repainted with glue to eliminate mosquitoes, other small insects, and dirt. The water as well as the fruits and seedpods were changed daily.

Of the 26 tested fruit, the following were purchased from the market in Sevare and were locally produced: Guava, *Psidium guajava* (Myrtaceae); honey melon, *Cucumis melo* (Cucurbitaceae); papaya, *Carica papaya* (Caricaceae); dates, *Phœnix dactylifera* (Arecaceae); sugar cane *Saccharum officinarum* (Poaceae); dwarf banana, *Musa acuminata* (Musaceae). The following fruit originated from southern Mali or were imported: orange, *Citrus sinensis*, and bitter orange, *Citrus aurantium* (Rutaceae); pineapple, *Ananas comosus* (Bromeliaceae); cooking banana, *Musa paeadisiaca* (Musaceae); water melon, *Citrillus lanatus* (Cucurbitaceae); apple *Malus domestica* (Rosaceae). The following edible fruit and seedpods were collected by the Mali team on wild trees in the delta: *Ziziphus mauritiana* (Rhamnaceae); *Balanites aegyptiaca* (Zygophyllaceae); *Diospyros*

Figure 1 Picture of the type of glue trap used for testing mosquito attraction to local fruits/seedpods and flowering plants in Mali. The three pictures show how traps are mounted, how flowering plants are inserted, and how the outside of the trap is painted with glue.

mespiliformis (Ebenaceae); *Grewia bicolor* (Tiliaceae); bush melon, *Citrullus colocynthis* (Cucurbitaceae); African fig, *Ficus thonningii* (Moraceae); tamarind, *Tamarindus indica* (Fabaceae); *Piliostigma reticulatum* (Fabaceae); *Acacia albida* (Fabaceae); the following seedpods are not used for human consumption but for animal fodder: *Acacia sieberiana, Acacia nilotica, Acacia seyal, Acacia macrostachya* (Fabaceae) and the fruits of *Solanum vescum* (Solanaceae).

Attraction to flowering plants

The attraction of *An. gambiae* s.l. to 26 different types of flowering plants was determined using the glue trap described above and shown in Figure 1. In each cylinder about 0.5 kg of fresh cut flowers (40-80 cm in length) were placed, then the cylinders were painted with glue (Tangle Foot, Tel Aviv). The experiments were conducted over seven consecutive days in late October 2008. A total of 30 traps, separated by 20 m, were placed along a road between rice fields. Every night two controls (two from each type: glue trap baited with a water-soaked sponge and glue trap baited with sugar-soaked sponge; each, totaling 14 replicates) and 28 baited traps were employed (with two to three samples of the same plant species per night totaling six replicates). In the morning the glue traps were picked clear with forceps, mosquitoes were counted and sexed, samples were stored in ethyl alcohol. The *An. gambiae* s.l. mosquitoes were identified morphologically [18] and a portion tested by standard PCR methods [19]. To remove dirt and small non-target species the traps were repainted with glue every afternoon.

The 26 flowering plant species were collected in a radius of about 20 km around the test site. The plants were: *Ziziphus mauritiana* (Rhamnaceae); *Acacia albida, Acacia nilotica, Acacia macrostachya* (Fabaceae); *Acacia senegal*; *Crotalaria* sp.; *Parkinsonia acculeata* (Fabaceae); *Leptadenia pyrotechnica* (Apocynaceae); *Boscia angustifolia* (Capparaceae); *Guiera senegalensis* (Combretaceae); *Gynandropsis gynandra* (Capparidaceae); *Mitracarpus scaber* (Rubiaceae); *Cassia tora, Cassia occidentalis, Cassia siamea* (Leguminosae); *Sesamum indicum* (Pedaliaceae); *Striga hermontheca* (Scrophulariaceae); *Rogeria adenophylla* (Pedaliaceae); *Monechma ciliatum* (Acanthaceae); *Hybiscus sabdarifa* (Malvaceae); *Calotropis procera* (Asclepideaceae); *Indigofera astragalina* (Papilionaceae); *Hyptis suaveolens* (Lamiaceae); *Ricinus communis* , *Croton zambesicus* (Euphorbiaceae); *Jacquemontia tamnifolia* (Convolvulaceae).

Nocturnal periodicity of attraction

Sixteen miniature CDC light traps (Model 512, John W. Hock, Gainesville, Florida) hung on tripods at a height of about 1 m were set up in a transect along rice fields at distances of 20 m. They were baited alternately with flowering branches, control branches without flowers, human scent and light. The plant traps were baited each with three bundles of 70 cm long *Acacia macrostachya* branches, weighing approximately 1.5 kg, with their cut ends in beakers of water surrounding the trap. This plant was selected because it was the most attractive plant in the above-described attraction to flowering plants study. The human scent traps were baited with two pairs of socks (worn by the participants of the study for 24 hr) which were strapped with rubber bands around the body of the traps. The light baited traps were equipped with a single standard incandescent light bulb (standard CM-47 bulb) while the other traps were operated without light. To cover the time of mosquito activity, traps were operated from 1 hr before sunset (1730 hr) to 1 hr after sunrise (0630 hr), and the catch was recovered every half hour. For this, net bags were exchanged swiftly every thirty minutes. The mosquitoes in the recovered bags were anesthetized with ethyl acetate and packed in separate labeled vials with ethyl alcohol and identified as described above. The study was done over two consecutive days in late October 2008 and involved 8 replicates per type of bait.

Results

Determination of *Anopheles gambiae* complex

PCR testing of *An. gambiae* s.l. mosquitoes collected during the studies demonstrated that the species composition at the Bandiagara site (fruits/seedpod study) included 85% *An. gambiae* (n = 55) and 15% *An. arabiensis* (n = 10). At the Mopti site (flowering plant study), the species composition included 97% *An. gambiae* (n = 83) and 3% *An. arabiensis* (n = 3).

Attraction to fruits/seedpods

Table 1 summarizes the attractiveness of fruits and seedpods for female and male *An. gambiae*. For females, five of 26 fruits and seedpods were significantly attractive. These included: *P. guajava, C. melo, P. reticulatum, F. thonningii*, and *S. officinarum*. The males were also significantly attracted to the first four that proved attractive to females, plus they were attracted to *A. albida*. The maximum attractiveness index (Table 1), 5.83 for females and 5.63 for males, was for *P. guajava*. Pictures of these most attractive fruits and seedpods are shown in Figure 2.

Attraction to flowering plants

Table 2 summarizes the attractiveness of flowering plants to female and male *An. gambiae* s.l. For females, nine of 26 flowering plants were significantly attractive. These included: *A. macrostachya, A. albida, B. angustifolia, Z. mauritiana, G. senegalensis, A. senegal, A. nilotica, L. pyrotechnica*, and *Crotalaria sp*. The males were

Table 1 Mean number of *An. gambiae* s.l. (± SE) females and males caught in seven replicates using fruits and seedpods of 26 plant species as attractants

	Females			Males		
Plant species	Mean ± SE	P	Attraction index[a]	Mean ± SE	P	Attraction index[a]
P. guajava	14.00 ± 2.52	< 0.01	5.83	9.00 ± 2.16	< 0.01	5.63
C. melo	8.00 ± 1.60	< 0.01	3.33	5.57 ± 1.27	< 0.05	3.48
P. reticulatum	7.43 ± 1.71	< 0.05	3.10	5.71 ± 1.43	< 0.05	3.57
F. thonningii	6.29 ± 1.05	< 0.05	2.62	4.14 ± 1.12	< 0.05	2.59
D. mespipiformis	6.00 ± 1.62	NS	2.50	3.43 ± 0.78	NS	2.14
B. aegyptiaca	5.86 ± 1.48	NS	2.44	3.86 ± 0.98	NS	2.41
T. indica	5.71 ± 1.29	NS	2.38	4.00 ± 1.00	NS	2.5
S. officinarum	5.57 ± 0.81	< 0.05	2.33	3.71 ± 0.87	NS	2.32
A. albida	5.00 ± 0.97	NS	2.08	4.71 ± 1.54	< 0.05	2.94
Z. mauritiana	4.57 ± 0.91	NS	1.90	3.29 ± 0.56	NS	2.06
C. papaya	3.71 ± 0.90	NS	1.55	3.14 ± 0.72	NS	1.96
G. bicolor	3.29 ± 0.81	NS	1.37	2.14 ± 0.50	NS	0.86
C. colocynthis	3.14 ± 0.76	NS	1.31	2.00 ± 0.53	NS	1.25
A. macrostachya	3.00 ± 0.78	NS	1.25	1.86 ± 0.55	NS	1.16
A. sieberiana	3.00 ± 0.62	NS	1.25	1.43 ± 0.46	NS	0.89
A. comosus	2.86 ± 0.55	NS	1.19	1.57 ± 0.40	NS	0.98
P. dactylifera	2.86 ± 0.44	NS	1.19	1.43 ± 0.66	NS	0.89
A. nilotica	2.71 ± 1.07	NS	1.13	2.29 ± 0.45	NS	1.43
M. paeadisiaca	2.57 ± 1.13	NS	1.07	1.71 ± 0.66	NS	1.07
C. sinensis	2.57 ± 1.13	NS	1.07	1.29 ± 0.39	NS	0.81
S. vescum	2.57 ± 0.81	NS	1.07	1.14 ± 0.44	NS	0.71
M. acuminata	2.43 ± 0.62	NS	0.94	1.71 ± 0.56	NS	1.07
M. domestica	2.29 ± 0.70	NS	0.95	1.86 ± 0.76	NS	1.16
A. seyal	2.29 ± 0.77	NS	0.95	1.43 ± 0.52	NS	0.89
C. lanatus	2.14 ± 0.80	NS	0.89	1.57 ± 0.62	NS	0.98
C. aurantium	2.14 ± 0.50	NS	0.89	1.14 ± 0.50	NS	0.71
Control						
water	2.40 ± 0.61	-	1.00	1.60 ± 0.52	-	1.00
sugar solution	2.75 ± 0.67	= 0.47	1.15	1.50 ± 0.55	= 0.84	0.94

[a] Attraction index: mean baited/mean baited by water-soaked sponge (control)
NS-not significantly different from water-soaked sponge mean catch (t-test).

also attracted to the first 8 flowering plants that were attractive to females. The highest attraction index, 19.24 for females and 14.76 for males, was for *A. macrostachya*. There was only one flowering plant that was significantly repellent for females and males, *H. suaveolens*. Pictures of the most attractive flowering plants are shown in Figure 3. A picture of the only repellent plant (*H. suaveolens*) determined in this study is shown in Figure 4.

Periodicity of *An. gambiae* s.l. activity

Figure 5 shows the periodicity of attraction for female *An. gambiae* s.l. to flowering plants, human-worn socks, and light from CDC traps, and the attraction of males to flowers and light from CDC traps. Light baited traps caught an average per night of 117 *An. gambiae* females (43.1 males) while flower baited, green plant, and human scent baited traps caught an average of 31.0 females (22.5 males), 1.38 females (1.0 males) and 37.9 females (0.75 males) respectively. Attraction of females to flowers peaked in the early evening and early morning. Attraction to human scent peaked around midnight. For males, there was also an early evening and early morning periods of attraction to flowering plants.

Discussion

The results of these studies show how *An. gambiae* s.l. are attracted to many types of fruits/seedpods and flowering plants in the natural environment of Mali. The high variation in their attraction highlights how available fruits/seedpods and flowering plants vary in their overall quality for these malaria vectors. Given the nature of *An. gambiae* s.l. to seek natural sugar sources for their survival [20,21], the plant-feeding choices these malaria

Figure 2 Pictures of the most attractive fruits/seedpods determined for *Anopheles gambiae* s.l. in Mali.

vectors make are apparently influenced very strongly by the diversity, abundance, and seasonal timing of attractive flowering plants and their products.

The attraction periodicity studies on the most attractive flowering plant provide interesting evidence on the timing of female and male *An. gambiae* s.l. sugar-feeding in nature. There were no major differences in sugar-feeding periodicity between female and male *An. gambiae* s.l. Clearly, there were pronounced early evening and early morning peaks of activity. This is likely due to behavioural patterns of mosquito attraction but could also be related to the interactions between mosquito behaviour and the timing of volatile release by plants. Not unexpectedly, the periodicity of sugar-feeding differed from the timing of catches in light traps and the use of human odor as bait. This is the first study of the *An. gambiae* s.l. sugar-feeding periodicity in Africa.

The methods employed using the glue trap proved highly successful for the African environment. In a very short time, a matter of days, it was possible to identify the variety of fruits/seedpods and flowering plants that were significantly attractive to *An. gambiae* s.l. The methods employed were simple and yielded rigorous data for determination of preferences in nature. Thus,

Table 2 Mean number of *An. gambiae* s.l. (± SE) females and males caught in six replicates using 26 flowering plant species as attractants.

	Females			Males		
Plant species	Mean ± SE	P	Attraction index[a]	Mean ± SE	P	Attraction index[a]
A. macrostachya	105.83 ± 16.12	< 0.01	19.24	41.33 ± 9.18	< 0.01	14.76
A. albida	76.83 ± 12.35	< 0.01	13.97	27.83 ± 7.30	< 0.05	9.94
B. angustifolia	64.00 ± 9.69	< 0.01	11.64	34.67 ± 6.88	< 0.01	12.38
Z. mauritiana	51.17 ± 12.64	< 0.05	9.30	22.33 ± 7.37	< 0.05	7.98
G. senegalensis	36.67 ± 8.29	< 0.05	6.67	18.83 ± 4.32	< 0.05	6.73
A. senegal	23.67 ± 4.16	< 0.05	4.30	13.17 ± 2.73	< 0.05	4.70
A. nilotica	19.83 ± 4.41	< 0.05	3.61	11.83 ± 2.42	< 0.05	4.23
L. pyrotechnica	16.17 ± 4.09	< 0.05	2.94	12.50 ± 2.91	< 0.05	4.46
Crotalaria sp.	9.17 ± 1.74	< 0.05	1.67	8.67 ± 2.57	NS	3.10
G. gynandra	8.50 ± 1.61	NS	1.55	5.33 ± 1.28	NS	1.90
M. scaber	7.83 ± 2.00	NS	1.42	3.83 ± 0.40	NS	1.37
C. tora	7.00 ± 1.24	NS	1.27	3.5 ± 0.72	NS	1.25
R. communis	6.50 ± 1.71	NS	1.18	3.67 ± 1.43	NS	1.31
C. occidentalis	6.33 ± 1.28	NS	1.15	4.00 ± 0.63	NS	1.43
C. siamea	6.17 ± 1.76	NS	1.12	2.50 ± 1.06	NS	0.89
S. indicum	6.00 ± 1.24	NS	1.09	3.17 ± 0.83	NS	1.13
S. hermontheca	5.67 ± 1.15	NS	1.03	2.00 ± 0.45	NS	0.71
R. adenophylla	5.67 ± 1.17	NS	1.03	3.00 ± 0.73	NS	1.07
P. acculeata	5.50 ± 0.92	NS	1.00	2.67 ± 0.49	NS	0.95
M. ciliatum	5.33 ± 1.33	NS	0.97	3.33 ± 0.61	NS	1.19
C. zambesicus	5.17 ± 1.14	NS	0.94	2.33 ± 0.67	NS	0.83
J. tamnifolia	4.83 ± 1.01	NS	0.88	2.33 ± 0.92	NS	0.83
H. sabdarifa	4.83 ± 0.65	NS	0.88	2.17 ± 0.48	NS	0.78
C. procera	4.67 ± 1.23	NS	0.85	2.50 ± 1.02	NS	0.89
I. astragalina	4.33 ± 0.99	NS	0.79	2.83 ± 0.60	NS	1.01
H. suaveolens	2.00 ± 0.58	< 0.05	0.36	0.33 ± 0.20	< 0.05	0.12
Control						
water	5.5 ± 0.90	-	1.00	2.79 ± 0.68	-	1.00
sugar solution	5.93 ± 1.12	= 0.63	1.08	3.21 ± 0.72	= 0.44	1.15

[a] Attraction index: mean baited/mean baited by water-soaked sponge (control)
NS-not significantly different from water-soaked sponge mean catch (t-test).

they can be considered very suitable for other environments in Africa.

In the semi-arid regional sites in Mali, there is typically a rainy season when a high diversity of flowering plants is present and a long dry season when the types and abundance of flowering plant is extremely limited. Therefore, for mosquitoes, there is apparently a great variation in the natural sugar sources that are available during the year. It is worthwhile mentioning that at least some of the identified attractive sugar sources like *P. reticulatum*, and the fig, *F. thonningii*, are available through most of the dry season and are often peridomestic. This is similar to the situation in Israel where the sugar-feeding field methods and ATSB approaches were developed [6-10]. A limitation of this study is that field-testing was done only at the end of the malaria transmission season in Mali and did not cover the entire flowering season. Further studies to link plant phenology and mosquito sugar-feeding are necessary to complement the picture of mosquito-plant relations.

These studies provide critical information for using ATSB methods for malaria vector control in Mali. First, the studies of *An. gambiae* s.l. attraction to fruits identify some of most highly attractive fruits that can be used for making attractive sugar bait solutions that are needed for ATSB bait stations [8,9] and plant spraying [7,10,12]. Some are locally available and abundant, and nearly all residents are familiar with them. The utility of using both guava and honey melons, two of the most attractive fruits, was recently demonstrated in the ATSB field trial in Mali [11]. Second, some of the seedpods identified as attractive (e.g. *A. albida, P. reticulatum, T. indica*) may serve as a key sugar source for mosquitoes; it appeared that the exudates of the fermenting liquid from the

Figure 3 Pictures of the most attractive flowering plants determined for *Anopheles gambiae* s.l. in Mali.

Figure 4 Picture of the only repellent plant (*H. suaveolens*) determined for *Anopheles gambiae* s.l. in Mali.

seedpods were particularly attractive. Seedpods that are highly attractive and readily available should be considered further for potential use in ATSB methods. However, the seedpods may not be readily available in the local markets. Third, studies of *An. gambiae* s.l. attraction to flowering plants identified the most attractive and available species of plants. Such information can serve to guide ATSB spraying on the most attractive plants in and around malaria endemic communities. Fourth, the index of attractiveness was higher for the most attractive flowering plant (i.e., *A. macrostachya*) than the most attractive fruit (i.e., *P. guajava*), indicating the nature of the plant-mosquito relation in Mali. This obviously may have longer-term implications for the ATSB approach because the semiochemicals responsible for attractiveness may be identified and chemical baits subsequently developed [22]. Ultimately, there is merit in using chemical baits instead of homemade brews of ATSB solutions. There may also be utility in chemically analyzing repellent plants such as *H. suaveolens* identified in this study or other repellent plants described from Africa [23].

Conclusion

These studies in Mali provide an assessment of the natural attractiveness of *An. gambiae* s.l. to fruits/seedpods and flowering plants. These data on mosquito-plant relations in nature are some of the first of their kind in Africa. Comparable studies employing the simple and effective field methods are needed in other ecosystems in Mali and elsewhere. Importantly, the results will help research teams adapt, improve, and optimize ATSB methods for malaria vector control in Africa.

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Authors' contributions

GCM, YS and JCB conceived and planned the study, interpreted results, and wrote the paper. GCM directed and performed the field experiments, and analyzed the data. SFT and SD facilitated field experiments by selecting study sites and obtaining local clearance from community leaders, and along with MBT, MMT, and SB assisted with the field and laboratory experiments, data management, and plant identifications. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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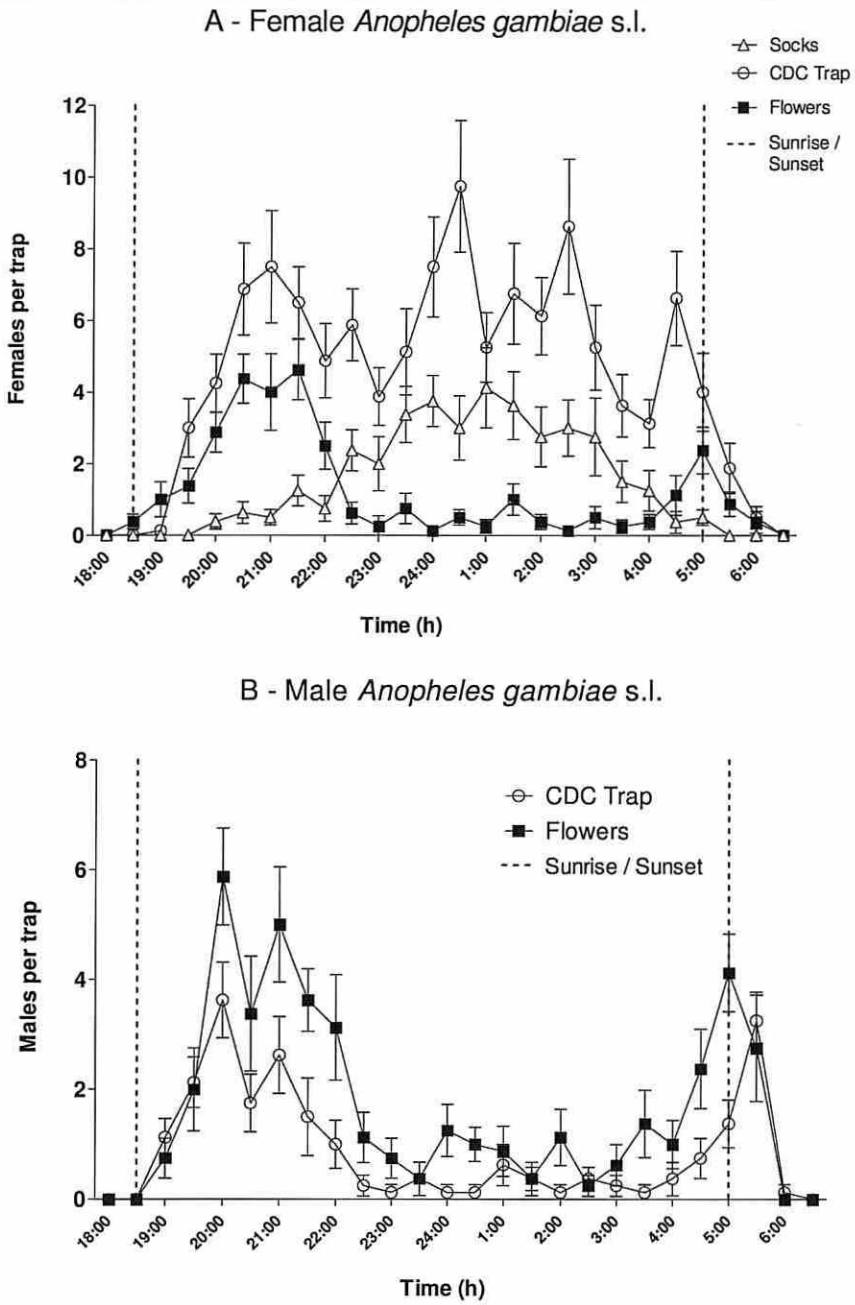

Figure 5 Nocturnal periodicity of *Anopheles gambiae* s.l. (± SE) females (5A) and males (5B) to 8 replicates each for the most attractive flowering plant (*A. macrostachya*), human odor from worn socks (females only), compared with catches from CDC light traps.

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Indoor use of attractive toxic sugar bait (ATSB) to effectively control malaria vectors in Mali, West Africa

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Abstract

Background: Attractive toxic sugar bait (ATSB) solutions containing any gut toxins can be either sprayed on plants or used in simple bait stations to attract and kill sugar-feeding female and male mosquitoes. This field study in Mali demonstrates the effect of ATSB bait stations inside houses as a vector control method that targets and kills endophilic African malaria vectors.

Methods: The studies were conducted in five villages located near the River Niger, Mali. Baseline village-wide assessments of densities for female and male *Anopheles gambiae* sensu lato were performed by pyrethrum spray collections (PSC) in ten houses in each of five villages. To determine the rate of mosquito feeding on bait stations, one bait station per house containing attractive sugar bait (ASB) (without toxin) plus a food dye marker, was set up in ten houses in each of the five villages. PSC collections were conducted on the following day and the percentage of female and male mosquitoes that had fed was determined by visual inspection for the dye marker. Then, a 50-day field trial was done. In an experimental village, one bait station containing ATSB (1% boric acid active ingredient) was placed per bedroom (58 bedrooms), and indoor densities of female and male *An. gambiae* s.l. were subsequently determined by PSC, and female mosquitoes were age graded.

Results: In the five villages, the percentages of *An. gambiae* s.l. feeding inside houses on the non-toxic bait stations ranged from 28.3 to 53.1% for females and 36.9 to 78.3% for males. Following ATSB indoor bait station presentation, there was a significant reduction, 90% in female and 93% in male populations, of *An. gambiae* s.l. at the experimental village. A 3.8-fold decrease in the proportion of females that had undergone four or more gonotrophic cycles was recorded at the experimental village, compared to a 1.2-fold increase at the control village.

Conclusion: The field trial demonstrates that *An. gambiae* s.l. feed readily from ATSB bait stations situated indoors, leading to a substantial reduction in the proportion of older female mosquitoes. This study demonstrates that ATSB inside houses can achieve impressive malaria vector control in Africa.

Keywords: *Anopheles gambiae*, Sugar feeding, Malaria, Attractive toxic sugar baits (ATSB), Bait stations, Indoor mosquito control, Mali

Background

Over the last decade, shortcomings of accepted vector-control methods have highlighted the need for integrated vector management (IVM) strategies that can be fully embraced and implemented by national malaria control programmes [1–3]. Current options for malaria vector control are limited, and usually consist of long-lasting, insecticide-treated nets (LLINs) and/or indoor residual spraying (IRS) [4, 5]. While these methods can reduce malaria parasite transmission rates and incidence of new

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infections, they do not consistently reduce malaria prevalence [3]. Moreover, sustained use of LLINs and IRS is problematic due to insecticide resistance, costs, inappropriate use, and lack of community acceptance [6]. In lieu of such drawbacks, development of additional tools and practical operational solutions which will complement existing methods for malaria vector control is of high priority [7].

A highly promising method for this purpose is attractive toxic sugar baits (ATSB)—a novel vector control approach that targets the sugar-feeding and resting behaviour of mosquitoes [8–14]. Developed and field-tested in the Middle East, the USA and Africa, this method was shown to effectively control local populations of anopheline, aedine and culicine mosquito species [8, 9, 11, 12, 14–21]. Notably, outdoor application of ATSB in a field evaluation conducted in Mali had caused a 90% decrease of the *Anopheles gambiae* sensu lato population, and particularly affected older, more dangerous females [12].

ATSB solutions can be applied to vegetation or used in bait stations to attract and kill sugar-seeking mosquitoes. A key principle is that ATSB includes a safe oral toxin that is ingested [9, 22] thereby circumventing problems associated with use of contact insecticides [23]. ATSB can be used with any type of insect gut-active, low-risk toxin, including some US Environmental Protection Agency materials that are exempt from registration because of their low toxicity to mammals [24]. Indeed, the high efficacy of ATSB has been demonstrated in field trials using a wide range of active ingredients, including spinosad [9, 11, 13], boric acid [12, 14, 17, 22], eugenol [19, 20], dinotefuran [18], pyriproxyfen [21], and micro-encapsulated garlic oil (unpublished data). The use of one or more low-risk ingestible toxins makes ATSB a potentially valuable new tool to fight rising resistance against conventional contact insecticides [25].

ATSB methods are highly effective, technologically simple, low cost, and proven to work in controlling mosquitoes outdoors, so it is reasonable to determine whether this method additionally works for indoor control. This study tests the effectiveness of ATSB bait stations inside houses against highly endophilic African malaria vectors.

Methods

Study sites

The study was conducted in 2010 in five villages (Saredere, Semina, Sarebambara, Papara, and Sambere) located near the margins of the inland delta of the River Niger in Bandiagara District, approximately 650 km northeast of Bamako, Mali. The population of each of the five villages exceeds 200 inhabitants, and all are situated in close proximity to rice paddies but separated by at least 1 km. The villages consist of compounds enclosing multiple buildings and bedrooms that house members of extended families. The rainy season in this semi-arid area occurs from July to September. Peak malaria transmission rates occur during the month of October, with *An. gambiae* s.l. representing 99.8% of the malaria vectors, out of which 86% are *An. gambiae* sensu stricto and 14% are *Anopheles arabiensis* [26]. Malaria transmission is seasonal, with up to 25 infective bites per person per month during peak periods of transmission and virtually undetectable transmission during the dry season [27]. The prevalence of *Plasmodium falciparum* infection in children varies from 45% during the dry season to >65% at the end of the rainy season [27]. In Mali, LLINs are the main tool for malaria vector control with a household coverage rate >90%.

Preparation of attractive sugar bait (ASB) and ATSB solutions

The attractive sugar bait (ASB) solution included juices of ripe/over-ripe fruits that are known to be enriched with attracting plant volatiles. The solution was prepared by mixing 30% guava juice, 30% honey melon juice, 25% water, 12% brown sugar W/V, 2% local millet beer, and 1% (W/V) BaitStab™ concentrate (Westham Innovations, Ltd, Israel) for preservation and stabilization of the bait. Guava and honey melons were selected for use since they are locally available and were previously demonstrated to be highly attractive for *An. gambiae* in comparative field tests of 26 different types of local fruits in Mali [28]. ATSB was similarly prepared but it included 1% boric acid as the active ingredient. As baits are typically invisible after application, a (1:200) blue (blue food dye no. 1) or red (Azorubine) food dye (Stern, Natanya, Israel) was added to the ASB and ATSB solutions, respectively, allowing identification of insects which have fed on the bait solutions by visual inspection of dye-stained guts [10, 16].

Bait station design

The bait stations were constructed from a plastic soft drink bottle (1.5 L), in which a 2-cm hole was cut about two-thirds of the way up (Fig. 1a). Cotton wicks were inserted through the holes so that both ends of the wick reached the bottom of the bottle. The bottles were then inserted into large, light-coloured cotton flannel socks, which were subsequently soaked in either ASB or ATSB solutions. The bottles were then filled with 0.9 L of the same solution, allowing for continuous seeping of the solution from the bottle through the wick as the external flannel coat dried [29].

Fig. 1 **a** Example of bait stations made from plastic drink bottles. Holes were cut in the middle of the bottles for placement of a cotton wick to absorb the attractive mixture. *White* socks covered the bottles and were coated in the attractive (non-toxic or toxic) mixture. **b** A field technician hanging a 1.5-L bait station in one of the houses in the Malian village. **c** Male and female mosquitoes feeding on the bait stations. The *colour dye* is ingested and stains the abdomen of the mosquitoes allowing for easy detection of mosquito feeding.

Methods for determining mosquito sugar feeding on indoor bait stations

Pyrethrum spray catches (PSC) were used to monitor indoor-resting male and female *An. gambiae* s.l. populations in the five villages [30]. Pre-treatment population densities were determined in at least ten houses per village, with a portion of the samples kept for subsequent identification by PCR [31]. White cloths were placed on the floors and windows, and doors were sealed prior to PSC collection. ASB bait stations (one per house) were placed the following day in ten randomly selected houses per village not initially sprayed with PSC (Fig. 1b), and PSC collections were performed 24 h after presentation of the bait stations. Mosquitoes that were knocked-down were removed from the white cloth, sexed [32] and the proportion of dyed, bait-fed specimens was determined visually (Fig. 1c) [10, 16].

Study design and methods for the ATSB field trial

The insecticidal efficacy of indoor-situated ATSB bait stations was evaluated in experiments performed in two compounds, situated in different villages. The two villages were selected based on large anopheline populations in the previous study. The experimental compound, Saredere, consisted of 19 houses of extended family members with multiple buildings and bedrooms, and the control compound, Semina, encompassed 33 houses. For pre-treatment evaluation, food dye-marked ASB stations were hung in all bedrooms (58) at the experimental village. On days 1, 4, 7, and 10 pre-treatment, six houses were randomly selected in each village to be sampled by PSC (described above) to determine the indoor mosquito populations. Following the ten-day pre-treatment evaluation, food dye-marked boric acid ATSB stations were hung in all 58 bedrooms (one per bedroom) at the experimental village compound. No bait stations were placed at the control village compound. Mosquito populations were then monitored in both villages twice a week for 40 days by randomly selecting six houses per sampling period. All rooms within the designated six houses were sampled using PSC (a total of 78 rooms sampled) to evaluate the effect of the ATSB boric acid indoor bait stations on mosquito populations. Bait stations were refilled with the ATSB solution three times during the evaluation.

All mosquitoes collected were sexed. Pre-treatment and post-treatment collections in both villages were visually inspected with a dissection microscope for the presence of food dye to determine if the mosquitoes had fed on the ASB (pre-treatment) and ATSB (post-treatment; experimental compound only) station. All the mosquitoes collected pre-treatment and at the post-treatment control site were evaluated for their sugar-feeding status by anthrone testing of the dissected guts [33]. In the absence of food dye in the abdomen of the mosquitoes collected at the experimental site post-treatment, the dissected guts were evaluated for their sugar-feeding status by anthrone testing. It should be noted that boric acid-induced mortality occurs ca. 48 h post-feeding [22, 25], therefore, visual inspection for presence of food dye was performed in the post-treatment collections to identify mosquitoes that had fed on the bait station but had not succumbed to the slow-acting toxin. Additional dissections were performed for age grading using the dilatation method [34]. The per cent reduction in the mosquito populations was calculated by determining the pre-treatment populations compared to post-treatment populations [100 − [(pre-treatment control village numbers/pre-treatment experimental village numbers) × (post-treatment control village numbers/post-treatment experimental village numbers)] × 100].

Statistical analysis

Counts of male and female mosquitoes were analysed with a generalized linear model with fixed effects for town, time (pre/post) and their interaction. A negative binomial regression was used because of overdispersion. Planned comparisons were made between pre- and post-measures within the villages. The per cent of stained males and females was calculated for each town. For ATSB indoor evaluation separate generalized linear models were used to analyse the female and male mosquito counts over the 50-day field trial. The model included group (experimental/control), day, and the interaction of group and day. A negative binomial regression model was used because of marked overdispersion. Counts of female mosquito in age groups were analysed with a generalized linear model with fixed effects for group (experimental/control), time (pre/post), and age group (0–3 and ≥4) plus all two-way interactions and the three-way interaction. A Poisson regression model was used because no overdispersion was evident. Planned comparisons between pre- and post-measures were made for each age group within experimental and control groups.

Results

Species identification

PCR testing of female *An. gambiae* s.l. showed that 96% (192/200) of samples from the five villages were *An. gambiae* s.s. and 4% were identified as *An. arabiensis*.

Mosquito feeding on bait stations inside houses

Initial baseline village-level densities of *An. gambiae* s.l. inside houses averaged 22.0 ± (SE) 5.2 females and 12.4 ± 3.0 males per house. In the presence of ASB stations the means among the houses were similar (average 19.3 ± 4.6 females and 12.3 ± 3.0 males), and the food dye marker labelled 40.4% (433/1,071) of the females and 59.4% (405/682) of the males, ranging from 28.3 to 53.1% females and 36.9 to 78.3% of the males in the five villages. Table 1 presents the range of means among the villages.

ATSB field trial of bait stations inside houses

There were no significant differences in pre-treatment female and male *An. gambiae* s.l. population densities ($P > 0.05$) between experimental and control villages. Females averaged 25.7 ± 8.8 and 21.6 ± 7.4 per house at the experimental and control village, respectively. Males averaged 18.5 ± 6.3 and 11.3 ± 5.4 per house at the experimental and control village, respectively. Of the females that were collected at the experimental village, 45.6% (202/443) of them were marked by bait food dye and 27.1% (120/443) were sugar positive. At the control

Table 1 The range of mean number of female and male *Anopheles gambiae* s.l. caught inside houses by pyrethrum spray catch among the five villages in Mali pre-and post-bait station presentation inside houses, and the per cent stained with food dye marker

Village	Pre-bait catch (mean number/house)			Post-bait catch (mean number/house)			Per cent stained	
	No. houses	Female	Male	No. houses	Female	Male	Female	Male
Saredere	10	35.3	14.3	10	29.1	17.5	30.92	42.29
Semina	10	24.1	18.5	10	19.5	14.0	34.87	50.71
Sarebambara	10	14.3	9.9	10	17.3	12.3	46.82	74.80
Papara	14	28.9	16.6	14	23.4	13.6	31.71	47.37
Sambere	12	5.9	3.1	12	7.0	4.5	58.33	81.48

village 36.1% (136/377) of the females collected were sugar positive. For males collected at the experimental village houses prior to ATSB presentation, bait food dye marker labelled 42.9% (265/617) of the male mosquitoes and 19.6% (121/617) were sugar positive. The number of sugar positive males at the control village represented 26.6% of the collections (138/518).

A significant reduction in *An. gambiae* s.l. populations at the experimental village was observed following indoor placement of ATSB bait stations, with a 90% reduction in female and 93% reduction in male populations. The population reduction was significantly higher from day 25 on for females and from day 16 on for males (Fig. 2) compared to the control village.

At the end of the 50-day field ATSB evaluation, female population densities averaged 5.9 ± 1.8 per house compared to 17.7 ± 5.4 at the control village. This reduction was a four-fold decrease in female populations compared to the pre-treatment populations. Male numbers post-ATSB exposure averaged 1.9 ± 1.0 per house at the experimental village compared to 18.7 ± 2.2 at the control site. This was a 13.5-fold decrease at the experimental site while concurrently in the control village there was a 1.1-fold increase.

A total of 7.7% (33/426) of the recovered females and 3.4% (4/147) of the recovered males were marked with the ATSB food dye suggesting that these would have died following full metabolism of the ATSB solution. Of the females collected at the experimental site, 39.8% (169/426) were sugar positive, which was significantly different to the number collected at the control village (21.1%; 292/1,382). Of the male mosquitoes collected at the experimental site 47.6% (70/147) were sugar positive which was significantly different to the number collected at the control village (27.9%; 336/1,204).

Table 2 shows that the decrease of the population following exposure to ATSB altered the initial proportion of different age groups of the females (classified according to gonotrophic cycles 0, 1, 2, 3, and >4). There was a significant reduction of female mosquitoes in the >4 age group

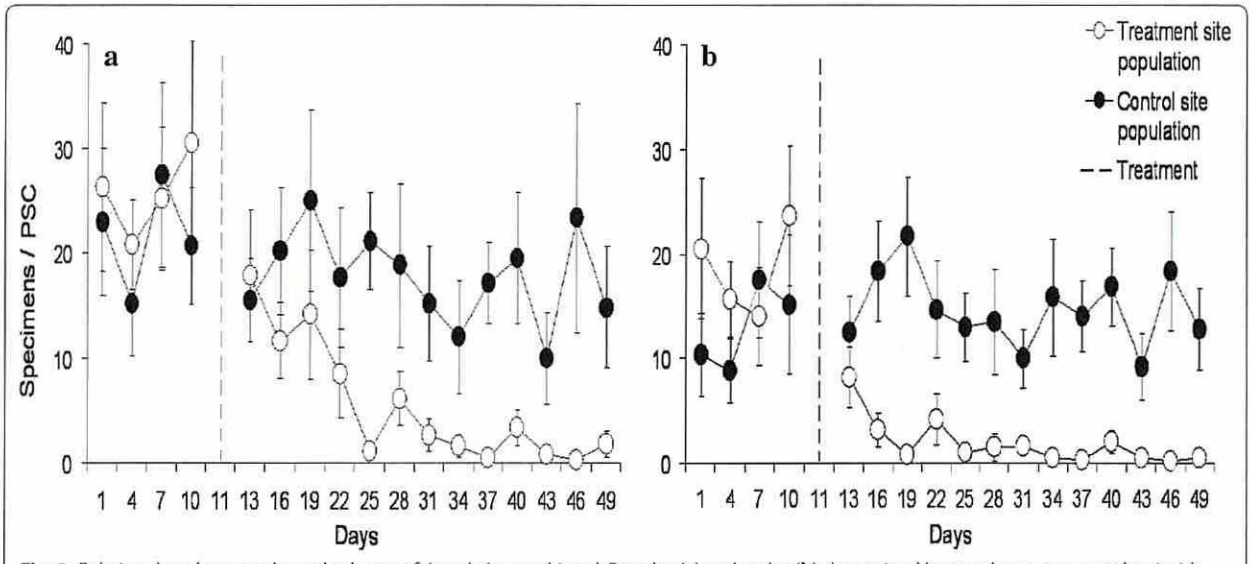

Fig. 2 Relative abundance and standard error of *Anopheles gambiae* s.l. Females (**a**) and males (**b**) determined by pyrethrum spray catches inside houses receiving ATSB bait stations in the village of Saredere compared to the control site of Semina in Mali.

Table 2 Age-group classification of *Anopheles gambiae* s.l. females collected indoors, before and after an application of ATSB bait stations indoors (experimental) and houses without bait stations (control)

Site and time	Females examined	% females by observed numbers of dilatations in dissections of ovaries				
		0	1	2	3	>4
Control pre-treatment	200	35.50	16.50	12.00	10.00	26.00
Control post-treatment	277	24.55	16.25	14.44	13.36	31.41
Experimental pre-treatment	200	22.00	17.50	16.00	11.50	33.00
Experimental post-treatment	277	43.32	25.27	16.61	6.14	8.66

for the experimental village ($P < 0.05$). In the diminished population there was a relative reduction in the proportion of older more epidemiologically dangerous mosquitoes (>4 gonotrophic cycles) from 33% (66/200) to 17.3% (48/277). At the same time in the control group there was a 1.2 increase in the proportions of older females with >4 gonotropic cycles. Comparison of pre-treatment and post-treatment female population structures in the experimental village showed similar differences.

Discussion

This field trial in Mali demonstrates that ATSB bait stations placed inside of houses can effectively reduce densities of both female (90%) and male (93%) *An. gambiae* s.l. Results also indicate the treatment disproportionately affects older females that are more likely to be infectious, with a 3.8-fold reduction in the number of female mosquitoes that had undergone four or more gonotrophic cycles observed at the experimental village, compared to a 1.2-fold increase at the control village. The use of a dye marker in the ASB bait stations, in both the initial indoor-feeding study and the ATSB indoor evaluation are in agreement with previous studies in Israel and Mali [10, 11], which demonstrated that a high proportion of the local *An. gambiae* populations were making daily contact with and feeding from the indoor bait station. This is further supported by the decline in anopheline populations after presentation of ATSB bait stations in the experimental village. Notably, the few stained mosquitoes that were collected could be subtracted from the number of survivors, as boric acid has been demonstrated to be a slow-acting gut toxin at 1% with optimal mortality at 48 h post-feeding [22, 25].

Importantly, this study establishes that anopheline mosquitoes will feed on ATSB indoors. Significantly more mosquitoes were sugar positive in the treatment village where ATSB bait stations were present, highlighting the attractive nature of the ASB. Currently, new mixtures of ASB have been developed and have been reported to attract mosquitoes from up to 8 m and are highly attractive to both male and female mosquitoes (unpublished data). These findings are important especially when considering the feasibility of ATSB application both indoors and outdoors in environments where competition from natural sugar sources is more likely. In Israel, it was shown that ATSB using BaitStab™ decimated mosquito populations because of the high frequency of sugar feeding by mosquitoes, regardless of sugar availability [14]. The authors associated the high frequency of sugar feeding to the increased probability that mosquitoes will be attracted and killed by the ATSB methods. The fact that anopheline mosquitoes are attracted to artificial sugar sources and potentially feed on baits indoors increases the likely success of using the attract and kill method for malaria control in Africa.

Furthermore, the presentation of ATSB in a bait station continues to validate the versatility of ATSB and its effectiveness in reducing mosquito populations. In the current study, a 1% boric acid solution was incorporated into the bait stations as a proof of concept that ATSB applied indoors can reduce malaria vector populations. Using field data collected in Mali, Marshall et al. modelled the impact of ATSB on outdoor anopheline populations and found that 50% of females fed on the ATSB per day [35]. In addition, the model suggested that a high LLIN coverage rate in combination with ATSB could result in a reduction in exophilic transmission. Indoor use of ATSB bait stations in combination with LLINs would be likely to increase the reduction in endophilic anopheline populations, further impacting malaria transmission. In a semi-field hut study, indoor bait stations made with a guava-based ATSB were as effective as LLINs [36]. In the semi-field study, three different active ingredients in the crude ATSB formulation were evaluated and the treatments were effective in knocking down 41–48% of *An. arabiensis* and 36–43% of *Culex quinquefasciatus*.

In this study, a crude ATSB mixture in plastic bottles needed three refills for successful control of anopheline populations during the 50-day evaluation. These studies were conducted in 2010 at which time the attractants used in the ATSB formulation were prepared with local materials and stabilized with Baitstab™. Thus, the ATSB baits varied greatly in their attraction for mosquitoes. Regardless, *An. gambiae* s.l. were continually attracted to the bait stations and the populations continued to decline throughout the 50-day evaluation. Similar results have been obtained in one study in Israel where anopheline populations were controlled for >6 weeks after ATSB application to vegetation [15]. However, ideally, the application of ATSB indoors should increase the residual activity of the bait due to less environmental exposure and the incorporation of a protective bait station design. The bait station prototype presented some problems in the current study, including contamination of the bait with dust. For successful incorporation into IVM programmes, the ATSB strategy will need to evolve with the development of universal baits and durable bait stations.

Importantly, the findings of the current study support a previous study in which ATSB application to vegetation had a dramatic impact on reducing the number of older more dangerous mosquitoes [12]. In the current study mosquitoes with >4 gonotrophic cycles represented <10% of the already diminished population compared to >30% of the population in control sites. Because this strategy targets sugar-feeding behaviour, which usually takes place before a blood meal [37], mosquitoes may be killed

prior to ever taking a blood meal. Although the numbers of older mosquitoes dropped significantly, there were still relatively high numbers of older mosquitoes; improved bait and bait-station design will need to address this to further reduce the number of older mosquitoes.

One important consideration worth noting is that the ATSB bait station approach has minimal risks to humans and to non-target organisms. Just like the ATSB application strategy to non-flowering vegetation, which has been demonstrated to reduce non-target impacts [18–20], bait station strategies are currently being developed to ensure little to no non-target impacts. However, in the current study, cockroaches, ants, houseflies, and other indoor pest insects were found dead after feeding on the indoor bait stations. Overall, the villagers were receptive to the ATSB bait stations placed indoors and especially receptive to the ATSB bait stations that were found to reduce the number of nuisance pests. In comparison to the use of LLINs which require proper placement of the net each night, ATSB bait stations will require no behaviour modification by the user which will likely result in greater acceptance of this method and less misuse. The findings of the current study of ATSB bait stations inside houses in rural villages in Mali begins to explore some of the ultimate impacts of the ATSB approach for malaria vector control in Africa. It is now clear that both outdoor and indoor use of ATSB can control malaria vectors and preliminary field studies demonstrate that ATSB indoor application is as effective as LLINs [36].

Beyond this initial field trial and for inclusion as an IVM strategy for malaria control, the full impacts of ATSB need to be determined by field assessments on a larger scale and of longer duration at the village and/or district levels with designs that measure impact not only on vector densities and vector longevity, but also measures of malaria parasite transmission (e.g., entomological inoculation rates), and malaria burden in human populations (e.g., incidence of malaria cases) [38]. Evidence continues to highlight the range of environments in Africa where ATSB methods can be used effectively. Future research should focus on the combination of both indoor and outdoor ATSB applications to determine if there is a synergistic effect. Importantly, ATSB methods differ from and potentially complement LLIN and IRS methods and they have so far proven effective in outdoor [11, 12, 14] and indoor environments for killing mosquitoes.

Conclusions

This study provides evidence that ATSB methods employed indoors can successfully attract and kill indoor anopheline mosquitoes. More importantly, *An. gambiae* populations collected indoors after exposure to ATSB bait stations included significantly fewer older females when compared to mosquitoes at the control site. This suggests that ATSB-induced mortality of indoor mosquitoes is dramatically skewing the adult age distribution towards younger mosquitoes, leading to potential reductions in both sporozoite rate and entomological inoculation rates beyond the effect of population decrease. Overall, this proof of concept study with crude bait and preliminary bait station design operationally controlled populations of anopheline mosquitoes suggesting that indoor ATSB bait stations can be a promising strategy for indoor vector control. These study findings should encourage further research to improve bait station design for incorporation into IVM programmes.

Authors' contributions

GCM, JCB, VDK, MMT, SFT, SD, and YS devised the study and objectives. GCM and VDK provided the data. KLA analysed the data. WAQ, GCM, JCB, and RDX wrote the manuscript. All authors commented on the final manuscript. All authors read and approved the final manuscript.

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Compliance with ethical guidelines

Competing interests

The authors declare that they have no competing interests.

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Short communication

Implications for operational control of adult mosquito production in cisterns and wells in St. Augustine, FL using attractive sugar baits

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ABSTRACT

The aim of this study was to further investigate the use of attractive sugar baits as an effective, inexpensive, and environmentally friendly tool for integrated mosquito management programs. Mosquitoes were offered dyed sugar bait in wells and cisterns in an urban tourist area in St. Augustine, FL. Exit traps were constructed to cover the well and cistern openings so the number of resting and emerging mosquitoes stained by feeding on the sugar bait could be monitored. Four mosquito species were collected from these structures: *Aedes albopictus* (Skuse), *Anopheles crucians* (Wiedemann), *Culex quinquefasciatus* Say, and *Toxorhynchites rutilus rutilus* (Coquillett). Overall, 90% (1482/1644) of the mosquitoes trapped were stained. In general, the number of mosquitoes stained was significantly greater in wells ($P < 0.0001$) and cisterns ($P < 0.0001$) than the numbers that were not stained by the colored bait. Based on the number of mosquitoes stained, we would have expected considerable mosquito mortality had the sugar bait contained an oral toxin. The results of this study support the concept of using attractive toxic sugar baits as an effective tool for integrated mosquito management.

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1. Introduction

Saint Augustine, FL most notably known for being the oldest European settled town in the Americas, is home to a wide variety of mosquitoes ranging from fresh floodwater to salt marsh mosquito species. However, a major focus of the mosquito control program in this area is on the control of container-inhabiting species such as *Aedes albopictus* (Skuse) and *Culex quinquefasciatus* Say. These species serve as important vectors for arthropod-borne viruses such as dengue fever (Family Flaviviridae; genus *Flavivirus*) and West Nile virus (Family Flaviviridae; genus *Flavivirus*) (Vinogradova, 2000).

On average about two million tourists visit the downtown area of St. Augustine including St. George Street which has a number of cisterns and wells that have been preserved and maintained as a natural heritage (The St. Augustine, Ponte Vedra, and Beaches Visitor and Convention Bureau). Historically the water used in St. Augustine, including drinking water, came from numerous ground water wells and deep cisterns dug in the coral rock that collected surface runoff water. Later in the 19th century, houses were also supplied with water from barrel shaped over ground built concrete or brick cisterns. During the last couple of decades most cisterns and wells were filled with soil, dismantled or sealed except for those preserved as a natural heritage. While few in number in the downtown St. Augustine area, these structures represent an important urban habitat for resting and developing mosquito populations (Lardeux et al., 2002; Caprara et al., 2009).

The use of attractive toxic sugar baits (ATSB) is a promising control method that exploits the dietary staples used to sustain the daily activities of mosquitoes (Müller and Schlein, 2008; Müller et al., 2008, 2010a,b). Female and male mosquitoes obtain energy for their day- to-day activities from floral nectar, nectaries on plant leaves and stems, and from homopteran honeydew (Yuval, 1992; Foster, 1995; Gary and Foster, 2004). In previous studies we have shown dramatic reduction of important mosquito vectors in cistern (Müller et al., 2008) and storm drain systems using ATSB (Müller et al., 2010a,b). We report here on our effort to evaluate the use of ATSB against mosquito populations resting and emerging in the cistern and well systems in St. Augustine, FL by targeting the sugar feeding behavior for their control.

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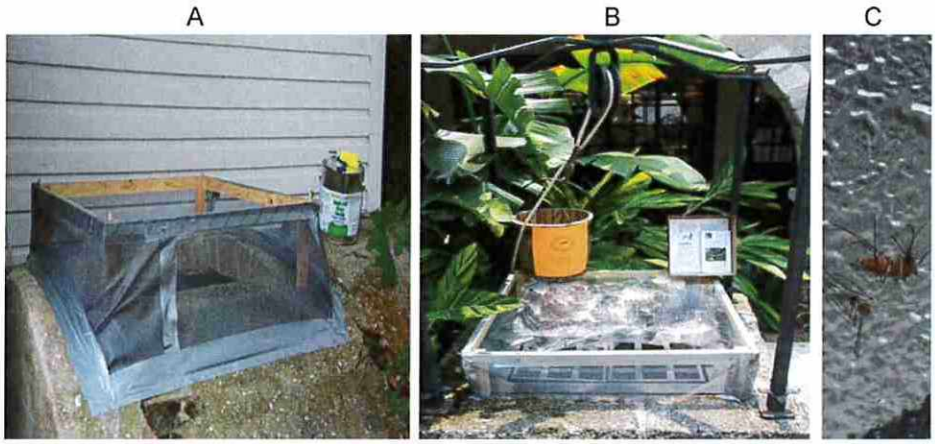

Fig. 1. (A) Exit trap covering the opening of one of the cisterns (without sticky cover). (B) Exit trap covering the opening of one of the wells (with sticky cover). Anastasia Mosquito Control District of St. Johns County used this as an opportunity to provide public education on mosquito control by placing educational material on the exit trap. The Noseeum mesh was duct taped down to the well and cistern structure to allow the mosquitoes to come in contact with the glue covered acrylic top. (C) Two mosquitoes trapped on the glue, upper specimen stained orange by food dye. For a full description, see Section 2.

2. Materials and methods

2.1. Study site

This study was conducted from mid-April to mid-May in the downtown St. Augustine area in 2007. Three wells were in the experiment that were approximately 2 m deep with square or rounded openings of about 1 m diameter and covered by loosely fitting iron bars. The wells contained from 0.5 to 1 m water. Two concrete and brick, above ground cisterns were also included in this study. The cisterns are square in shape with a rounded roof (3 m long, 1.5 m wide and 1.6 m high) with a square opening (0.4 m × 0.4 m top). The cisterns contained about 0.2 m water. Wells and cisterns harbored numerous mosquito larvae and resting adult mosquitoes at the start of the study.

2.2. Marking and sampling of cisterns and wells for resting and emerging mosquitoes

An exit trap was specifically designed to cover the openings of the wells and cisterns used in this study (Fig. 1). A structure made out of wood (121 cm × 121 cm with 22 cm legs) covered by an Optix® acrylic sheet (Plaskolite, Inc., Columbus, OH) top (121 cm × 121 cm) was constructed to cover the openings. After placing the table-like structure over the exit, fine Noseeum (Diptera: Ceratopogonidae; genus *Culicoides*) mesh (The Home Depot®) was duct taped to the wood structure and the mesh was draped over the exit trap. This ensured that exiting mosquitoes would fly up and come into contact with the acrylic sheet. Tangle-Trap® Sticky Coating Paste Formula (The Tanglefoot Company®, Grand Rapids, MI) was painted on the bottom side of the acrylic sheet in a thin coat. This allowed the mosquitoes to adhere to the acrylic sheet as they were exiting. The mosquitoes could then be counted. Any holes in the cisterns were filled in with a cloth material.

Before placing the exit traps over the cistern or well opening a sugar bait was hung in the structure (Fig. 2). Plastic soft drink bottles (0.5 L), with a ~2 cm hole about two-thirds of the way up, were prepared. Cotton wicks were inserted through the holes so that both ends of the wick reached the bottom of the bottle. The bottles were then inserted, bottom first, into large, light-colored, cotton flannel socks that had been thoroughly washed in water and dried. The socks were wetted by dipping them into the sugar solution and 0.3 L of solution was poured into each bottle. Thus, fluid from inside the bottle was sucked out by the wick as the external layer of the flannel dried. Each bottle was covered with a 19 cm diameter, fish-bowl shaped plastic cover. Each bait station was hung at the opening of the cistern or well.

The sugar solution used in the bait consisted of the following: ~95% juice of over-ripe to rotting plums (*Prunus Americana* Marshall: Rosacea), 5% (v/v) red wine (Veo Grande Cabernet Sauvignon; Viñedos Errázuriz Ovalle SA, Santiago, Chile). To this solution was added 10% (w/v) brown sugar (Nature Sugar, brown: Louis Dreyfus Commodities Ashdod, Israel), 10% (w/v) of a mixture of slow-release substances and preservatives (BaitStab™; Westham Ltd, Tel Aviv, Israel), and 0.5% (w/v) orange food dye (Carmoisine; Stern Inc., Natanya, Israel). The entire solution was ripened for 48 h in covered buckets and left outdoors in the sun in temperatures up to ~30 °C. At the end of this procedure another 10% (w/v) brown sugar was added.

The studies were carried out for six days and replicated by habitat. After each 24 h trapping period the mosquitoes that were stuck to the Tanglefoot® covered Optix® acrylic sheet were removed and sex, species, and stained status was recorded. The orange dye allowed for the mosquitoes that fed on the sugar bait to be stained.

Fig. 2. Orange dye sugar bait station constructed for use in this study. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Visual observation of the mosquitoes' abdomens that were stuck to the acrylic sheet was used to determine the efficacy of the bait system in the cistern and wells. Once all the mosquitoes were removed for that 24 h trapping period the acrylic sheet was placed back on top of the exit trap.

Catches of the first day were a combination of resting mosquitoes and mosquitoes that had emerged overnight. The catches from the following day were only emerging mosquitoes since the habitats were closed off to entering (resting) mosquitoes. The average daily number of resting individuals was calculated by using the following equation: [(average catch day 1) – (average catch day 2)] (Müller et al., 2011).

2.3. Data analysis

Data were analyzed using Stats Direct (Stats Direct Ltd., Cheshire, UK). A Chi square analysis with untransformed data was used for sample comparison and differences were considered significant at $P < 0.01$.

3. Results

Males and females of four mosquito species were trapped throughout the study evaluation: *Ae. albopictus*, *Anopheles crucians* (Wiedemann), *Cx. quinquefasciatus*, and *Toxorhynchites rutilus rutilus* (Coquillett). The total number of mosquitoes recovered from wells and cisterns amounted to 746 females and 898 males; 93% of the females and 94% of the males were stained orange. There was no significant difference between the number of orange-stained males and females ($P > 0.01$). Overall, 90% (1482/1644) of the mosquitoes trapped were stained. The number of mosquitoes stained was significantly greater in wells ($x^2 = 625.1$, df = 1, $P < 0.0001$) and cisterns ($x^2 = 829.5$, df = 1, $P < 0.0001$) compared with the numbers not stained.

The daily resting average of both males and the females of each species trapped throughout this evaluation by structure type is shown in Table 1. On average 41 mosquitoes were considered to be resting and 15 mosquitoes were considered to be hatching daily in these structures. Both resting and emerging mosquitoes were equally as likely to feed on the sugar bait. In fact, significantly more resting ($x^2 = 418.9$, df = 1, $P < 0.0001$) and emerging ($x^2 = 1023.4$, df = 1, $P < 0.0001$) mosquitoes were stained compared with those that were not stained by the colored bait.

The predominate species trapped in the cisterns and wells was *Cx. quinquefasciatus*. There were no significant differences between the numbers of stained males and females or stage (resting or emerging) of this species in wells and cisterns ($P > 0.01$). Of the total *Cx. quinquefasciatus* trapped in the wells, significant significantly greater number were stained, 89%(403/452; $x^2 = 3320$, df = 1, $P < 0.0001$) compared with those that were not stained by the orange sugar solution. This was also true for the *Cx. quinquefasciatus* trapped in the cisterns, 96% (609/631; $x^2 = 1685$, df = 1, $P < 0.0001$).

Ae. albopictus and *Tx. rutilus rutilus* (Coquillett) were trapped from the wells but were not trapped from the cisterns. No significant difference existed between the number of stained males and females or stage (resting or emerging) of both species ($P > 0.01$). Of the *Ae. albopictus* trapped, significantly more, 95% (140/148; $x^2 = 2450$, df = 1, $P < 0.0001$), were stained orange from feeding on the sugar solution. Of the *Tx. rutilus rutilus* trapped, significantly more, 91% (108/119; $x^2 = 1061$ df = 1, $P < 0.0001$) were stained orange from feeding on the sugar solution.

An. crucians was trapped only in the cisterns. There was no significant difference between the numbers of stained males and females or stage (resting or emerging) ($P > 0.01$). Of the *An. crucians* trapped, significantly more mosquitoes (95%) were stained orange (252/264; $x^2 = 5292$, df = 1, $P < 0.0001$).

4. Discussion

We have demonstrated that regardless of species, sex, or stage (resting or emerging) the majority of the mosquitoes trapped in these habitats would readily feed on the dyed sugar bait. With >90% of the mosquitoes stained as they were exiting the wells or cisterns, we can infer from these results and those of other studies that mosquito mortality would have been equally as high had the sugar bait been spiked with a toxin (Schlein and Müller, 2008; Müller et al., 2010a,b; Xue et al., 2011). In a previous study, work by Müller and Schlein (2008) where ATSB placed near the openings of cisterns nearly eliminated *Anopheles claviger* (Meigen) from an experimental area in Israel. Another evaluation of ATSB in a storm drain system that utilized non-toxic control sugar bait like the one described in this study, demonstrated similar results (Müller et al., 2010a,b). Furthermore, Müller et al. (2010a,b) demonstrated that mosquitoes contained in a storm drain system will readily feed on the sugar bait system. Although, the low capture rate of mosquitoes in the storm drain system that received the ATSB compared with the number of stained mosquitoes that were captured on the side of the storm drain that was treated with the colored sugar bait strongly suggests that control of subterranean mosquitoes can be achieved.

Given the limitations of traditional methods used in subterranean mosquito control, there is a need for development of innovative control strategies (Dhillon et al., 1984; Metzger et al., 2008). Mosquitoes that develop in or find shelter in these types of structures are extremely difficult to control (Hazelrigg and Peslsue, 1980).

Moreover, mosquitoes that develop in or find shelter in these types of structures cause considerable public health concerns from the standpoint of arbovirus transmission (Kay et al., 2000; Marfin et al., 1993). Another added benefit of the ATSB method would be a potential decline in older more epidemiologically dangerous mosquitoes (Gu et al., 2011; Beier et al., 2012). ATSB treatment in sugar-rich and sugar-poor oases located in the African-Syrian Rift Valley showed a dramatic reduction of the proportion of older (3 or more gonotrophic cycles) *Anopheles sergentii* (Sergent) populations which resulted in a decrease in malaria vectorial capacity (Beier et al., 2012). Even in a highly competitive sugar source environment, the attract and kill nature of the ATSB decimated these *Anopheline* populations because of this mosquitoes high frequency of sugar feeding. Since this sugar-feeding behavior

Table 1
Mean number of resting mosquitoes (±SD), by species, caught by emergence traps, in wells and cisterns on the first and second day.

Habitat	Species	Average 1st day	Average 2nd day	Daily resting
Well	*Ae. albopictus*	17 (26)	12 (19)	5 (7)
	Cx. quinquefasciatus	18 ± 6 (29 ± 8)	13 ± 5 (14 ± 7)	5 (15)
	Tx. rutilus rutilus	16 (22)	4 (9)	12 (13)
Cistern	*An. crucians*	34 (45)	20 (21)	14 (24)
	Cx. quinquefasciatus	43 ± 17 (52 ± 17)	28 ± 11 (29 ± 16)	15 (23)

Daily resting rate = [(average catch day 1) – (average catch day 2)].

commonly occurs before and after a bloodmeal (Foster, 1995) and older ovipositing or resting mosquitoes are repeatedly exposed to the baits when entering the wells or cisterns; it is therefore probable that a high percentage of these individuals with vector competency would be killed. Further studies evaluating the sugar bait feeding tendencies of mosquitoes in different physiological states and ages needs to be addressed since mosquitoes may be migrating into an area to oviposition or rest and were not initially exposed to the sugar baits.

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Formulation of attractive toxic sugar bait (ATSB) with safe EPA-exempt substance significantly diminishes the *Anopheles sergentii* population in a desert oasis

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Abstract

Attractive toxic sugar bait (ATSB) is a highly effective method which targets mosquitoes based on their sugar foraging behavior, by presenting baits of attractive compounds in combination with sugar and oral toxin to local mosquito populations. Environmental concerns and insecticide selection-pressure have prompted investigations of novel, ecologically-harmless substances which can be used as insecticides. This study examined the efficacy of microencapsulated garlic-oil as the oral toxin component of ATSB for controlling *Anopheles sergentii* populations inhabiting desert-surrounded wetlands in Israel. ATSB solution containing 0.4% encapsulated garlic oil was applied to local vegetation around a streamlet located in the lower Jordan Valley. To determine the propensity of bait ingestion, and assess the potential ecological impact of the method, mosquito and non-target specimens were collected and tested for the presence of natural plant- or attractive sugar bait (ASB)-derived sugars. Over the experimental period, biting-pressure values in the

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actatropica.2015.06.018

ATSB treatment site decreased by 97.5%, while at the control site, treated with non-toxic ASB, no significant changes were observed. Approximately 70% of the mosquitoes collected before both treatments, as well as those captured following the application of ASB at the control site, were found to have ingested sugar prior to capture. Non-target insects were minimally affected by the treatment when ATSB was applied to foliage of non-flowering plants. Of the non-Diptera species, only 0.7% of the sampled non-target insects were found to have ingested ASB-solution which was applied to green vegetation, compared with 8.5% which have foraged on ASB-derived sugars applied to flowering plants. Conversely, a high proportion of the non-target species belonging to the order Diptera, especially non-biting midges, were found to have ingested foliage-applied ASB, with more than 36% of the specimens collected determined to have foraged on bait-derived sugars. These results prove that food-grade, EPA-exempt microencapsulated garlic oil is a highly effective insecticide which can be utilized for mosquito population control. The relatively short half-life of this active ingredient makes it a suitable for use in areas where repeated application is possible, limiting the accumulation of deleterious compounds and ensuring minimal environmental impact when applied in accordance with label recommendations.

Keywords

Culicidae; *Anopheles sergentii*; Sugar-feeding; ATSB; Vector-control; Non-targets

1. Background

Mosquito-borne diseases are responsible for a significant portion of human morbidity and mortality (Tolle, 2009). Consequently, increased attention has been given for development of vector control strategies which aim to reduce mosquito population numbers or their contact with potential human host, with some methods proving highly effective in lowering the incidence of such ailments in many affected areas (Beier et al., 2008; Enayati and Hemingway, 2010). However, the efficacy of current vector control methods is mostly limited to low-transmission environments, such as arid locations or isolated islands (Bhattarai et al., 2007; Kleinschmidt et al., 2007; Keating et al., 2011), whereas they have negligible or no impact in areas where entomological inoculation rates (EIRs) are more substantial (Beier et al., 1999; McKenzie et al., 2001; Shaukat et al., 2010). These shortcomings can be attributed to several factors. Insecticide-based methods such as long lasting insecticide-treated nets (LLINs) and indoor residual spraying (IRS) have been shown to cause selection-pressure and contribute to the emergence of mosquito populations which are resistant to one or several classes of chemical compounds (Oxborough et al., 2008; Ranson et al., 2009, 2011; Beier et al., 2012). In addition, most strategies target host-seeking and blood feeding mosquitoes, relying on potential human hosts to attract the vectors to the vicinity of the insecticide, which is exclusively used within residences. Consequently, the efficacy of such treatments is behavior-dependent, with endophilic species which tend to rest and feed indoors affected more than exophilic, outdoor-feeding vectors (Fornadel et al., 2010; Russell et al., 2011; Derua et al., 2012). Accordingly, a shift in the disease transmission dynamics may perpetuate the incidence of the disease, as a readily available pool of the pathogen is maintained within the latter, impeding attempts to reduce and sustain EIRs below the desired thresholds for prolonged periods (Beier et al., 1999; McKenzie et al.,

2001; Shaukat et al., 2010). Hence, while vector control remains a central aspect in the efforts to eradicate mosquito-borne diseases, such drawbacks have highlighted the need to find additional, complementing methods to be utilized in integrative vector management (IVM) for controlling vector populations.

In view of the above, several studies have explored the efficacy of alternative strategy which targets mosquitoes based on physiological requirements or behavioral responses other than the search for blood meals. Both male and female mosquitoes ingest sugars to meet energetic demands, (Yuval, 1992), and are highly-selective in choosing their source (Müller and Schlein, 2006; Schlein and Müller, 2008), which can include fruit, floral nectar, or honeydew. Increased knowledge of mosquito feeding-habits and preferences has enabled targeting the vectors by using attractive toxic sugar bait (ATSB) – a mixture of sugar and oral insecticide dissolved in water, which was initially sprayed on plants known to be highly-attractive to local mosquito populations (Müller and Schlein, 2006; Schlein and Müller, 2008; Müller et al., 2010; Gu et al., 2011). Following studies have improved the method by adding plant-derived attractants to the bait mixture, allowing its application on non-attracting plants (Müller and Schlein, 2008), and in portable bait-stations (Müller et al., 2008; Naranjo et al., 2013).

Most of the trials which tested the efficacy of ATSB as a vector-control strategy employed one of several chemical compounds (such as spinosad, boric acid, and dinotefuran) as oral insecticides (Müller et al., 2008; Khallaayoune et al., 2013; Naranjo et al., 2013). Possible selection-pressure and development of resistant mosquito populations, as well as ecological and environmental concerns, have prompted studies which tested alternative substances, such as eugenol and garlic oil, which were shown in several studies to possess effective insecticidal properties (Amonkar and Reeves, 1970; Amonkar and Banerji, 1971; Isman, 2000; Cetin et al., 2004; Aboelhadid et al., 2013; Zhao et al., 2013; Khater, 2014; Qualls et al., 2014). This was done in the aim to increase the range of compounds which are used as oral toxins, thereby reducing selection-pressure for specific toxins. In this study we tested the efficacy of the first commercially available ATSB formulation containing a commercial attractant and encapsulated garlic oil as the active ingredient for controlling the population size of *Anopheles sergentii*, the most common *Anopheles* species in Israel, and a main vector of malaria in the Afro–Arabian zone (Farid, 1956; Zahar, 1974).

2. Materials and methods

2.1. Study sites

The study was conducted in the Lower Jordan Valley, part of the Dead Sea Rift, which flanks the eastern part of Israel and the Palestinian territories. The climate in the region is arid, with annual precipitation ranging between 50 mm to 100 mm, and an average relative humidity of 20–30%. Local flora and fauna is typical of the Sahara–Arabian phyto-geographical zone, shifting to tropical conditions and associated biota around sporadic natural and anthropogenic water-sources.

Two sections of streamlets (ca. 1.5 km long) with similar vegetation, situated 10 km apart, were chosen as the experimental and control sites. The watercourses of both sites are

encompassed by riparian vegetation, which varies between 10–40 m in width (ca. 25 m average), and mainly consists of reeds and *Tamarix* spp. thickets. A short distance from the water-flow the vegetation abruptly changes to grassland with scattered shrubs and semi-shrubs. The two sites are "island-like" isolated ecological pockets suitable for mosquito breeding, and are predominantly inhabited by the malaria vector *A. sergentii*, the principle species found in this area.

2.2. Preparation and field application of ATSB solutions

Commercially available industrial-grade bait concentrates were purchased from Terminix®(Memphis, TN, USA). ATSB concentrate containing 0.4% (w/w) Garlic oil encapsulated in beta-cyclodextrin was diluted in tap water (1:3 ratio) containing blue food-dye (0.4% E132, Indigotine "Food Blue No. 1"; Stern, Netanya, Israel). For preparation of ASB (attractive but non-toxic sugar bait), concentrates lacking the active ingredient were similarly diluted in tap water containing green food-dye (0.4% Tartrazine 19140 "Special green"; Stern, Netanya, Israel). ATBS and ABS solutions were applied to a double perimeter of vegetation growing near the banks of either streamlets utilizing a Back-Pack Sprayer (Killaspray, Model 4526, Hozelock, Birmingham, UK) in accordance with label recommendations.

2.3. Experimental design and methods of ATSB field trials

The field trials were conducted over a period of 47 days, starting on September 30th, and ending on November 16th, 2013. Adult mosquitoes were sampled every two days for the first 11 days of the experiments, and every three days following the ATSB/ASB application, which was performed 13 days into the experiment. Female mosquitoes were captured in the evening with a Power Vac Back-Pack unit (John Hock, Gainesville, FL) while attempting to land on the legs of human baits, or in the morning from the surrounding vegetation using entomological sweep-nets. Human bait samples were collected between 20:00 and 22:00, and pooled into 5 min intervals, constituting nine repetitions per day for both the treatment and the control sites. Daily catches collected on human bait and on surrounding vegetation were stored at −70°C until further use.

2.4. Analysis of frequencies of ASB and natural sugar presence in gut

Mosquitoes captured on the surrounding vegetation were pooled according to gender, and random samples of 100 male and female mosquitoes were selected from the total daily catches obtained on four days prior to and six days following the treatment application, totaling 2000 mosquitoes from the control site, and slightly less ($n = 1734$) from the treatment site due to the gradual reduction in biting-pressure. Random samples of 100 female mosquitoes captured on the human baits were selected from the total daily catches obtained on four days prior to and six days following the bait application, totaling 1000 mosquitoes for the control site and 734 from the treatment site. The presence of natural plant-derived sugars in the gut contents of *A. sergentii* which were captured before the application of the bait solutions was confirmed by performing cold anthrone assays for fructose (Schlein and Jacobson, 1994). Specimens captured following the treatments were initially inspected visually under a dissecting microscope for the presence of ingested ASB-

or ATSB-derived food-dye in the gut tissue, followed by anthrone testing of samples in which food-dye was absent. Each mosquito was placed in the well of a flat-bottomed microtiter plate and soaked with 20 μl of 100% ethanol. Aliquots of 200 μl reaction solution, containing 0.15% anthrone (Sigma, St Louis MO, USA) w/v in 71.7% sulphuric acid were added to the wells and the specimens were homogenized with a glass rod, followed by incubation of the samples for 1 h at 25°C.

2.5. Ingestion of ASB by non-target insects

Experiments investigating the impact of ATSB on non-target insects were performed at a third site, similar to the two described above. ASB solutions containing no oral-toxins were pre-mixed with either green or yellow food-dyes E102, Tartrazine 19140 (Special green) and E110, Sunset yellow FCF 15985, (Stern, Natanya, Israel) which were then applied to flowerless or blossoming plants, respectively. Target and non-target insects were collected by a variety of methods, including two 6 m Malaise traps (BioQuip, CA), 40 pitfall traps (500 ml plastic cups), and 16 yellow plates (yellow disposable plastic plates, 20 cm diameter) which were used on 15 days throughout the experiments. In addition, collection with entomological nets was performed by two technicians everyday for one hour per day during the entire 15 day period. Six small UV tray traps (Müller et al., 2010) were deployed on eight nights, and a single large light-trap (white cloth with generator-powered 250 Watt mercury Vapor bulb) was used on six nights of the experimental period. All specimens collected were stored at −70°C before being subjected to taxonomic classification and visual inspections of gut-contents with the aid of a dissecting microscope. Presence of green or yellow food–dye in the digestive tissue indicated ingestion of bait from green or blossoming plant-material, respectively.

2.6. Statistical analysis

Statistical analysis was conducted with SPSS software version 20.0 (SPSS inc., Chicago, IL). Biting pressure count data recorded at the control and experimental sites before and after the ATSB treatments was analyzed by performing a general linear model for negative binomial distribution with a log link function to adjust for overdispersion of the data. Wald chi square statistics were used to assess significance, which was taken at $P < 0.05$. Values used throughout the text represent mean ± S.E.

3. Results

As shown in Fig. 1, application of garlic-oil ATSB greatly diminished the average biting pressure of *A. sergentii* in the treatment site over the experimental period. A significant reduction ($P < 0.01$) of more than 81% was seen four days after introduction of the toxic bait, at which the average biting pressure decreased from ca. 14 landings/minute before the treatment (mean ± S.E of all pre-treatment data = 69.7 ± 5.7 landings/5 min) to a transiently-stable 3 landings/minute (15.3 ± 2.1 landings/5 min) four to ten days following the application of the ATSB. Population densities continued to decline throughout the remainder of the experiment, to an average biting pressure of 0.35 landings/minute at the end of the field trials (1.5 ± 0.7 landings/5 min), constituting a decrease of 97.5% in the population density of *A. sergentii*. In contrast with the reduction seen at the treatment site, no

significant changes in biting pressure were observed following application of non-toxic ASB at the control site.

The extent of sugar-feeding and ATSB/ASB ingestion by the mosquito population were assessed by anthrone test for sucrose and by visual inspection of guts for the presence of food dye. Field samples were grouped by gender, collection time, location and capturing method (Table 1). Results show the majority of both female and male mosquitoes which were collected from the surrounding vegetation had ingested sugar-meals prior to the application of the treatment in both the experimental (70.7 ± 6.7% of the females and 72.75 ± 2.9% of the males; $n = 400$ for both groups) and the control sites (females −72 ± 3.7% of the females and 70.5 ± 3.33% of all males; $n = 400$). Moreover, the intensity of color-reaction indicated meal sizes were relatively large in many of the tested samples. In contrast, only 8.3 + 1.1% and 11.25 ± 2.66% of the female mosquitoes captured on human bait at the treatment and control sites, respectively, were found to have consumed sugars. The consistently low intensity of the anthrone color-reaction may indicate that these specimens had ingested (from natural sources) relatively small sugar-meals prior to capture, in line with reports that extensive ingestion of sugar by female mosquitoes can inhibit their blood-seeking and blood-feeding behaviors (Straif and Beier, 1996; Gary and Foster, 2001). Alternatively, ingestion of the toxic bait may inhibit both blood-feeding and sugar-feeding activities (Junnila et al., submitted), such that previously ingested sugars had been largely digested by the time of capture, considering the relatively long duration required for the insecticide to take effect.

Specimens collected after application of the ATSB and ASB were investigated visually for the presence of food-dye, and samples in which coloration was absent were subjected to cold-anthrone assays for sucrose. The fraction of sugar-fed *A. sergentii* captured on the surrounding vegetation was not affected by the treatment of ASB in the control site, with 71.8 ± 3.8% of the females and 75.5 ± 3.8 of the males testing positive in either assays. ASB-derived sugars accounted for 35% of the sugar-fed females (27.25 ± 0.9% of the total) and 49% of the sugar-fed males (37 ± 2.9% of the total), corresponding to ABS/natural sugar-fed ratios of 0.61 and 0.96 for females and male specimens captured at the control site, respectively. These values show that despite the abundance of natural plant sugar sources, bait-derived sugar was readily ingested by both male and female mosquitoes, indicating the commercial formulation has effectively attracted the target species to the applied bait. As most mosquito species seek and feed on plant sugars at least once a day, exposure to the added insecticide becomes highly likely as time progresses.

The application of ATSB had seemingly lowered the incidence of sugar-feeding in the treatment site by ca. 47% ($P < 0.01$), with only 41.5 ± 4.3% of the females and 39 ± 3.8% of the males captured on the surrounding vegetation testing positive for traces of food-dye or sucrose, compared with 70.7 ± 6.7% of the females and 72.75 ± 2.9% of the males captured at the same location prior to the application of the treatment. ATSB-derived sugars accounted for 20% of the sugar-fed females (8.2 ± 1.3% of the total) and 28% of the sugar-fed males (11.2 ± 2.8% of the total) captured after the application of ATSB in the treatment site. These correspond to ATBS/natural sugar-fed ratios of 0.25 and 0.39 for females and male specimens captured at the treatment site, respectively. However, consequent mosquito

mortality following the ingestion of ATSB likely explains these seemingly low values, since such feeding rates were sufficient for the near annihilation of the mosquito population at the end of the study period. Moreover, these results may be biased due to consequent behavioral changes of the mosquitoes, which are likely less active following ingestion of the garlic oil and are thus less prone to be captured by standard methods. This is further supported by the fact very few blood-seeking females were found to be stained by ASB at the control site, and even fewer were captured following the application of ATSB in the treatment site.

Samples of female *A. sergentii* which were collected on human bait following the applications of ATSB and ASB in the treatment and control sites, respectively, were also subjected to visual inspection and cold anthrone assays for sucrose. In agreement with the results described above, a smaller fraction of the blood-seeking females were found to be stained by ATSB than by ASB following the application of the treatments. Out of 600 specimens sampled at the control site, 11.7 ± 2.7% tested positive for sugar by cold anthrone assay for sucrose, compared to only 7 ± 2.5%, which were found to have ingested ASB by visual inspection. In contrast, while 13.2 ± 3.9% of the females captured at the treatment site ($n = 324$) tested positive for sucrose by cold anthrone assays, only 2.4 ± 1.8% were stained by ATSB. The ratio of ABS/natural sugar-fed females in the control site (0.59) was significantly higher ($P < 0.01$) than the ratio of ATBS/natural sugar-fed females which were captured in the treatment site (0.2). These results may be partially due to ATSB-induced mortality of the target insects, but may also reflect changes in feeding behavior of *A. sergentii* females following ingestion of the microencapsulated garlic oil, which likely inhibits host-seeking and blood-feeding activities.

As many non-target insects depend on plant-derived sugars for their energetic requirements, application of ATSB may have detrimental effects on the ecology of treatment sites. To test the extent of such impacts, additional trials were performed to determine what qualitative and quantitative proportions of the insect populations are prone to ingest ASB/ATSB following its application on different plant-types. Green or yellow food-dyes were added to non-toxic ASB solutions, which were subsequently applied to non-flowering or flowering plants, respectively. Insects were captured using several of techniques on 15 days and 8 nights throughout the experimental period (see methods). Two thousand mosquitoes from nine different morpho-species/ 4 genera and 20,399 non-target insects belonging to nine orders (including non-target Diptera species) and comprising 659 morpho-species were captured, classified, and visually inspected for the presence of green or yellow food dye in the gut tissue with the aid of a dissecting-microscope. Results presented in supplementary Table 1 and described below indicate ASB solutions were mainly ingested by the targeted mosquitoes and non-biting midges, both belonging to the order Diptera.

Out of the 2000 mosquitoes captured at the non-target trials (nine morpho-species 4 genera), 14.75% were found to contain green food-dye, while 18.5% were found to contain yellow food-dye, totaling 33.3% of the population found to ingest ASB from either type of plants.

Of the 4500 specimens of non-target insect belonging to order Diptera, 25% of the higher Diptera species, and 81% of the non-biting midge samples were found to contain food-dye, the former mainly foraging from flowering plants (22.9% containing yellow food-dye

compared to only 2.3% which tested positive for green food-dye), while in samples of the latter the proportion of green and yellow-stained specimens were almost equal (36.5% and 44.5%, respectively).

In contrast with target- and non-target insects from the order Diptera, a relatively small fraction of non-target insects belonging to other orders were found to have ingested bait material. Of the 15,899 non-target insects (excluding non-target Diptera), only 1462 (9.2%) were found to have ingested ASB. Out of these, 1356 (93%) ingested bait material which was applied to flowering-plants, while only 106 (7% of the sugar-positive insects, representing 0.65% of the total non-target insects which were sampled) were found to have foraged on ASB applied to the foliage of green plants. These results prove that specific application of the ATSB treatment exclusively to flowerless vegetation, as stated on the product label, can dramatically reduce the impact on non-target insects, while still maintaining a significant effect on target-insects and nuisance pests such as and biting non-biting midges, the latter proliferating into swarms which can impose significant economic burdens to many urban areas around the globe (Richard and Arshad Ali, 2006).

4. Discussion

In agreement with earlier reports (Aboelhadid et al., 2013; Amonkar and Reeves, 1970; Amonkar and Banerji, 1971; Zhao et al., 2013), data obtained in this study confirms the insecticidal properties of microencapsulated garlic-oil. The commercial ATSB formulation with garlic-oil as an active ingredient nearly exterminated the *A. sergentii* population at the study site, as inferred by the dramatic 97.5% drop from the biting-pressure values recorded prior to the treatment. The impact of garlic-oil ATBS on the mosquito population densities appears to be biphasic: a rapid, significant decrease in biting-pressure over a period of four days, followed by a transient stable period of six days, after which the population gradually declines over 25 days, reaching a mere 2.5% of the biting-pressure recorded prior to the application of the treatment. As larval stages are unlikely to encounter the plant-applied insecticide, this pattern may reflect the duration required for toxin exposure by the entire mosquito population, including all juvenile stages.

An important point demonstrated in this study is that when utilizing an efficient attractant, ATSB ably attracts sugar-feeding mosquitoes despite the availability of competitive natural-sugar sources, as a considerable fraction of the mosquito population was found to have ingested the bait solution. It should be noted that a larger proportion of both male and females consumed ASB in the control site than ATSB at the treatment site. These differences may be caused by mosquito mortality or behavioral changes, as laboratory trials have demonstrated mosquitoes show no aversion to ATSB when compared with ABS solutions devoid of garlic oil. An added advantage of this method is that older mosquitoes are more greatly impacted, since exposure to the toxin becomes more likely as time progresses. Maturation of infective sporozoite stages of *Plasmodium* sp. parasites within mosquitoes require several days (Beier, 1998), enhancing the vectorial capacity of older, mature adults. The ability of ATSB treatments to remove the more competent vectors from the environment makes it highly suitable for use in malaria-affected areas.

New vector control strategies must have low ecological-impact, and pose minimal risks to non-target insects. Consequently, the ATSB method may be of some concern, as insecticides are applied to plant species which may be important sugar-sources of a wide variety of species. Indeed, while a relatively low number of insects were found to be stained by either dye (9.2% of all non-target specimens, excluding those belonging to order Diptera), some non-target insects, especially specimens belonging to order Hymenoptera, were similarly prone to ingest ASB-derived sugars (23.6–31.4%, depending on the family). However, all of the non-target insects were found to have foraged almost exclusively from flowering plants (8.5% of the total, constituting 92.3% of the non-target insects which tested positive for food dye). While mosquitoes are also more likely to feed on bait sprayed on flowering plants (18.5%), a significant proportion of the target insects were found to have foraged from solutions applied to flowerless vegetation (14.5% of the total, constituting 44% of the target-insect which tested positive for food dye). These findings that are in agreement with recent studies, which also demonstrated ATSB application has little or no effect on predatory insects (Khallaayoune et al., 2013; Aboelhadid et al., 2013; Revay et al., 2014). Our report further demonstrates that selective targeting of desired vectors with can be greatly enhanced by following simple label guidelines of treatment application. Specifically, application of ATBS exclusively on green, non-flowering plants would cause negligible environmental impact while having minimal effect on the efficacy of vector-control. However, it is noteworthy that collecting methods were focused on adult insects, and foliage-feeding larvae were under sampled in our study. While we expect no major differences in ATSB-sensitivity between phytophagous larvae and foliage-feeding beetles and sap-feeding hemipterans (Table 1), more research is needed to assess the overall impact to the non-target species populations, e.g., through insects surveys 0–2 years following ATSB application.

5. Conclusions

The enduring threats of mosquito-borne diseases and the enormous impact they have on human health in developing countries underline the need for new vector-control strategies which can be easily implemented in affected areas by fairly simple means. A relatively new approach, ATSB is a highly effective method which targets the vectors based on their plant sugar foraging behavior, by combining oral toxins and sugars with potent mosquito attractant of plant origin. The results obtained in this study indicate that the now commercially available ATSB with EPA-exempt, microencapsulated garlic oil can be effectively used for *A. sergentii* control. The results presented above indicate that when ATSB is applied on flowerless vegetation it has a negligible effect on non target, particularly pollinating insects, making it an ecologically-safe alternative for conventional insecticides. Furthermore, as the efficacy of the treatment is mainly dependent on the attractant rather than the active ingredient which is of lesser importance, this method enables utilization of a wide range of insecticides which to date were deemed ineffective for mosquito control, minimizing resistance development due to intensive application of currently used insecticides. An additional benefit is that ATSB disproportionally impacts older adult mosquitoes which possess the highest vectorial capacity, and should thus greatly decrease the entomological inoculation rates when applied in affected areas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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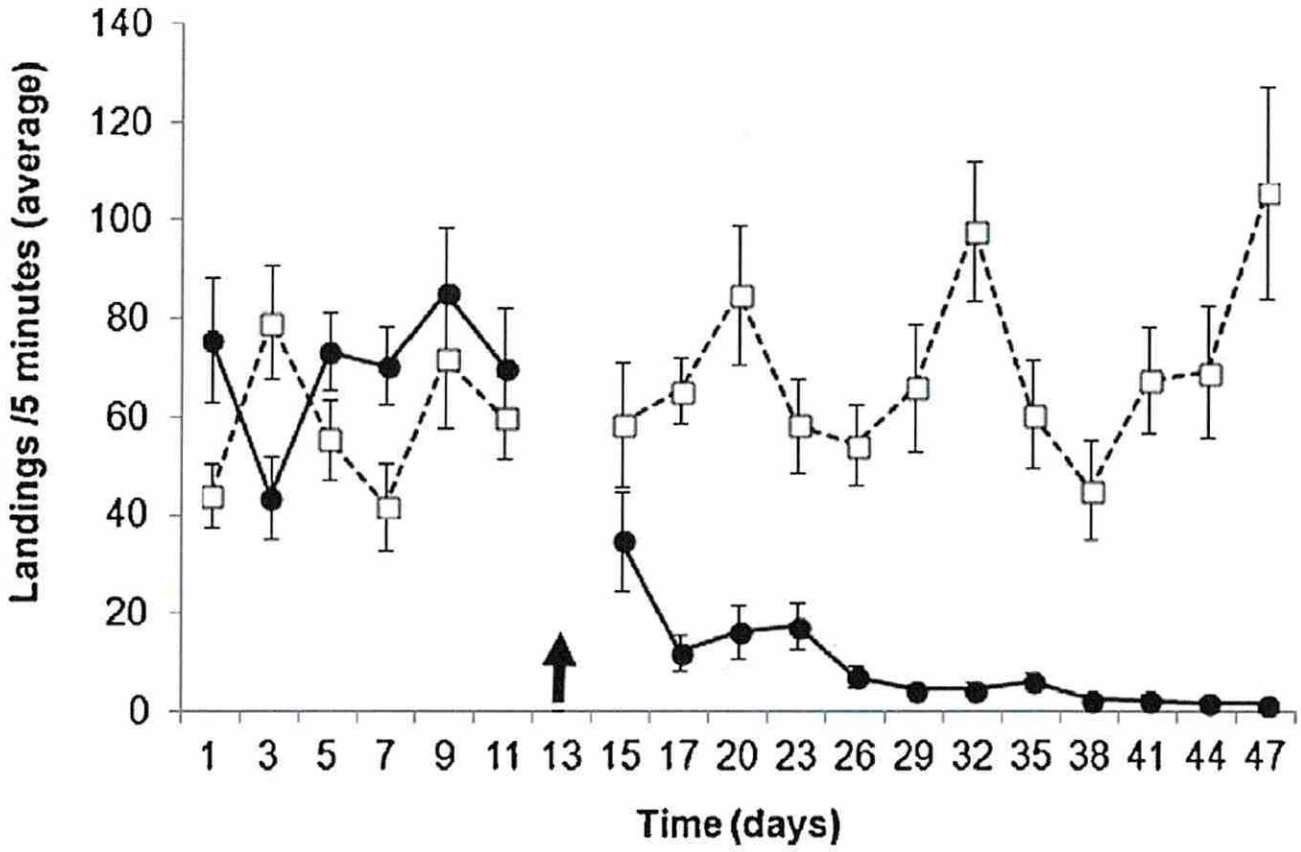

Fig. 1.

Effect of ATSB and ASB on the biting pressure of female *A. sergentii* in the treatment site (closed circles) and control site (open squares), respectively. Application of the solutions on the surrounding vegetation was performed on day 13 (black arrow). Values represent mean ± SE of females caught attempting to land on human bait during 5 min intervals over a period of 90 min, constituting 9 repetitions.

Table 1

Percent of the samples testing positive for sugar in anthrone assays for sucrose or by visual inspection for the presence of food-dye. Values represent mean ± S.E, sample size is indicated by superscript.

		Pre-treatment		Post-treatment[a]	
Location	**Site**	**Female**	**Male**	**Female**	**Male**
Vegetation	Control	72 ± 3.7%[b]	70.5 ± 3.3%[b]	71.8 ± 3.8%[c] (27.25 ± 0.9%)	75.5 ± 3.8%[c](37 ± 2.9%)
	treatment	70.7 ± 6.7%[b]	72.7 ± 2.9%[b]	41.5 ± 4.3%[d](8.2 ± 1.3%)	39 ± 3.8%[e] (11.25 ± 2.8%)
Human	Control	11.3 ± 2.7%[b]		18.7 ± 3.5%[c](7 ± 2.5%)	
bait	treatment	8.3 + 1.1%[b]		15.6 ± 3.8%[f](2.4 + 1.8%)	

[a] Values expressed in prentices are the percentages of the samples found to contain ATSB or ASB-derived food-dye in the gut tissue.

[b] ($n = 400$).

[c] ($n = 600$).

[d] ($n = 515$).

[e] ($n = 392$).

[f] ($n = 324$).

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PONTIFICIA UNIVERSIDAD CATÓLICA DEL ECUADOR
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Developing a Novel Attractive Toxic Sugar Bait (ATSB) Device for Intra-domiciliary Control of *Aedes aegypti*

Disertación previa a la obtención del título de Licenciado en Ciencias Biológicas

GALO E. RIVERA

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A mi madre.

Developing a Novel Attractive Toxic Sugar Bait (ATSB) Device for Intra-domiciliary Control of *Aedes aegypti*.

Short Title: *Aedes aegypti* Control with an ATSB Device.

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Abstract

On account of vector control being the most successful approach towards prevention of the major arboviral concerns worldwide and the imperative pursuit of alternatives to traditional pesticides, we developed a novel attractive toxic sugar bait (ATSB) device. The device incorporates seemingly inexpensive (cost < 1 USD) olfactory and visual cues attractive to *Aedes aegypti* L. We incorporated 1% boric acid in 10% sucrose solution as toxic component on the devices and this showed to be effective killing female *A.aegypti* in controlled laboratory conditions (0% survival probability after 48h).

In addition, we evaluated the biological action of the device and concluded that boric acid acts as a stomach poison. Using transmission electron microscopy we further determined that it disrupts the continuity of the epithelial tissue of the posterior midgut. The device is effective poisoning *A. aegypti* females in two different physiological statuses (recently blood fed and parous). Finally, devices were effective after 180 days of being assembled. These features provide us with information that will be useful for future semi-field and field trials.

Introduction

The diseases caused by dengue, chikungunya, and Zika viruses are becoming increasingly important public health concerns around the globe. Dengue virus (DENV) alone presents an estimate of more than 300 million new infections worldwide every year [1]. In the Americas only, dengue renders an average economic burden of USD 2.1 billion per year [2]. Chikungunya virus (CHIKV) and Zika virus (ZIKV) have caused major outbreaks in the Americas in 2013 and late 2015, respectively [3-5]. Furthermore, there have even been clinical reports of patients presenting co-infection between the three viruses in the region [6].

In Ecuador all three viruses have been reported and, according to health officials, the incidences of both chikungunya and dengue during 2015 (33,621 and 42,505 cases, respectively) more than doubled the incidence of these diseases during any of the previous four years [7].

Although there are promising efforts aimed at developing a vaccine against DENV [8-14], at the moment there are no commercially available vaccines or antivirals for any of these diseases leaving patients with the sole option of symptomatic treatment. As a consequence of this lack of direct approaches, vector control has become the main focus towards preventing these diseases.

Aedes aegypti L. is the principal vector of these three arboviruses [15-17] throughout its geographic distribution range. Originally from Africa, this mosquito species is, at present, widely distributed around the globe [18]. *A. aegypti* is both highly anthropofilic and highly synanthropic [19, 20, 21].

Female *A. aegypti*'s strong preference towards human blood hosts (i.e. anthropophily) has been vastly studied [20]. It is now known that this behavior is genetically fixed on at least one odorant receptor (*AaegOr4*), located on the antennae, which is highly sensitive to a component (Sulcatone) present in human odor at particularly high levels [19].

Synanthropy indicates the tight connection of species to human households. Several aspects of the biology of *A. aegypti* benefit from conditions provided by human habitations [21]. For instance, aquatic stages of their life cycle develop in artificial water containers in or around human habitations [22]. In addition, adults tend to rest on clothing or surfaces inside houses [23, 24]. These ecological and behavioral traits have made intradomicile control a priority [21].

Historically, pesticide spraying has been the main approach towards *A. aegypti* control. The intense application of dichlorodiphenyltrichloroethane (DDT), promoted by the PAHO during the

decades of the 1940s to the 1960s, achieved mosquito eradication in more than 18 countries in the Americas [25]. This intention to eradicate the mosquito at a continental scale rapidly fell apart after a lack of political interest on continuing eradication programs and strong selection of vectors resistant to DDT and other organochlorine insecticides [26]. This cleared the way for reinfestation on the course of the following decades. At present, long-term overuse of traditional pesticides has widespread selection of resistance alleles in mosquito populations which, consequently, has made the pursuit of new control methods imperative [27].

At present, a variety of novel mosquito control methods exist under different phases of development. These methods include, among other, the use of genetically modified mosquito strains, infection of mosquito strains with the intracellular parasite *Wolbachia*, and the use of attractive toxic sugar baits (ATSBs) [27].

ATSBs target the sugar feeding behavior of mosquitoes [27-29] by using sugary solutions laced with a toxic component [28, 30]. Boric acid has proven to be highly effective as the toxic component of ATSBs used against different species of mosquitoes, including: *Anopheles gambiae* Meigen [31], *Aedes albopictus* Skuse [30], and *Culex quinquefasciatus* Say [30]. In addition, boric acid presents a relatively low toxicity to humans and other vertebrates. To the best of our knowledge, there is no published information evaluating boric acid's toxicity on *A. aegypti.*

Most studies published on the use of ATSBs for mosquito control have consisted on aerosol dispersal of toxic sugar solution on outdoor vegetation and have been focused on the control of malaria vectors [31-36]. As far as we are aware, only a few studies have trialed ATSB's in intradomiciliary conditions, and were carried out in Africa to control malaria vectors as well [37,38].

The efficiency of ATSBs depends on the sugar feeding behavior of mosquitoes. Some controversy has arisen regarding sugar consumption of female mosquitoes following blood ingestion

[39]. Since this would directly affect the performance of the devices, it is worth mentioning that in field conditions, female mosquitoes readily feed on sugar [40, 41]. However, there is a relevant requirement for further information to clarify potential effectiveness of ATSB's in different physiological statuses and for novel means of enhancing ATSB's attractiveness.

It is known that olfactory, visual, and thermal cues are key for mosquitoes in detecting potential hosts [42]. Regarding visual cues, black and white contrasts have proven to be strong long-range attractants for *A. aegypti* [43, 44] and have been previously used on successful mosquito traps [45] therefore we consider visual cues to be of vast importance for the development of novel traps and baits. We think that ideally the sum of these cues will play an important role on improving the attractiveness of ATSBs nonetheless simplicity is required for logistical purposes.

In this study we present the design of a simple ATSB device, which has the potential to be used as a tool for the reduction of indoor adult populations of *A. aegypti*. We assessed the efficiency of this device under laboratory conditions, provide insight into the biological mode of action of the devices, and evaluated parameters relevant for future field trials. Given the fact that devices will potentially be utilized inside households of vulnerable sectors of the population we feel it is important for the device to be safe for humans and affordable as well.

Methods

Mosquitoes

Two Ecuadorian strains of *Aedes aegypti* (Ae. aeg-2: Ecuador, Guayas province, Guayaquil city, acquired in 2014; T-COCA 02.1: Ecuador, Orellana province, Puerto Francisco de Orellana city, acquired in 2015), generously provided by the National Service for Malaria Eradication (SNEM) and maintained at the Center for Research on Heath in Latin America (CISeAL), were used in the experiments. Mosquitoes were reared and maintained under standard insectary conditions: 28 ± 2 °C temperature; 80

± 10 % relative humidity; 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 15 x 15 x 15 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 (*Mus musculus* L.). 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" were deprived of access to sugar during 48 hours previous to their use in experiments (but allowed continuous access to water throughout this time).

Attractive Sugar Bait Devices

The devices consisted of two concentric foam sheet circles: an inner white circle (5 cm diameter) and an outer black circle (10 cm diameter). Before assembly, both foam circles were individually submerged for 24 hours in either a non-toxic sugar solution (10% sucrose, prepared using distilled water and brown sugar) or a toxic sugar solution (1% boric acid, prepared using 10% sucrose solution as solvent). Henceforth in this manuscript, devices coated with non-toxic sugar solution will be called "attractive sugar baits" (ASBs) in order to differentiate them from ATSBs, which are coated with the toxic sugar solution.

After 24-hour submersion, the foam circles were air-dried for 24 hours and subsequently stapled together. A bamboo stick was fixed to the back of the device, to serve as a stand (Fig. 1B).

Fig 1. Attractive Toxic Sugar Bait Devices. (A) 3D model of the attractive toxic sugar bait devices. Diameters for each of the foam sheet circles are shown. (B) Photography of an attractive sugar bait device. Photographed by M. Neira.

Survival Assessment of Mosquitoes Exposed to the Device

To determine whether exposure to the ATSB devices has an influence on adult mosquito survival probability, we conducted an experiment in which groups of 30 adult female mosquitoes, placed in a 15 x 15 x 15 cm cage, were exposed during 48 hours to either an ATSB device (for experimental treatments) or an ASB device (for control treatments). Mortality in each cage was recorded every 24 hours. The test was replicated four times. The assessment was repeated using each of the two strains.

For each treatment, interval censored survival data and subsequent non-parametric maximum likelihood estimate (NPMLE) was plotted and analyzed using the 'survival' package [39] in R version 3.2.2 (R Core Team, www.r-project.org). A log-rank hypothesis test was used to compare the survival distributions of the two treatments.

Appraisal of the Biological Mode of Action of the Devices

Uptake Mechanism of the Toxic Component. To establish whether the toxic component of ATSBs needs to be ingested by the mosquitoes in order to exert its effect, we presented the devices to cohorts of adult females which were unable to ingest food due to the surgical ablation of their mouthparts. To establish these cohorts, individuals 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 (Fig. 2). Following intervention, mosquitoes were left to rest for 24 hours before being used in any experiment.

Fig 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.

To control for the potentially 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.

For each experiment two cages were set up, each containing 20 starved ablated mosquitoes. Individuals in one of these cages were exposed to an ATSB device, and individuals in the other cage were exposed to an ASB device. Two more cages containing 20 non-ablated, starved mosquitoes each were set up likewise, making a total of four cages (a summary of the experimental set-up is shown in Table 1). Mortality in all groups was assessed at 24 and 48 hours of exposure to the devices. The experiment was replicated three times. Only one strain (Ae.aeg-2) was tested. Normal distribution of the data was determined with Kolmogorov-Smirnov and Shapiro-Wilk tests. Analysis of variance (ANOVA) was performed to evaluate differences between treatments and a post-hoc Tukey's test was used to determine ranks. These analyses were performed in R version 3.2.2 (R Core Team, www.r-project.org).

Table 1. Names assigned to treatments used to evaluate the mechanism of ingression of the toxic component

	Whole proboscis	**Cut proboscis**
Toxic Device	"Whole toxic"	"Cut toxic"
Non-toxic device	"Whole non-toxic"	"Cut non-toxic"

Histopathological Effects on the Midgut. Two cages were set up, each containing 30 adult starved female mosquitoes. Specimens in one of these cages were exposed to a toxic device (ATSB), and specimens in the other cage were exposed to a non-toxic device (ASB). Cages were monitored during the next 24 hours, and dead mosquitoes were removed by aspiration every hour from the cages. Using a dissection microscope, the legs, head and wings of every dead specimen were removed on a drop of 70% ethanol. The abdominal cuticle was gently disrupted in order to permit the exposure of internal tissues to

the fixative. Afterwards, individuals were fixed in a solution containing 2.5% glutaraldehyde, 2.5% paraformaldehyde in 0.1M cacodylate buffer (pH 7.4), and stored at 4 °C for 72 hours. Specimens were then washed 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 (Epon 812, Araldite 502, dodecenyl succinic anhydride, benzyl dimethylamine) mixture for one hour, and later, 1 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 embedded tissues were cut using an ultramicrotome and mounted on copper grids. Later, mosquitoes were stained using 2% uranyl acetate. Specimens were observed using a transmission electron microscope and micrographs of tissues of interest were obtained.

Evaluation of Parameters Relevant for Future Field Trials

Effects of the Physiological Status of the Mosquitoes on the Performance of the Device. These tests were performed using strain TCOCA 02.1. Two different physiological statuses were evaluated using mated starved female adult mosquitoes: blood fed and parous. Females deemed as "blood fed" were established by selecting blood-engorged individuals immediately after a blood meal. Females deemed as "parous" were first blood fed and subsequently maintained for 7 days under insectary conditions, in order to ensure that they had oviposited before being used for experimentation.

Two cages for each of the defined physiological statuses were set up with 30 mosquitoes each. One cage exposing them to an ATSB and the other to an ASB. Survival data was gathered at 24 and 48 hours. The test was replicated 3 times. Interval censored survival data was plotted and analyzed using

the 'survival' package [39] in R version 3.2.2 (R Core Team, www.r-project.org). A log-rank hypothesis test was used to compare the survival distributions of the two treatments.

Shelf Life of the Device. In order to determine the shelf life of ATSB devices, toxicity tests were performed using ATSB and ASB 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 inside an incubator at 28 ± 2 °C and 80 ± 10% relative humidity.

The protocol for performing the bioassays was identical to that previously described to assess survival of mosquitoes exposed to the device. For each group of mosquitoes exposed to an ATSB device, we set up a matching control group exposed to an ASB device stored during an equivalent amount of time.

For each storage time, three replicates of the experiment were set up. Interval censored survival data was plotted and analyzed using the 'survival' package [39] in R version 3.2.2 (R Core Team, www.r-project.org). A log-rank hypothesis test was used to compare the survival distributions of the two treatments.

Results

Survival Assessment of Mosquitoes Exposed to the Device

Mosquitoes exposed to toxic devices presented 55% survival probability reduction in the first 24 hours post-exposure, and 45% reduction between 24 and 48 hours post-exposure, resulting in a 0% survival probability by the end of the trials. On the other hand, mosquitoes exposed to control devices presented 0.83% survival probability drop during the first interval (0h-24h] and 1.67% reduction during the second interval (24h-48h], resulting in 97.5% survival probability by the end of the experiment (Fig.

3). Differences between the survival curves of toxic and non-toxic treatments were highly significan ($p<0.001$).

Fig 3. Survival Assessment of Mosquitoes Exposed to the Device. Survival and NPMLE of individuals exposed to toxic (dotted line; n=120) or non-toxic devices (solid line; n=120). Interval-censored survival data collected at two time points (24h and 48h).

Appraisal of the Biological Mode of Action of the Devices

Uptake Mechanism of the Toxic Component. After 48 hours, mosquitoes which could still feed (i.e. mosquitoes with an intact proboscis), presented 100% mortality when exposed to the toxic device, and 3.33% mortality when exposed to the non-toxic device. Mosquitoes which were intervened in order to block feeding presented 38.33% mortality regardless of the toxic or non-toxic condition of the devices. Significant differences were found between the four treatments ($p<0.001$). Post-hoc pairwise comparison determined only intervened treatments were not significantly different between each other (Fig. 4).

Fig 4. Uptake Mechanism of the Toxic Component. Mortality after 48 hours of exposure to devices and mosquito conditions summarized in Table 1. The letters above the bars show ranks of statistical significance. Different letters mean a $p<0.05$.

Histopathological Effects on the Midgut. Mosquitoes that had ingested toxic sugar solution presented histological abnormalities in the posterior midgut. Electron micrographs depict a disruption of the continuity of the epithelial tissue (Fig. 5A, 5C). Due to the distribution of bacteria in the gut lumen, we suggest this disruption cannot be considered a microscopy artifact. In addition, we found abnormal adipocytes that we hypothesize are undergoing a process of necrosis (Figs. 5E, 5F). We suggest these

two affections are the probable cause of death of these individuals. Microscopic images of individuals that were only exposed to sucrose solution presented none of these pathologies on the posterior midgut (Figs. 5B, 5D).

Fig 5. Histopathological Effects on the Midgut. Longitudinal sections of *Aedes aegypti* posterior midgut. (A,C,E,F) Mosquitoes exposed to toxic devices. (B, D) Normal posterior midgut of mosquitoes exposed to non-toxic devices. Abbreviations: LM, gut lumen; AC, adipocyte; ED, epithelial disruption.

Evaluation of Parameters Relevant for Future Field Trials

Effects of the Physiological Status of the Mosquitoes on the Performance of the Device. Both physiological statuses evaluated ("blood fed" and "parous") presented a lower survival probability when exposed to toxic devices than when exposed to non-toxic devices.

Blood fed females' survival probability dropped 13.33% during the (0h-24h] interval, 22.22% during the (24h-48h] interval, and 55.56% during the last interval (48h-72h]. This results in 8.89% survival probability by the end of the experiment after 72 hours of exposure. On the other hand, the non-toxic control for this physiological status resulted in 90% survival probability by the end of the 72 hours after having dropped 8.89%, 1.1%, and 0% during the (0h-24h], (24h-48h], and (48h-72h] intervals, respectively. Differences between control and toxic treatment survival curves are highly significant ($p<0.001$).

Parous females presented 65.6% decline on their survival probability during the first interval (0h-24h] and 0% survival probability after 48 hours of being exposed to toxic devices. These results are significantly different ($p<0.001$) to the non-toxic control, which showed 2.2% survival probability drop during the (0h-24h] interval, resulting in 97.8% survival probability after 48 hours of exposure had passed (Fig. 6B).

Fig 6. Effects of the Physiological Status of the Mosquitoes on the Performance of the Device. Survival of individuals exposed to toxic (dotted line; n=90) or non-toxic devices (solid line; n=90). Survival curves and NPMLE for (A) blood fed individuals exposed for 72 hours and (B) parous mosquitoes exposed to the device 48 hours.

Shelf Life of the Device. Mosquitoes exposed to toxic devices stored for 38 days showed 0% survival probability after the (0h-24h] interval. On the contrary, non-toxic treatment showed 96% survival probability after the (24h-48h] interval was concluded. Highly significant differences were found between treatments (Fig. 7A).

Mosquitoes exposed to toxic devices stored for 80 days showed 16% survival probability after the (0h-24h] interval, and 0% survival probability at the end of the experiment. On the other hand, non-toxic treatment showed 97% survival probability after the (24h-48h] interval was concluded. Highly significant differences were found between treatments (Fig. 7B).

Mosquitoes exposed to toxic devices stored for 118 days showed 95% survival probability after the (0h-24h] interval, 64% survival probability during the (24h-48h] interval, and 35% survival probability by the end of the trials. On the contrary, non-toxic treatment showed 96% survival probability after the (24h-48h] interval was concluded. Highly significant differences were found between treatments (Fig. 7C).

Fig 7. Shelf Life of the Device. Survival and NPMLE of individuals exposed to toxic (dotted line; n=90) or non-toxic devices (solid line; n=90) that had been stored for: (A) 38 days, (B) 80 days, and (C) 118 days. Interval-censored survival data collected in two time points (24h and 48h).

Discussion

Our results provide strong evidence that toxic sugar bait devices loaded with boric acid as active component are highly toxic to *A. aegypti* when tested under our experimental conditions.

Our appraisal of the biological action of the devices provides an insight into how low concentrations of boric acid (1% boric acid in 10% sucrose solution) affect *A. aegypti* and have the potential of causing mortality in these insects. Although boric acid's toxicity has been previously reported for other mosquito species [28-38], to the best of our knowledge, this is the first report of the effect of this compound on this species of mosquito. By determining that the mechanism by which the toxic component enters the body of the insect is ingestion, we provide further evidence to support the notion that this inorganic pesticide acts as a stomach poison, which has been previously suggested [29, 30]. Because the digestive system of insects is well adapted to avoid intoxication and infection, an effective stomach poison needs to be able to cause cellular damage [47, 48]. Our electronic microscopy analysis confirms that insects which ingested boric acid, display tissue abnormalities similar to those previously reported in other insect species which ingested stomach poisons [47, 48]. Based on our experimental results, we suggest that in *A. aegypti* these effects are altering the integrity and normal performance of the midgut, to the point of becoming lethal to the mosquitoes.

The evaluation of the influence of the physiological status of the females becomes relevant for the eventual use of the devices in the field, where female mosquitoes are likely to have access to various sources of food, including blood and sugar. There has been some debate about whether female *A. aegypti* do in fact consume sugar when they have blood available as a food source [39, 40]. The fact that significant mortality is observed when recently blood-fed mosquitoes are exposed to the ATSB devices supports the notion that blood feeding does not completely inhibit sugar feeding behavior in this species.

Under our experimental conditions, exposure of blood fed females to ATSB devices does result in significant mortality, albeit at a somewhat reduced rate when compared to the mortality observed in

starved individuals. Interestingly, the largest drop in survival probability in blood fed females is observed between 48h and 72h post-exposure to the ATSB (Fig. 6), suggesting that after 48 hours 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 deployed in the field, ATSB devices would be efficient in killing female mosquitoes of various physiological statuses, including females which have already ingested blood – a particularly important group from an epidemiological standpoint.

One other parameter considered important for future field trials was the shelf life of the devices. The fact that toxic devices are still effective after 118 days storage (in conditions similar to those found in the field), enormously simplifies potential logistical challenges that might occur during future field and semi-field trials. As far as we can tell, the extended duration of the devices (at least 118 days) is probably a consequence of the boric acid's relatively high stability.

Further investigation on evaluating the device's attractiveness is suggested. Our research does not quantify how attractive to mosquitoes are the devices. We suggest additional semi-field trails in order to evaluate this and various other parameters.

Finally, we would like to remark the need for developing novel control methods which take into consideration geographic, social, and economical challenges affecting those individuals most vulnerable to mosquito-borne infections. The attractive toxic sugar bait devices we developed in this investigation resulted to be relatively inexpensive, having a cost of less than 1 USD. The actual cost might result to be even lower if the devices were to be mass-produced. Furthermore, the toxicity of boric acid to vertebrates is low in the concentrations which were used for the devices. This provides an enormous logistical asset.

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FIGURES

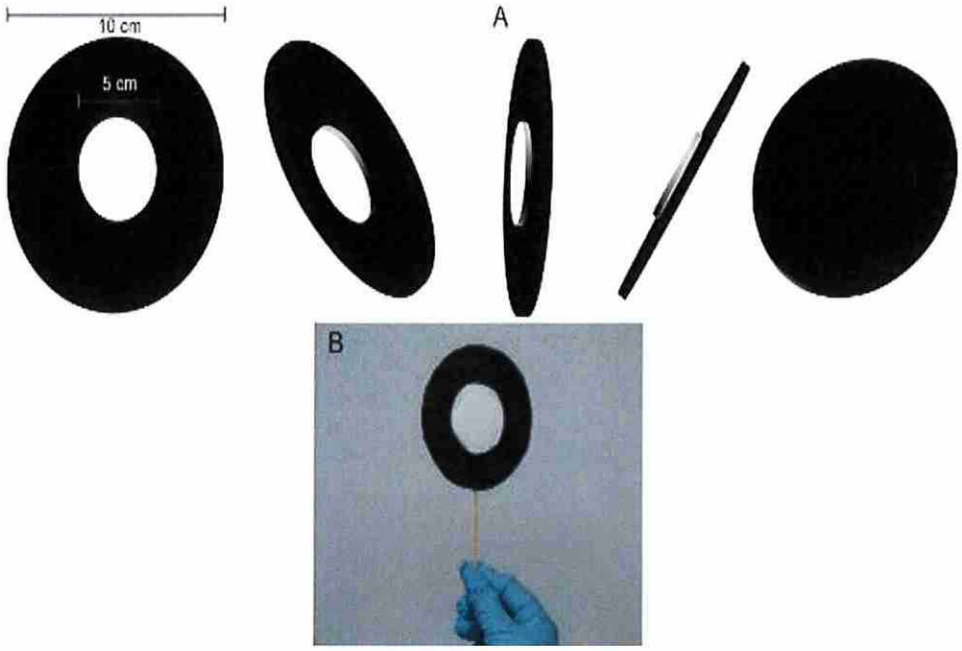
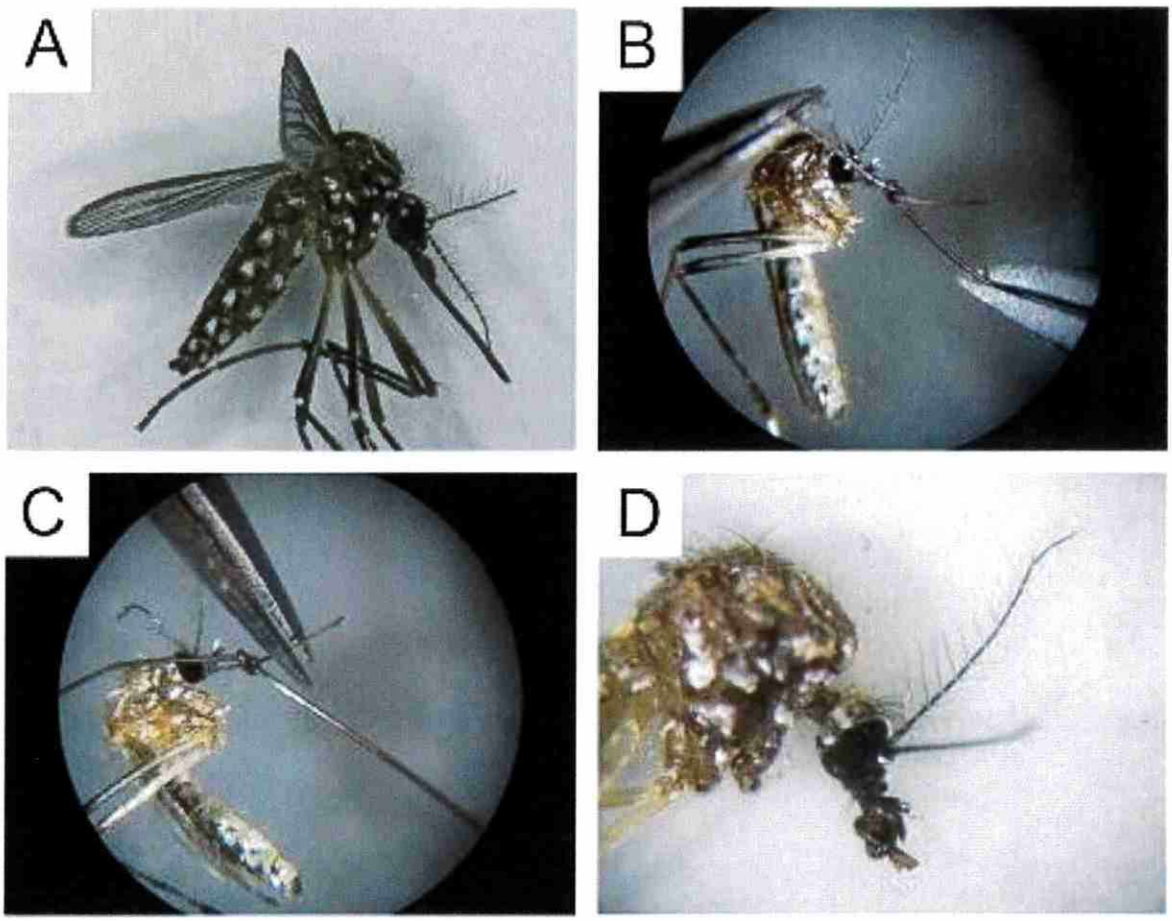


Fig 1. Attractive Toxic Sugar Bait Devices. (A) 3D model of the attractive toxic sugar bait devices. Diameters for each of the foam sheet circles are shown. (B) Photograph of an attractive sugar bait device.



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.

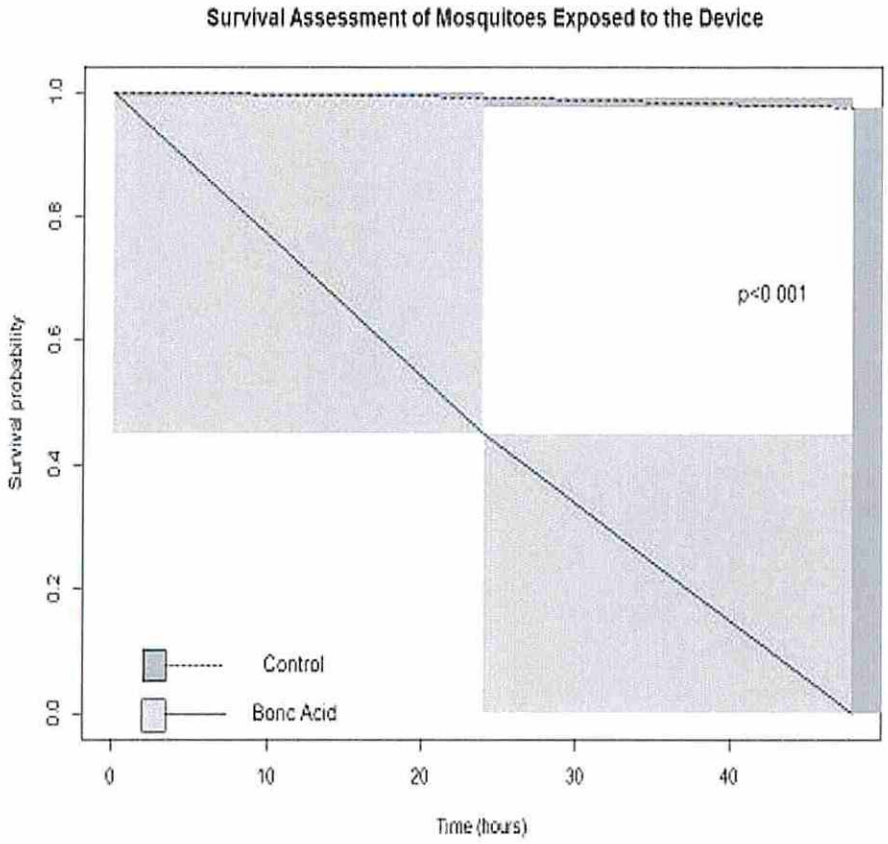


Fig 3. Survival Assessment of Mosquitoes Exposed to the Device. Survival and NPMLE of individuals exposed to toxic (dotted line; n=120) or non-toxic devices (solid line; n=120). Interval-censored survival data collected at two time points (24h and 48h).

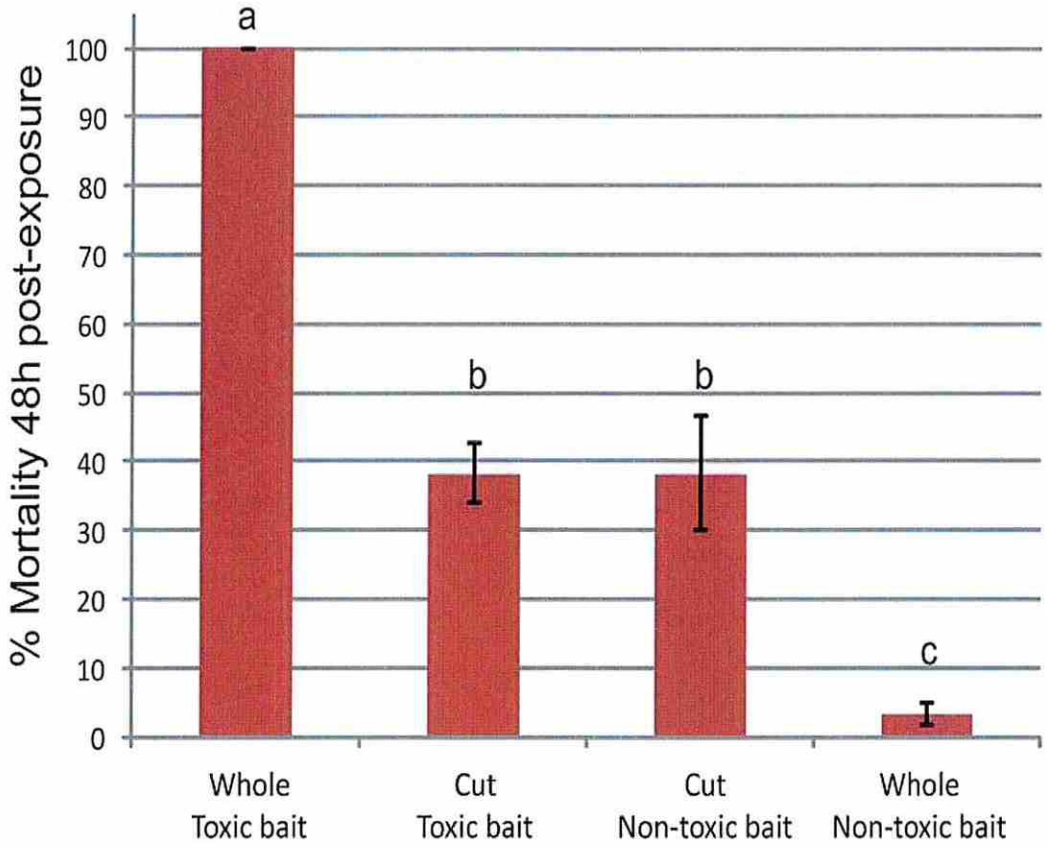


Fig 4. Uptake Mechanism of the Toxic Component. Mortality after 48 hours of exposure to devices and mosquito conditions summarized in Table 1.

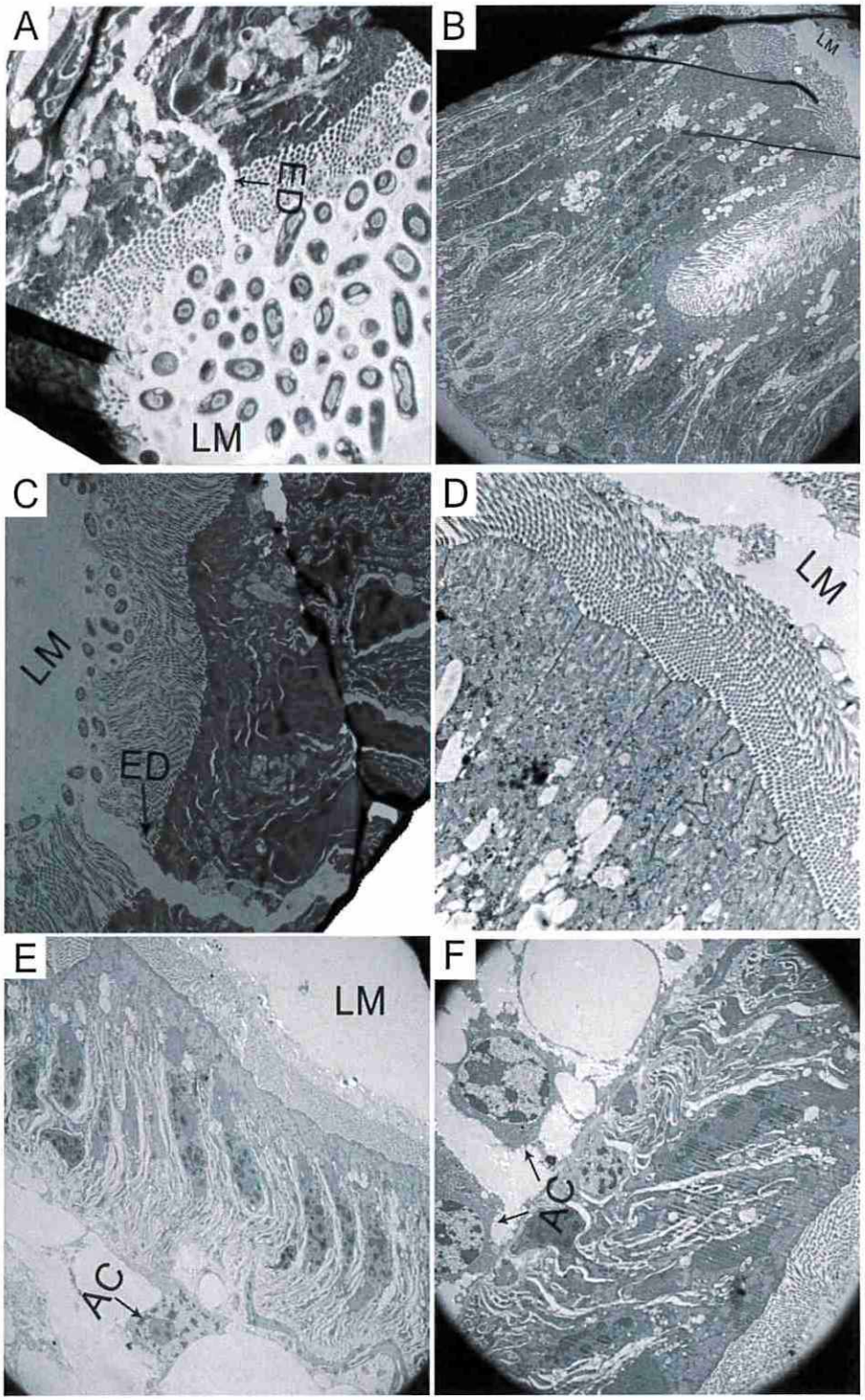


Fig 5. Histopathological Effects on the Midgut. Longitudinal sections of *Aedes aegypti* posterior midgut. (A,C,E,F) Mosquitoes exposed to toxic devices. (B, D) Normal posterior midgut of mosquitoes exposed to non-toxic devices. Abbreviations: LM, gut lumen; AC, adipocyte; ED, epithelial disruption.

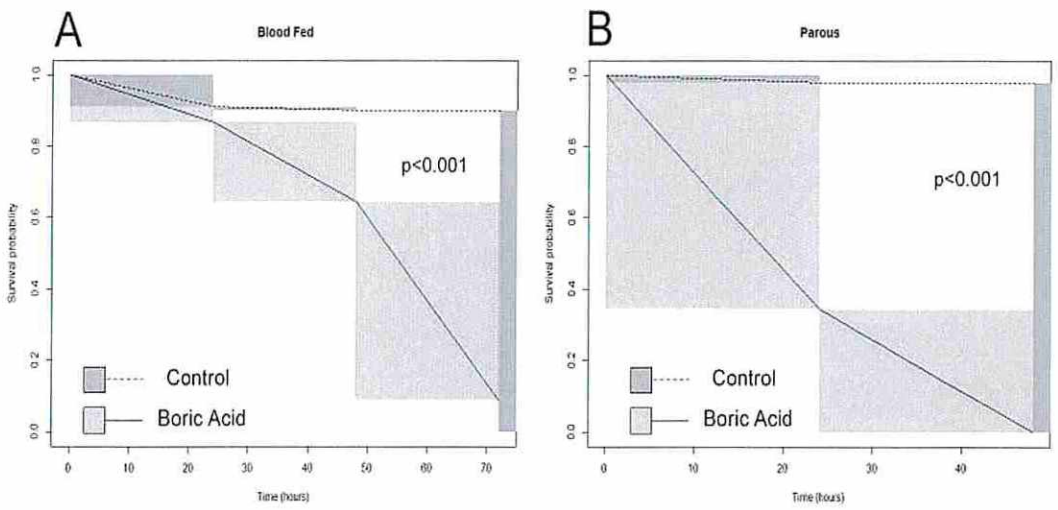


Fig 6. Effects of the Physiological Status of the Mosquitoes on the Performance of the Device. Survival of individuals exposed to toxic (dotted line; n=90) or non-toxic devices (solid line; n=90). Survival curves and NPMLE for (A) blood fed individuals exposed for 72 hours and (B) parous mosquitoes exposed to the device 48 hours.

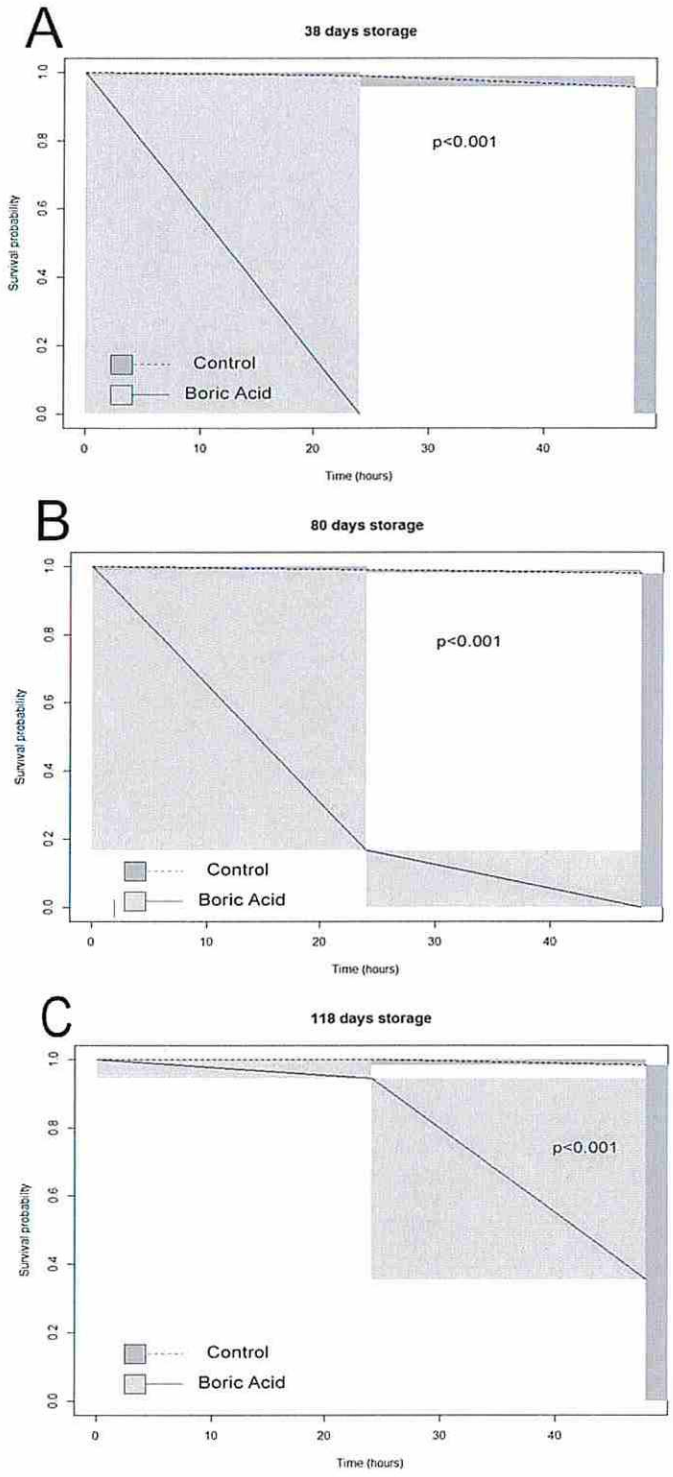


Fig 7. Shelf Life of the Device. Survival and NPMLE of individuals exposed to toxic (dotted line; n=90) or non-toxic devices (solid line; n=90) that had been stored for: (A) 38 days, (B) 80 days, and (C) 118 days. Interval-censored survival data collected in two time points (24h and 48h).

Scientific Note

Control of male *Aedes albopictus* Skuse (Diptera: Culicidae) using boric acid sugar bait and its impact on female fecundity and fertility

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Aedes albopictus (Skuse) is a serious threat to human health as a vector of arboviruses such as dengue, chikungunya (Lambrechts et al. 2010, Gubler 1998), and Zika (Wong et al. 2013). Adult *Ae. albopictus* are controlled primarily via ground and aerial ultra-low volume applications of insecticides. The chemical insecticides may potentially pollute the environment, and mosquito populations gradually develop resistance to them (Waits et al. 2017). The future for mosquito control requires safer and more effective alternatives to replace and improve currently used adulticides.

Male and female mosquitoes require carbohydrates after emerging for energy, survival, and reproduction (Yuval 1992, Foster 1995). Adult *Ae. albopictus* mosquitoes, like other mosquito species, need regular sugar meals (Muller et al. 2010a, Xue et al. 2010). Attractive toxic sugar bait (ATSB) is a novel method for adult mosquito control. This control method takes advantage of mosquito sugar-feeding behavior by mixing insecticides with their food (Lea 1965, Xue et al. 2013). The ingredients in ATSB are fruit juice (attractive), 10% sugar (feeding stimulator), and 1% boric acid (stomach toxin), and adult male and female mosquitoes usually die 24-48 h after ingestion of TSB or ATSB (Xue and Barnard 2003, Xue et al. 2008). The ingredients of toxic sugar bait (TSB) are usually 10% sugar and 1% boric acid, without fruit juice as an attractant. When applied to foliage, TSB or ATSB was successful in controlling *Ae. albopictus* in both semi-field conditions (Xue et al. 2006) and residential communities in St. Augustine, FL (Naranjo, et al. 2013). ATSB or TSB can be targeted to males when timed to their emergence. Male mosquitoes typically emerge at least 12 h earlier than females (Xue and Barnard 1997) and feed only on sugar for nutrition and survival (Xue et al. 2010). This early emergence time and complete dependence on sugar for survival suggests male mosquitoes can be affected by TSB or ATSB. If TSB or ATSB exposure is directed to this early emergence time, it may have an indirect effect on female mosquito fecundity and fertility.

In this study, we tested the hypothesis that mosquito reproductive capacity is negatively affected if male *Ae. albopictus* are exposed to TSB before females emerge. After a brief exposure period to TSB, male and female mortality were analyzed to see if the exposure period only affected males. Next, reproductive effects of females exposed to TSB were analyzed by counting the number of eggs laid and the hatch rate. Each assessment of TSB was compared to a 10% sucrose control.

The *Ae. albopictus* strain was obtained from the USDA, ARS, Center for Medical, Agricultural, and Veterinary Entomology, in Gainesville, FL and reared in the insectary at Anastasia Mosquito Control District (AMCD) in St. Augustine, according to the method described by Gerberg et al. (1994). The adult mosquitoes were fed a 10% sucrose solution *ad libitum*, and were maintained in a photoperiod of 14:10 (L:D) h at 80% relative humidity and temperature of 27° C.

The toxic sugar bait (TSB) formulation was prepared by adding boric acid at 1% W/V to a 10% sucrose solution. The TSB (treatment formulation) was mixed and heated at 60° C until the boric acid completely dissolved followed by cooling to room temperature. The control formulation was a 10% sucrose solution, and both treatment and control formulations were soaked in cotton balls and fed to mosquitoes.

Four hundred pupae were collected and equally distributed at 200 each between treatment and control groups. The pupae were housed inside a screened cage with either TSB (treatment) or 10% sucrose in reverse osmosis water (control). The treatment and control groups were provided their respective solutions after pupae began to emerge to adults, and the time of feeding was noted. Most of the males emerged and fed at the same time. The treatment and control group solutions were replaced with a 10% sucrose solution after TSB exposure for 24 h. After 96 h, the mosquitoes were fed chicken blood by gently holding a chicken's feet in the cage for at least 15 min. The procedure was in accordance with protocol # AC 2005 approved by the animal care and use committee at the Anastasia Mosquito Control District. Brown oviposition filter papers (25 x 8 cm) were placed in a black ovicup (200 ml) with 100 ml infusion water for the treatment and control group after 48 h, respectively. The blood-fed and ovipositing mosquitoes had access to a 10% sugar solution via a cotton strip.

The egg papers were collected from ovitraps and dried at room temperature. The numbers of eggs from the treated and control groups were counted under a microscope. The hatch rate was calculated by comparing the number of hatched larvae between treatment and control groups. Hatch rate was calculated as: [% hatch rate = total of larva ×100/ total of eggs]. This experiment was repeated three times with different cycles of mosquitoes from the same colony in identical laboratory conditions.

All statistical analyses were performed with SPSS 16.0 statistical software. Comparisons of the data between treatment and control groups were performed using the two-tailed Student's t test. Hatch rate comparisons between treatment and control groups were performed using the Chi-square test. Differences between treatment and control groups were considered statistically significant at $p \leq 0.05$.

The number of male and female mosquitoes that emerged from the water to be exposed to the TSB were calculated to determine

Table 1. The number of male and female *Ae. albopictus* that emerged between TSB treatment (1% boric acid sugar bait) and control groups (10% sucrose solution) (n = 200 pupae).

Repetition	TSB treatment group		Sucrose control group	
	No. of males	No. of females	No. of males	No. of females
1	112	74	108	71
2	104	69	111	65
3	118	61	121	66
Mean (SE)	111.3 (7.0)[a]	68 (6.6)[b]	113.3 (6.8)[a]	67.3 (3.2)[b]

[a,b]Means in each row followed by the same letter are not significantly different from each other ($P > 0.05$) using a Student's t-test (SPSS 16.0).

the overall health of each repetition. The emerging number of male mosquitoes (t=0.354, df=4, P=0.741) and female mosquitoes (t=0.158, df=4, P=0.882) were not significantly different between treatment and control groups (Table 1). This indicates that there were no significant health abnormalities between each cycle of mosquitoes used from the colony.

Mosquito mortality was calculated by counting the number of dead male and female mosquitoes at 24 and 48 h post-exposure. The mean number (51, 102) of dead males exposed to boric acid bait (treatment) was significantly higher than in control mosquitoes (5, 12) at 24 h (t=6.850, df=4, P=0.002) and 48 h (t=20.893, df=4, P=0.000), respectively. The mean number (3, 8) of dead female mosquitoes exposed to boric acid bait (treatment) was not significantly different, compared to the control (2, 9) at 24 h (t=1.000, df=4, P=0.374) and 48 h (t=0.802, df=4, P=0.468), respectively (Table 2). These results indicate that only males suffered significant population reduction following exposure to boric acid baits. Also, minimal loss of females to TSB at 48 h indicates that little to no females ingested TSB during the exposure period.

Eggs were counted between the treatment and control groups to determine if early male exposure to TSB reduced the capacity of the population to reproduce. The mean number (1,070) of eggs produced by females exposed to TSB was significantly reduced (t=10.834, df = 4, P=0.0001) compared to the control (1,858, Table 3). Next, the egg hatch rate was analyzed by comparing the percentage hatch rate between treatment and control groups to get a rate of hatch reduction. Hatch reduction rates indicate that females laid non-fertilized eggs and potentially mated with males that were compromised due to TSB exposure. The mean egg hatching rate (54%) was significantly reduced in females exposed to TSB (χ^2= 60.004, df=1, P=0.000) compared to the control (72%, Table 4).

Current control strategies against *Ae. albopictus* include trapping, chemical/biological insecticide treatment, and public health education for source reduction and prevention of mosquito bites. The major problems with several kinds of insecticides currently used for mosquito control are their persistence in the field, the rapid development of insecticide resistance, and their impact on non-targeted species. The majority of current mosquito control methods focus on females, while male mosquitoes are seldom targeted. In reality, the males contribute substantially to many aspects of female behavior and physiology and indirectly influence parasite transmission through their effects on the female (Klowden, 1999). This study on the control of male mosquitoes presents a new alternative strategy to control mosquito populations.

It is evident from this study that the male mosquitoes emerged earlier than female mosquitoes, and the emerging male mosquitoes fed on sugar for nutrition and survival. The TSB significantly reduced male survival under laboratory conditions with a morality rate at 91.3% at 48 h. On the other hand, females were not exposed to TSB to the same extent as males because they emerged later, and they suffered little to no mortality. Based on this study, we suspect that a lower abundance of male mosquitoes may reduce the mating rate with females due to a high mortality caused by the exposure to TSB or ATSB. The female mosquitoes in this study still laid eggs, and some of their eggs hatched while others could not due to a lack of insemination. Therefore, the fecundity and fertility (number of total laid eggs and the egg hatch rate) of female mosquitoes in the treated group was significantly reduced. The mean egg production was reduced by 42.4%, and the mean egg hatching rate was reduced by 23.9%, compared with the control groups. The higher mortality of males in the population is what likely contributed to the decrease in fecundity and fertility.

Several experiments have been conducted using TSB around mosquito larval habitats to control adult mosquitoes and significantly reduced the mosquito population (Muller et al. 2010b, Hossain et al. 2014). Treatments with ATSB or TSB around mosquito larval habitats may kill the early emerged male mosquitoes and potentially impact female fecundity. This new strategy of targeting males with ATSB or TSB as a means to reduce subsequent generations of mosquitoes is similar to sterile insect technology (SIT). This method may be environmentally safe and economical and has the potential for controlling mosquitoes by the treatment of mosquito breeding sites. The study was only conducted in the laboratory, but future studies should include semi-field and field tests using this approach to target male mosquitoes.

Table 2. The number of dead male and female *Ae. albopictus* after exposure to TSB treatment (1% boric acid sugar bait) and 10% sucrose (control) at 24 h and 48 h.

Sex	Repetition	TSB treatment group		Sucrose control group	
		24 h	48 h	24 h	48 h
Males	1	62	109	4	9
	2	51	95	6	12
	3	39	101	5	14
Mean (SE)		50.6 (11.1)[a]	101.7 (7.0)[b]	5 (1)[a]	11.7 (2.5)[b]
Females	1	4	9	3	10
	2	3	8	2	7
	3	2	6	2	9
Mean (SE)		3 (1)[c]	7.7 (1.5)[d]	2.3 (0.6)[c]	8.7 (1.6)[d]

[a,b]Row means for males with the same letter are significantly different ($P< 0.05$) using the Student's t-test (SPSS 16.0).
[c,d]Row means for females with the same letter are not significantly different ($P> 0.05$) using the Student's t-test (SPSS 16.0).

Table 3. The number of eggs produced by female *Ae. albopictus* after exposure to TSB treatment (1% boric acid sugar bait) and 10% sucrose (control).

Repetition	TSB treatment	Sucrose control	Reduction rate (%)
1	1034	1930	46.4
2	1192	1811	34.2
3	983	1833	46.4
Mean (SE)	1069.7 (109)[a]	1858 (63.3)[a]	42.4

[a]Means in each row followed by the same letter are significantly different ($P< 0.05$) using the Student's t-test (SPSS 16.0).

Table 4. The hatch rate from eggs produced by female *Ae. albopictus* mosquitoes exposed to TSB treatment (1% boric acid sugar bait) and 10% sucrose (control).

Test	TSB treatment			Sucrose control		
	Total eggs tested	larvae	Hatch rate (%)	Total eggs tested	larvae	Hatch rate (%)
1	243	106	43.6	253	163	64.4
2	368	235	63.9	353	287	81.3
3	308	172	55.8	316	224	70.9
Mean (SE)	306.3 (62.5)	171 (64.5)	54.4 (10.2)[a]	307.3 (50.6)	224.6 (62)	72.2 (8.5)[a]

[a]Means followed by the same letter are significantly different ($P<0.05$) using the chi-square test (SPSS 16.0).

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Boric Acid Bait Kills Adult Mosquitoes (Diptera: Culicidae)

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ABSTRACT The toxicity of boric acid solutions to adult *Anopheles quadrimaculatus* Say, *Culex nigripalpus* Theobald, and *Aedes albopictus* Skuse was evaluated in the laboratory. Median lethal concentrations (LC_{50} in %) at 24-h exposure for male and female *An. quadrimaculatus* were 0.317 and 0.885, respectively; for *Cx. nigripalpus*, 0.273 and 0.560, respectively; and for *Ae. albopictus*, 0.174 and 0.527, respectively. The LC_{50} values at 48-h exposure for male and female *An. quadrimaculatus* were 0.101 and 0.395, respectively; for *Cx. nigripalpus*, 0.098 and 0.255, respectively; and for *Ae. albopictus*, 0.078 and 0.244, respectively. In laboratory tests, access for 48 h to sucrose (10%) water containing 1% boric acid (boric acid bait) resulted in 98% mortality in blood fed, gravid, and parous *Ae. albopictus*. When offered a choice between boric acid bait and sucrose water, 52% of male and 33% of female *Ae. albopictus* ingested sufficient boric acid bait in 24 h to cause death; after 48 h, respective percent mortalities were 88 and 58%. In outdoor tests, in a walk-in screened cage (156 m^3) containing 1,250 female *Ae. albopictus*, mosquito biting rates on the exposed forearm of a human subject in 3-min exposure were reduced ≥78% for the boric acid bait treatment, compared with a sucrose water control.

KEY WORDS toxicity, *Aedes albopictus*, *Anopheles quadrimaculatus*, *Culex nigripalpus*

CONVENTIONAL CONTROL OF ADULT mosquitoes is based primarily on aerial and ground ultra-low volume application of insecticides. But these techniques risk environmental pollution because of the large areas that are treated and the quantities of insecticides that are applied. There is also the potential for development of insecticide resistance in the mosquito population. Each of these consequences compels us to find new ways to reduce the use of adulticides and to develop safe and effective alternative control methods for adult mosquitoes.

Because adult mosquitoes require carbohydrates for energy, survival, and reproduction (Yuval 1992, Foster 1995), one alternative to aerial/ground application of insecticides is to provide mosquitoes a poison mixed with their food. A bait formulation of boric acid has been used in this way to control pest ants (Klotz et al. 1997) but not to kill mosquitoes. Sucrose is an excellent candidate for use in a bait product for mosquitoes because it is a component of the sugars they acquire in nature from honeydew, plant sap, and nectar (Schlein and Muller 1995, Burkett et al. 1999).

The study reported herein was performed to determine the potential usefulness of boric acid as a mosquito control agent. In devising it, we sought answers to three questions. First, will a mixture of boric acid, sucrose, and water kill adult mosquitoes and, if so, what is the lethal concentration? Second, does the effectiveness of a bait solution vary with mosquito species, sex, or the physiological status of the female? Finally, will mortality in a mosquito population caused by boric acid ingestion result in a reduction of the mosquito biting rate on humans?

Boric acid baits could be used to reduce adult mosquito populations if they are determined to be lethal to mosquitoes. In this regard, bait stations augmented with a chemical feeding attractant and designed for economy and portability would provide a safe, nonpesticidal, point-source control technology for mosquitoes in urban and suburban environments and in some types of livestock housing.

Materials and Methods

Mosquitoes. *Anopheles quadrimaculatus* Say, *Culex nigripalpus* Theobald, and *Aedes albopictus* Skuse were reared and maintained in the laboratory under controlled temperature (27°C), relative humidity (70%), and a photoperiod of 14:10 (L:D) h. Adults emerged in stock cages (≈500 cm^3), were provided 10% sucrose in water ad libitum, and were blood fed weekly on restrained 5–7-wk-old chicks or membrane fed on bovine blood. Methods for rearing mosquito larvae followed those described by Gerberg et al. (1994).

Both sexes of each species were evaluated in acute toxicity tests in the laboratory. We used *Ae. albopictus* to determine the effect of boric acid on blood en-

Written informed consent was obtained for all human subjects used in this study in accordance with protocol IRB-01 #445-96 as currently approved by the University of Florida, Health Sciences Center, Institutional Review Board for Human Subjects. Approval to use animals in this research has been granted by the Institutional Animal Care and Use Committee at the University of Florida under project #A622.

Table 1. Toxicity of boric acid baits to male and female *Anopheles quadrimaculatus*, *Culex nigripalpus*, and *Aedes albopictus* in the laboratory after 24- and 48-h exposure

Species and sex	Exposure time					
	24 h			48 h		
	LC_{50} (%)	95% C.I.	Slope	LC_{50} (%)	95% C.I.	Slope
Aedes albopictus						
Male	0.174	0.146–0.199	4.143	0.078	0.069–0.089	4.961
Female	0.527	0.479–0.577	3.036	0.244	0.220–0.268	4.876
Culex nigripalpus						
Male	0.273	0.117–0.397	5.047	0.098	0.085–0.112	3.905
Female	0.56	0.498–0.626	9.92	0.255	0.233–0.277	5.337
Anopheles quadrimaculatus						
Male	0.317	0.289–0.344	4.568	0.101	0.019–0.196	3.719
Female	0.885	0.800–1.005	3.252	0.395	0.328–0.461	5.193

gorged, gravid, and parous females, whereas both male and female *Ae. albopictus* were used in choice tests between boric acid in sucrose water (i.e., boric acid bait) and sucrose water alone. Nulliparous 5–7-d-old *Ae. albopictus* were used to determine the effect of boric acid bait on mosquito biting rates on a human subject in an outdoor cage.

Acute Toxicity Tests. Treatments in toxicity bioassays comprised five concentrations of boric acid (0.0001, 0.001, 0.01, 0.1, and 1%) dissolved in deionized water containing 10% sucrose (i.e., sucrose water). Ten percent sucrose in deionized water was used as the control. Before each test, 50 ml of one of the five treatments, or sucrose water control, was transferred to a 60-ml plastic cup containing a single cotton ball. A cup with either treatment or control solution was then placed inside a mosquito cage (45 × 38 × 38 cm) that held 100 male and 100 female mosquitoes. In separate sets of bioassays that ended after 24 h and after 48 h, the cups were removed from each cage and the number of dead mosquitoes in the cage collected, sexed, and their numbers recorded. Each toxicity bioassay was replicated four times for each mosquito species.

Boric Acid Bait-Induced Mortality in Blood Fed, Gravid, and Parous *Aedes albopictus*. In these tests, the effect of boric acid (1%) bait with sucrose water on mortality in blood fed, gravid, and parous *Ae. albopictus* was compared. Blood fed mosquitoes were obtained by feeding 7-d-old females on restrained 5–7-wk-old chicks and then testing them immediately (blood fed), after holding for 3 d (with sucrose water available ad libitum) (gravid), or after 7 d (with sucrose water available ad libitum, and after oviposition) (parous). Blood fed and gravid females were tested using 25 mosquitoes per cage (45 × 38 × 38 cm), and parous mosquitoes using 200 females per cage. Mortality was assessed after 48 h using the methods described above. Each test was replicated four times.

Choice Tests of Boric Acid Bait versus Sucrose Water with *Aedes albopictus*. These tests were made to determine the preference of male and female mosquitoes for boric acid (1%) bait or sucrose water when both were simultaneously provided in a cage. Two cages were used in each test, each containing 100 male and 100 female, 5–7-d-old *Ae. albopictus*. One cage (treatment) received one cup each of boric acid bait and sucrose water. The second cage (control) received two cups of sucrose water. Choice was determined by assessing mortality in males and females in each cage after 24 h. Choice tests were repeated four times.

Effect of Boric Acid Treatment on *Aedes albopictus* Biting Rates on Humans. An outdoor, walk-in, screened cage (156 m^3) was used for this study. A total of 1,250 female *Ae. albopictus* was released into the cage and 1 h later the number of mosquito bites in 3 min on the exposed forearm of a human volunteer was recorded. Afterward, four 200-ml black plastic cups, each containing 100 ml of boric acid (1%) bait, were placed in each corner of the cage 1 m above ground level on a wood rack. After 48 h, the same human volunteer reentered the cage and recorded the number of mosquito bites received on an exposed forearm in 3 min. The control scenario used the same regimen with sucrose water substituted for the boric acid bait. The same cage and human subject were used each time to assess mosquito biting rates. Treatment and control tests were each replicated six times.

Data Analysis. All tests were organized using a completely randomized design. Acute toxicity data were analyzed using probit analysis (Finney 1971). Response data for percent mortality in blood fed, gravid, and parous mosquitoes, choice tests, and the mosquito biting rates in outdoor cage tests were analyzed using Student's *t*-test or analysis of variance (SAS Institute 1988) with means separation in the latter case using Tukey's honestly significant difference test.

Results

Acute Toxicity Tests. Boric acid baits were more toxic to male than to female mosquitoes and were more toxic to *Ae. albopictus* and *Cx. nigripalpus* than to *An. quadrimaculatus* (Table 1). In general, LC_{50} values decreased 50–70% after 48-h exposure, compared with 24-h exposure. There was attenuation of toxicity (i.e., decreased slope of the fitted regression line) after 48 h for male *An. quadrimaculatus* and for *Cx. nigripalpus*, with the opposite effect for female *An. quadrimaculatus* and *Ae. albopictus*.

Table 2. Mean percent mortality (SE) in blood fed, gravid, and parous *Aedes albopictus* after 48-h exposure to boric acid bait or sucrose water in laboratory tests

Physiological state	Boric acid bait[a]	Sucrose water
Blood fed	99.0 (1.7)a	1.0 (0.4)b
Gravid	99.0 (1.7)a	4.0 (2.8)b
Parous	98.0 (2.4)a	4.0 (2.6)b

[a] Means in each row followed by same letter are not significantly different ($P = 0.05$) using Student's t-test (SAS 1988).

Boric Acid-Induced Mortality in Blood Fed, Gravid, and Parous *Aedes albopictus*. Access to boric acid bait significantly increased mortality in blood fed ($t = 84.47$, df = 24, $P < 0.001$), gravid ($t = 49.61$, df = 24, $P < 0.001$), and parous *Ae. albopictus* ($t = 42.52$, df = 196, $P < 0.001$), compared with females that had access to sucrose water only (Table 2). Percent mortality for treatment and control groups was ≥98% and ≤4%, respectively.

Choice Tests of Boric Acid Bait versus Sucrose Water with *Aedes albopictus*. When presented with a choice between boric acid bait and sucrose water, 52% of males ($t = 6.53$, df = 99, $P < 0.01$) and 33% of females ($t = 5.68$, df = 99, $P < 0.05$) fed at least once from the boric acid bait cup and died in the first 24-h exposure (Table 3). After 48 h, mortality increased to 88% for males ($t = 16.67$, df = 99, $P < 0.01$) and 58% for females ($t = 8.85$, df = 99, $P < 0.01$). Conversely, 12% of males and 42% of females did not ingest sufficient boric acid bait in 48 h to induce mortality.

Effect of Boric Acid Treatment on *Aedes albopictus* Biting Rates on Humans. Availability of boric acid bait in the outdoor cage test significantly ($F = 29.45$, df = 3,20, $P < 0.001$) reduced biting rates (≥78%) by *Ae. albopictus* on the human subject (Table 4). Biting rates before introduction of boric acid bait or sucrose water and 48 h after introduction of sucrose water were not significantly different.

Discussion

Boric acid has been tested as a toxic bait for house flies and ants. In laboratory tests, LC_{50} values for boric acid in 10% sugar water for adult house flies, *Musca domestic* (L.), ranged from 0.37% to 0.88% (Hogsette and Koehler 1992). For the red imported fire ant, *Solenopsis invicta* Buren, the LC_{50} decreased from 1.27% at 3 d, to 0.11% at 8 d (Klotz et al. 1997). The results of our study show that boric acid bait has toxicity to mosquitoes similar to house flies and is more toxic to mosquitoes than to fire ants.

Our results also showed that blood fed, gravid, and parous mosquitoes ingested sufficient boric acid bait in 48 h to cause mortality. This observation is important because it reveals that the toxicity of boric acid baits to mosquitoes is unaffected by the physiological state of the female.

Choice tests showed that boric acid bait was not ingested at lethal levels in 24 h by 12 and 67% of male and female mosquitoes, respectively; however, these percentages were halved after 48 h of exposure. High concentrations of boric acid are known to inhibit ingestion of baits by house flies, cockroaches, and ants (Hogsette and Koehler 1994, Strong et al. 1993, Klotz et al. 1997). The reason for such inhibition is not presently understood and the basis for repellency of high concentrations of boric acid to mosquitoes needs further study. One objective, in this regard, would be to determine whether baits can be formulated to reduce boric acid concentration while maintaining or improving efficacy and palatability to mosquitoes.

Use of boric acid bait may be an effective strategy to control adult mosquitoes. Boric acid mixed with sucrose and made available to mosquitoes in nature could mitigate some of the safety and environmental concerns associated with conventional mosquito adulticides. Furthermore, it should be possible to formulate and deliver boric acid baits in the environment in an effective way. Possible delivery systems include various types of point-source bait containers, such as hanging bait stations, or traps, that are augmented with mosquito feeding attractants. Targeted spray applications of boric acid baits could also be made to vegetation (provided phytotoxicity is not a limiting factor) or to inanimate surfaces, including those in mosquito refugia.

Table 3. Mean percent mortality (SE) of male and female *Aedes albopictus* in choice tests after 24- and 48-h exposure to boric acid bait and/or sucrose water in laboratory tests

Exposure period and sex	Boric acid bait[a]	Sucrose water
24 h		
Male	51.8 (11.3)a	3.5 (3.8)b
Female	33.5 (10.1)a	0.3 (0.4)b
48 h		
Male	88.0 (2.4)a	7.5 (5.6)b
Female	58.0 (10.5)a	1.0 (0.7)b

Tests comprised the choice of boric acid bait or sucrose water in one cage and sucrose water only in the second cage (see text for details).

[a] Means in each row followed by same letter are not significantly different ($P = 0.05$) using Student's t-test (SAS 1988).

Table 4. Number of bites in 3 min by *Aedes albopictus* on exposed forearm of a human subject in an outdoor cage before and after 48-h exposure of mosquitoes to boric acid bait or sucrose water

Test	Boric acid bait		Sucrose water	
	Before	After 48 h	Before	After 48 h
1	1.8	0.2	2	1.2
2	2.2	0.2	1.8	1.4
3	2.3	0.5	1.9	1.4
4	2.2	0	1	1.4
5	1.9	0.2	2.3	1.7
6	1.2	0.2	2.6	1.8
Mean (SE)[a]	1.9 (0.4)a	0.2 (0.1)b	1.9 (0.5)a	1.5 (0.2)a

[a] Means (as percent of 1,250 female *Aedes albopictus*) followed by same letter are not significantly different ($P = 0.05$) using Tukey's HSD test (SAS 1988).

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APPLICATION OF BORIC ACID BAITS TO PLANT FOLIAGE FOR ADULT MOSQUITO CONTROL

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ABSTRACT. Boric acid (1%) in 5% sugar water bait solution was applied as a spray to the foliage, stems, and other surfaces of plants for control of adult *Aedes albopictus, Culex nigripalpus*, and *Ochlerotatus taeniorhynchus*. Initial studies outdoors in small (1.42-m³) screened cages showed that exposure of male and female mosquitoes to 1% boric acid bait for 48 h resulted in 80 to 100% mortality in *Ae. albopictus* and ≥98% mortality in *Cx. nigripalpus* and *Oc. taeniorhynchus*. At 48 h posttreatment, in large (1,178-m³) outdoor screened cages, 1% boric acid bait applied as a spray to plant surfaces significantly reduced the landing rates of *Ae. albopictus* and *Cx. nigripalpus* on a human subject as well as the numbers of these two species captured in mechanical traps, compared with responses for adults exposed to 5% sugar water solution only (control). Boric acid bait treatments in large screened cages did not significantly reduce landing rates or trap captures of *Oc. taeniorhynchus*. The application of boric acid baits to plant surfaces may be an effective adulticidal method for selected species of pest and disease vector mosquitoes.

KEY WORDS *Aedes albopictus, Culex nigripalpus, Ochlerotatus taeniorhynchus*, adulticide, stomach poison, mosquito control

INTRODUCTION

To survive as adults, and to provide energy for feeding, flight, and other activities, mosquitoes must regularly consume carbohydrates (Nayar and Sauerman 1971, Yuval 1992, Foster 1995). Several studies have shown that both male and female mosquitoes ingest sugars of plant origin for this purpose (Bidlingmayer and Hem 1973, Schlein and Muller 1995, Burkett et al. 1999). This feeding behavior presents some interesting possibilities with respect to the use of sugar attractant-based toxic baits for mosquito control.

Boric acid has been used as a toxic bait for the control of cockroaches and pest ants (Klotz et al. 1997). When dispensed by cotton wick, it also kills adult mosquitoes (Xue and Barnard 2003). In the study reported here, which was made outdoors in screened enclosures, we sought to determine the effects of boric acid bait, when applied to leaves, stems, and other plant surfaces, on mortality in *Aedes albopictus* Skuse, *Culex nigripalpus* Theobald, and *Ochlerotatus taeniorhynchus* (Say). These mosquitoes are potential vectors of West Nile virus in the USA (Turell et al. 2001).

The information obtained in this study will help us judge the usefulness of boric acid baits for mosquito control. It also will allow us to assess the technology as an alternative to the use of conventional pesticides for pest and disease vector abatement.

MATERIALS AND METHODS

Mosquitoes

All mosquitoes were reared and maintained at 27°C, 80% RH, and a 14:10-h light-dark cycle in the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE) insectary by using methods described by Gerberg et al. (1994). *Aedes albopictus* were induced to oviposit on wet filter paper; oak leaf infusions were provided for this purpose for *Cx. nigripalpus*. *Ochlerotatus taeniorhynchus* eggs were collected from salt-marsh peat or sphagnum wrapped in cheesecloth, hatched in shallow trays, and the larvae were reared in saltwater (1% NaCl). Pupae were collected from rearing trays, allowed to emerge into holding cages, and the adults were provided a 10% sucrose solution (in water) ad libitum. *Aedes albopictus* and *Cx. nigripalpus* were blood-fed by using

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[5] Written informed consent was obtained for all human subjects in this study in accordance with protocol IRB-01 #445-96 as approved by the University of Florida, Health Sciences Center, Institutional Review Board for Human Subjects. Approval to use animals in this research was granted by the Institutional Animal Care and Use Committee at the University of Florida under project #A622.

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Table 1. Mean percentage of mortality (±SE) at 48 h posttreatment in 3 species of mosquitoes exposed to *Raphiolepis indica* treated with 1% boric acid bait in small screened cages.

Species	Sex	Mean % mortality (±SE)[1]	
		Treatment	Control
Aedes albopictus	Male	100.0 (±0.0)a	3.8 (±0.9)b
	Female	99.5 (±0.3)a	1.3 (±0.9)b
Culex nigripalpus	Male	99.5 (±0.3)a	4.0 (±0.7)b
	Female	98.0 (±0.0)a	0.8 (±0.5)b
Ochlerotatus taeniorhynchus	Male	99.8 (±0.3)a	2.3 (±0.3)b
	Female	97.0 (±0.6)a	1.0 (±04)b

[1] Row means followed by the same letter are not significantly different (ANOVA, LSD, $P = 0.05$).

restrained 5- to 7-wk-old chickens; *Oc. taeniorhynchus* was membrane fed bovine blood. All mosquitoes were 5–7 days old when used in tests.

Small screened cage studies

These tests were conducted outdoors using a screened cage within a screened cage arrangement. The outer cage was 8 × 6.5 × 3 m (length × width × height) (~156 m³) with a zippered door that allowed walk-in access. Inside the outer cage were placed two small (92 × 92 × 168 cm) (~1.42-m³) screened cages, each with an access sleeve on the front. Inside each of these small cages, we placed two nonflowering specimens of *Raphiolepis indica* that were grown separately in 12-liter (3-gal) clay pots. The foliage and stems of both plants in one of the two small cages in a large cage received 20 ml of 1% boric acid in a 5% sugar solution applied using a 100-ml capacity hand-pump sprayer. Plants in the remaining cage served as the control and were treated (sprayed) in the manner described above with 20 ml of 5% sugar solution only. Treatment and control cages were selected at random. One-hundred male and 100 female mosquitoes were released into each cage 1 h after application of the treatment. Mortality was determined 48 h later and was based on the number of males and females alive in each cage. Each treatment was replicated four times.

Large screened cage studies

We used two large outdoor screened cages (18.3 × 9.2 × 7 m) (~1,178 m³) for these tests. One cage served as the treatment cage, and the other cage as the control; the status of each cage was assigned at random before a test. Ten species of plants (114 total) (each plant in a 12- to 15-liter [3–5 gal] steel container) were placed in each cage: *Rhodendron hybrid, Raphiolepis indica, Ligustrum lucidum, Ilex cornuta, Viburnum odortissuiumum, Liriope muscari, Crossandra* spp., *Duranta repens, Cuphea hyssopifolia*, and *Ilex vomitoria*. Plants were allocated among each of the four quadrants in a cage, in a semicircle, to provide an even distribution of the species and vegetative cover in each quadrant (as determined by visual inspection). Plants in the treatment cage received 4 liters of 1% boric acid in 5% sugar solution applied evenly by spray to all foliage and stems by using a 10-liter hand pump pressure sprayer. The same procedure was used to apply 4 liters of 5% sugar solution to all plants in the control cage. Separate tests were made to determine the effect of these boric acid–bait treatments on *Ae. albopictus, Cx. nigripalpus*, and *Oc. taeniorhynchus* populations, and tests were replicated four times for each species.

Before each test, we released approximately 5,000 adult mosquitoes into a cage, 1/2 of which were females. Mosquito mortality was estimated 48 h later using mechanical traps to capture adult mosquitoes and by observing the mosquito landing rate on a human subject. Responses (percentage of mortality and percentage of landing rate) in each case were calculated as a proportion of the starting population in each cage (2,500 ♀♀, 2,500 ♂♂) × 100. An omnidirectional trap (Jensen et al. 1994), supplemented with CO_2 (flow rate 5 ml/min) was used to capture *Ae. albopictus* mosquitoes; a counterflow geometry trap (Kline 1999) was used to capture *Cx. nigripalpus* and *Oc. taeniorhynchus*. Traps were operated 1 time in each test from 1600 to 2000 h at 48 h posttreatment. Immediately before this time (between 1530 and 1600 h), the mosquito landing rate was determined by counting the number of mosquitoes landing on the exposed forearm skin of a human volunteer in a 3-min period. The same human subject was used in all tests.

Data analysis

Small cage and large cage studies were made using a completely randomized design. Percentage of mortality and percentage of landing rate responses were analyzed separately by species and sex (after angular transformation; Steel and Torrie 1980) by using analysis of variance (ANOVA) procedures with means separation

Table 2. Mean percentage of mosquitoes landing (±SE) on a human subject and mean percentage (±SE) captured in traps at 48 h posttreatment for 3 species of mosquitoes exposed to 10 plant species treated with 1% boric acid bait in large screened cages.

	Mean % (±SE)			
	Landing[1]		Captured in traps[2]	
Species	Treatment	Control	Treatment	Control
Aedes albopictus	0.09 (±0.04)a	0.78 (±0.09)b	0.40 (±0.30)a	6.40 (±2.74)a
Culex nigripalpus	0.00 (±0.0)a	0.02 (±0.002)b	0.52 (±0.47)a	11.90 (±2.63)a
Ochlerotatus taeniorhynchus	0.05 (±0.02)a	0.60 (±0.26)a	16.86 (±13.57)a	34.25 (±9.53)a

[1] Row means followed by the same letter are not significantly different (ANOVA, LSD, $P = 0.05$).
[2] Row means followed by the same letter are not significantly different (ANOVA, LSD, $P = 0.05$).

($P = 0.05$) by using least significant difference (LSD) test (SAS Institute 2003).

RESULTS AND DISCUSSION

In small screened cage tests, the application of boric acid bait to plants resulted in ≥96% mortality of adult mosquitoes (Table 1). There was no significant difference in mortality rates between males and females of any species. In the large screened cage tests, boric acid bait treatment significantly reduced the landing rates of *Ae. albopictus* and *Cx. nigripalpus* but not *Oc. taeniorhynchus* (Table 2). Similarly, the capture rates of female mosquitoes in mechanical traps differed significantly among the treatment and control groups for the same two species, but not for *Oc. taeniorhynchus* (Table 2); males made up approximately 1% of the total catch in each case.

Ground and air application of synthetic and natural product-based mosquito adulticides leads to contact with, and penetration of, insecticide through the mosquito cuticle or the entry of insecticide into the insect's body via the tracheal system. Stomach poisons, such as boric acid, must be ingested to be effective. For liquid-feeding insects, such as mosquitoes, properly formulated stomach poisons (possibly incorporating a feeding stimulant or arrestant) could be placed in the environment or near nectar sources or other plant-based sources of carbohydrates. Although these stomach poisons can cause immediate death in mosquitoes, we have found that sublethal doses also reduce survival, host-seeking, bloodfeeding, and fecundity rates in mosquito populations (Xue and Barnard 2003).

Boric acid baits can be applied as a spray to vegetation in, or near, mosquito breeding sites, recreational areas, residences, and in urban environments. In such cases, treatment of the vegetation could provide an effective control mechanism for adult mosquitoes before they commence emigration, host finding, or bloodfeeding. In urban areas, boric acid bait treatment of vegetation may provide an effective and economical alternative to ground or air ultra-low volume application of insecticides for adult mosquito control.

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EFFECT OF APPLICATION RATE AND PERSISTENCE OF BORIC ACID SUGAR BAITS APPLIED TO PLANTS FOR CONTROL OF *AEDES ALBOPICTUS*

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ABSTRACT. The use of toxic bait to kill adult *Aedes albopictus* is a safe and potentially effective alternative to the use of synthetic chemical insecticides. This study was carried out to determine effective concentrations of boric acid needed in sugar bait solutions applied to plant surfaces, and to determine its residual effect in reducing adult mosquito densities. In outdoor tests in 1,100-m³ screened enclosures, landing rates of *Ae. albopictus* on a human subject and the number of female mosquitoes in mechanical traps were significantly reduced by a 1% boric acid bait compared with the other tested concentrations (0.25%, 0.50%, and 0.75%) and untreated control. Studies of the duration of boric acid activity on plant surfaces were made in 1.4-m³ cages in the laboratory and outdoors in 78-m³ screened enclosures. In the laboratory tests, 1% boric acid bait resulted in >96% mortality in male and female *Ae. albopictus* for 14 days, whereas in outdoor tests, mosquito landing rates in the treated enclosures were significantly lower than in the control enclosures for 7 days. Also, mosquito mortality responses to boric acid baits between plants with flowers and nonflowers (1.4-m³ cages in the laboratory) were not significantly different. The results of this study suggest that boric acid baits applied to plant surfaces may provide specific data related to the development of an effective point-source–based adjunct/alternative to the use of conventional adulticides for mosquito control.

KEY WORDS *Aedes albopictus*, boric acid, residual effect, plant, sugar bait

INTRODUCTION

Aedes albopictus Skuse is an important vector of dengue virus and a frequently encountered domestic/peridomestic pest species. Efforts to control *Ae. albopictus* with attractive toxic sugar baits and bait stations in and near human habitations are predicated on the carbohydrate requirement of adult mosquitoes for sustenance (Xue and Barnard 2003, Müller and Schlein 2006). Use of toxic baits and bait stations in such settings is a comparatively safe and potentially effective point-source–based alternative to the aerial and ground ultra-low volume (ULV) application of adulticides for mosquito control.

To date, a number of insecticides mixed with sugar baits have been tested for toxicity to adult mosquitoes (Lea 1965, Xue and Barnard 2003, Müller and Schlein 2008). A low-risk stomach poison, boric acid when mixed in a sugar solution and provided to adult mosquitoes by oral ingestion in the laboratory killed a significant number of male and female mosquitoes in several studies (Xue and Barnard 2003, Ali et al. 2006, Xue et al. 2006). Similarly, in semifield (outdoor screened enclosure) conditions, when applied as a 1% boric acid sugar solution to plant surfaces or when used in a bait station device (Xue et al. 2008), this mixture also killed a significant number of mosquitoes. However, neither the boric acid sugar bait dose-response relationship toward *Ae. albopictus* nor the duration of activity of boric acid–bait mixtures on plant surfaces has been documented. The purpose of this study was to characterize these 2 factors by measuring mosquito responses to varying concentrations of boric acid–bait treatments applied as topical sprays on plant surfaces in the laboratory and to evaluate these responses over time with respect to change in the numbers of mosquitoes that land on a human subject or are captured by a mechanical trap under semifield conditions.

MATERIALS AND METHODS

Mosquitoes

Aedes albopictus (Gainesville strain) were reared and maintained in an insectary at 27°C and 80% relative humidity in a 14:10 light:dark photoperiod using the methods described by Gerberg et al. (1994). Mosquitoes were 5–7 days old when used in each test.

Concentration

Tests were conducted in 5 outdoor screened enclosures each measuring 18.3 m long × 9.2 m wide × 7 m high (1,100 m³). The interior of each enclosure was provided with like numbers of specimens of locally available cultivars (e.g., *Rhaphiolepis indica*, *Viburnum odortissuiumum*, *Ilex vomitoria*, and *Rhododendron hybrid*) planted in beds surrounded by turf.

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Each test replicate consisted of boric acid spray treatment, at 1 of 4 concentrations (0.25%, 0.5%, 0.75%, 1%) in 5% sucrose-water solution or a 5% sugar solution control, to the branches and leaves of the plants in the 5 cages. The wetted plants were allowed to dry (60–120 min) and later 2,500 female 5–7 day old *Ae. albopictus* were released into each cage. At 48 h a human volunteer stood in the center of each cage for 3 min and recorded the number of mosquitoes that landed on both forearms. These observations were followed for 24 h by the collection of adult mosquitoes in each cage using a CO_2-baited counterflow geometry trap (Kline 1999). Concentration tests were replicated 4 times, and the same human volunteer was used for the whole experiment.

Duration of toxicity

The duration of boric acid bait solution toxicity on plant surfaces was studied initially in the laboratory using 1.4-m^3 cages (92 cm wide × 168 cm high × 92 cm deep) each containing a 12-liter single nonflowering plant specimen (*R. indica*). In the treatment cage, the plant was sprayed with 100 ml of a 5% sugar solution containing 1% boric acid, enough to wet all plant surfaces thoroughly, but without runoff. In the control cage, the plant was sprayed with 100 ml of 5% sugar water only. Once the spray was dry, 200 *Ae. albopictus* (100 females and 100 males) were placed in each cage. Forty-eight hours later, the dead mosquitoes in each cage and/or on the plant were removed and their numbers recorded. The remaining live mosquitoes in the cages were removed using a mechanical aspirator. Subsequently, at 7, 14, and 21 days after treatment, 200 adult mosquitoes were each placed in the treatment and control cages and the numbers of dead mosquitoes were recorded after 48 h. These tests were replicated 3 times.

A 2nd test to determine the duration of boric acid bait solution toxicity on plant surfaces was conducted using 2 outdoor screened 78-m^3 enclosures (4 m long × 6.5 m wide × 3 m high). Fourteen potted plants (*R. hybrid*; 1.5 m tall, 0.8 m wide each in 20-liter pots) were arranged in a circle (1.5 m radius) in the center of each enclosure. One liter of a 1% boric acid in 5% sugar solution was evenly applied (using a hand compression sprayer) to all plants surfaces but without runoff. Once the spray was dry, 200 female *Ae. albopictus* were released into each enclosure. At 48 h, the same human volunteer stood in the center of each enclosure and recorded the numbers of mosquitoes that landed on both forearms during a series of 3 min long observation periods, 5 min apart. These observations were followed for 12 h by collection of female mosquitoes in each enclosure, using a CO_2-baited mosquito trap mentioned earlier. Subsequently, at 7 and 14 days after treatment, 200 female mosquitoes were released into each cage. The number of mosquitoes landing on a human subject and the number of mosquitoes captured by the same trap were conducted. These tests were replicated 3 times.

Effect of flowers on boric acid bait efficacy

For this experiment, we used 4 cages measuring 1.4 m^3 in the laboratory. Two of these contained a single potted plant (*R. indica*) without flowers (1 to receive boric acid bait treatment, the other as a control). The other 2 cages each contained a single plant with flowers (1 to receive boric acid bait treatment, the other as a control). To the plant in each treatment cage, we sprayed 100 ml of 1% boric acid in 5% sugar solution, and to the plants in the control cages, we applied 100 ml of 5% sugar solution. Once the spray was dry, 200 *Ae. albopictus* (100 females and 100 males) were released into each cage. Forty-eight hours later, dead mosquitoes in the cage and/or on the plant were removed and their numbers were recorded. These tests were repeated 4 times.

Data analysis

Mosquito responses in each test were analyzed using multiple ways of analysis of variance (ANOVA) procedures (SAS, 2003); each count datum for landing mosquitoes and mosquitoes captured in mechanical traps was transformed to $\log_{10}(n + 1)$ before analysis. Mean values in each test were compared using Tukey's honestly significant difference (HSD) test.

RESULTS

Concentration

The effect of boric acid bait concentration on the number of female mosquitoes landing on a human subject and captured in the CO_2-baited counterflow geometry trap was significant in each case ($F_{4,19} = 18.1$, $P < 0.0001$ and $F_{4,19} = 3.7$, $P < 0.02$, respectively). Mean landing counts in the control and at the 0.25% concentration (Table 1) were significantly higher than those at the higher application rates, whereas the only significant difference in the numbers of mosquitoes captured by trap was between the 1.0% boric acid bait application rate and the control.

Duration of toxicity

Under laboratory conditions, contact with boric acid bait residues on plant surfaces caused >98% mortality in both male and female *Ae. albopictus* for at least 14 days (Table 2). Treatment effects were significant when compared with

Table 1. Mean number (±SD) of female *Aedes albopictus* landing on a human subject in 3 min and mean number of females captured in a CO_2-baited mechanical trap in 24 h following 48-h exposure to plant surfaces treated with different concentrations of boric acid bait in an outdoor screened enclosure.[1]

Concentration (%)	Landing count	Trap count
0 (control)	18.7 (5.1) a	280.7 (281.6) a
0.25	11.5 (5.7) a	119.25 (119.26) ab
0.50	4.0 (1.6) b	41.7 (23.1) ab
0.75	3.0 (2.0) b	35.5 (20.5) ab
1.00	3.5 (0.5) b	16.0 (17.1) b

[1] Tabulated means are based on raw data; data were transformed to $\log_{10}(n + 1)$ for statistical analysis. Means in each column followed by the same letter are not significantly different (Tukey's HSD test, $P = 0.05$).

control responses for males at 2, 7, 14, and 21 days after treatment ($P < 0.0001$ in all cases); females responded similarly at all intervals except 21 days after treatment ($P = 0.09$). The effect of posttreatment interval was significant for males ($F_{3,11} = 217.2$, $P < 0.0001$) and females ($F_{3,11} = 2{,}463.3$, $P < 0.0001$), attributable in each case to the responses on days 2, 7, and 14 compared with day 21 following treatment. There was no difference in the mortality of males or females in the control group at any posttreatment interval.

Under semifield conditions, contact with boric acid bait residues on plant surfaces significantly reduced the mean number of landing female *Ae. albopictus* for at least 7 days (Table 3). In tests of the duration of boric acid bait toxicity on plant surfaces in 78-m³ screened outdoor cages, counts of landing female *Ae. albopictus* indicated a significant effect due to boric acid bait on days 2 ($F_{1,5} = 131.9$, $P = 0.0003$) and 7 ($F_{1,5} = 9.4$, $P = 0.03$) after treatment but not on day 14 after treatment (Table 3). In contrast, the mean number of mosquitoes collected in traps indicated significant bait effects up to day 2 after treatment only ($F_{1,5} = 14.4$, $P = 0.01$). Toxicity decreased significantly over time ($F_{2,8} = 43.9$, $P = 0.0003$) as indicated by increased numbers of landing mosquitoes on day 7 versus day 2 after treatment, and on day 14 versus days 7 and 2 after treatment and for the trap collections ($F_{2,8} = 26.0$, $P = 0.001$), on day 7 and 14 after treatment compared with day 2. There was no difference in the mean numbers of mosquitoes landing or those captured in the trap in the 2 control groups at any time after treatment.

Effect of flowers on boric acid bait efficacy

There was no significant difference in the mean percentage mortality of both male and female *Ae. albopictus* exposed to boric acid bait on plants with or without flowers (Table 4). Mean percentage mortality in females in boric acid bait–treated cages without flowers was lower (albeit not significantly so) than in cages with flowers.

DISCUSSION

Previous studies have shown that adult mosquitoes die after feeding on boric acid bait (Xue and Barnard 2003). Sublethal application rates of boric acid baits are also known to reduce blood feeding rates and fecundity in *Ae. albopictus* (Ali et al. 2006). In 1.4-m³ cages placed outdoors, the exposure of male and female *Ae. albopictus*, *Culex nigripalpus* Theobald, and *Ae. taeniorhynchus* (Say) to 1% boric acid in 5% sugar water solution for 48 h resulted in 80% to 100% mortality in *Ae. albopictus* and ≥98% mortality in *Cx. nigripalpus* and *Ae. taeniorhynchus* (Xue et al. 2006). Subsequent studies in outdoor screened enclosures showed a significant reduction in the landing rates of *Ae. aegypti* L. and *Ae. taeniorhynchus* on human hosts when using a 1% boric acid bait and a 0.1% fipronil bait in multiple bait stations (Xue et al. 2008). However, no effect was observed when this treatment was tested against mosquito populations in northern Florida (Xue et al. 2008). The study in northern Florida was conducted in the midst of extensive wood and farmland, an open system, which enabled an undetermined number of mosquitoes from outside the treated area to migrate freely and to replenish the populations exposed to toxic baits. In similar studies in Israel and Mali, intentionally

Table 2. Mean percentage mortality (±SD) of male and female *Aedes albopictus* at different times following treatment after 48-h exposure to plant surfaces treated with 1% boric acid in 5% sugar solution (treatment) or 5% sugar solution (control) under laboratory conditions.[1]

	Treatment		Control	
Days after treatment	Males	Females	Males	Females
2	100 (0) aA	100 (0) aA	3.6 (1.1) aB	0.3 (0.5) aB
7	100 (0) aA	98.3 (2.1) aA	3.3 (0.5) aB	0 (0) aB
14	98.6 (2.3) aA	98.6 (1.1) aA	4.3 (1.5) aB	0.3 (0.5) aB
21	31.6 (7.6) bA	3.3 (2.3) bA	2.6 (0.5) aB	0.3 (0.5) aA

[1] Means followed by the same letter, lowercase for posttreatment interval by column and uppercase for sex in a row, are not significantly different (Tukey's HSD, $P = 0.05$).

Table 3. Mean number (±SD) of female *Aedes albopictus* landing on a human subject in 3 min and mean number of females captured in a CO_2-baited mechanical trap in 12 h following 48-h exposure to plant surfaces treated with 1% boric acid in 5% sugar solution (treatment) or 5% sugar solution (control) in a 78-m³ screened outdoor enclosure.[1]

Time after treatment (d)	Treatment		Control	
	Landing count	Trap count	Landing count	Trap count
2	1.3 (0.5) aA	7.3 (5.1) aA	20.6 (5.5) aB	86.6 (63.6) aB
7	8.0 (3.0) bA	99.6 (62.6) bA	18.6 (5.6) aB	146.6 (39.2) aA
14	19.3 (5.0) cA	106.6 (23.0) bA	19.0 (1.0) aA	138.3 (36.1) aA

[1] Tabulated means are based on raw data; each count datum was transformed to $\log_{10}(n + 1)$ for statistical analysis. Means followed by the same letter, lowercase for posttreatment days in a column and uppercase for collection method in arrow, are not significantly different (Tukey's HSD, $P = 0.05$).[1]

isolated, island-like habitats surrounded by arid land were selected to avoid possible migration of mosquitoes into the experimental sites (Müller et al. 2010a, 2010c). Other factors that may have contributed to the failure of the study in Florida were the composition of the sugar baits, competition from naturally occurring attractive plants (Schlein and Müller 2008) and the potency of toxins used in the baits. With respect to the latter factor, we determined in the present study (in screened outdoor enclosures) that 0.75% boric acid in 5% sucrose solution measurably reduced the number of mosquitoes landing on a human host and that 1% boric acid was required to reduce the numbers of *Ae. albopictus* captured in CO_2-baited traps.

In the laboratory, the toxicity of 1% boric acid sugar bait persisted for 14 days, while at the same time, under field-like conditions, the period of activity was reduced to 7 days. The only feasible difference between the laboratory and the field trials were the weather conditions which probably influenced the toxic baits. According to the product information of Sigma-Aldrich, Boric acid is very stable in its dry form and dissolves in water. It is therefore unlikely that boric acid degrades in a relatively short time. We therefore assume that it was the phago-stimulant sucrose that degraded after 1 wk outdoors and 2 wk indoors on the plant foliage. In field trials in Israel and Mali, the attractive toxic sugar baits contained 10% to 12% brown, unrefined sugar, varying amounts and types of fruit juices, and additional BaitStab™ to preserve and stabilize the baits (Schlein and Müller 2008, Müller and Schlein 2010, Müller et al. 2010a). In these field trials, the baits were attractive from 3 to 4 wk.

Adult mosquitoes need sugar for survival, reproduction, and day-to-day activity. Common sugar sources are floral nectar and honeydew, although adult mosquitoes feeding on plant juices and tissues have been documented (Schlein and Müller 1995). Boric acid bait sprayed on plants without flowers provided effective control for *Ae. albopictus*, *Cx. nigripalpus*, and *Ae. taeniorhynchus* (Xue et al. 2006). Similar results were obtained with the application of toxic sugar baits on nonflowering vegetation in and around resting and breeding sites of *Cx. pipiens* (Müller et al. 2010c), *Cx. quinquefasciatus* (Müller et al. 2010b) and *Anopheles gambiae* Giles (Müller et al. 2010a). In the present study, the presence of flowers did not impact the mortality caused by the boric acid sugar baits. In early experiments in Israel, the scent of flowers was used to attract mosquitoes to the baits (Schlein and Müller 2008), while in more recent studies the attractant was incorporated in the bait.

Based on the data in our study, application of attractive toxic sugar baits or bait stations could be considered for use in integrated mosquito management programs.

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Bijan Dehgan (University of Florida, IFAS) identified the plant species used in this study, and Brad Fuller provided technical assistance. Written informed consent was obtained for human sub-

Table 4. Mean percentage mortality (± SD) of male and female *Aedes albopictus* in 1.4-m³ cages in the laboratory following 48-h exposure to plant surfaces, with and without inflorescence, treated with 1% boric acid in 5% sugar solution (treatment) or 5% sugar solution (control).[1]

Sex	With inflorescence		Without inflorescence	
	Treatment	Control	Treatment	Control
Males	100.0 (0) aA	3.7 (1.7) bA	100.0 (0) aA	3.7 (1.2) bA
Females	99.5 (0.5) aA	1.2 (1.8) bA	93.7 (9.2) aA	1.2 (0.5) bA

[1] Means in each row followed by the same letter, lowercase for sex in a column and uppercase for inflorescence in a row, are not significantly different (Tukey's HSD test, $P = 0.05$).

jects in this study in accordance with University of Florida, Health Science Center, Institutional Review Board Human Subjects protocol 445-96.

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Carbon Dioxide, Odorants, Heat and Visible Cues Affect Wild Mosquito Landing in Open Spaces

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CO_2 and other chemicals affect mosquito blood meal seeking behavior. Heat, humidity and black color can also serve as orientation cues. However mosquito attraction does not necessarily mean that it will land. The sequence of the cues used for mosquito landing is unclear. We performed a field study with wild mosquitoes in an open space and found that no chemicals (except pyrethrins) could completely prevent mosquitoes from landing. CO_2 mimics cyclopentanone and pyridine attracted mosquitoes but did not lead to landing. No mosquito was caught in the absence of heat, although in the presence of CO_2. Mosquito females commonly explore visible black objects by eyes, which is independent of infrared radiation. Humidification around the heat source may increase the detection distance but it did not affect mosquito landing. If a black object was located distant from the CO_2 and heat, mosquitoes still explored the heat source. Relative to CO_2 and heat, odorants, humidity and black color show lesser effects on mosquito landing.

Keywords: mosquito landing, CO_2 mimics, heat, humidity, black color

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INTRODUCTION

Mosquitoes transmit pathogens such as the malaria parasite, Dengue virus and the Zika virus (Yuan et al., 2017). Female mosquitoes use multiple cues to identify and move toward the hosts. These include exhaled CO_2 (Lacey and Cardé, 2011; Turner et al., 2011; Tauxe et al., 2013; Lacey et al., 2014; McMeniman et al., 2014; van Breugel et al., 2015), skin odors (Eiras and Jepson, 1994; Hallem and Carlson, 2006; Saito et al., 2009; Syed and Leal, 2009; Carey et al., 2010; Turner et al., 2011; Tauxe et al., 2013; McMeniman et al., 2014; Gonzalez et al., 2015), heat (Burgess, 1959; Davis and Sokolove, 1975; Gingl et al., 2005; van Breugel et al., 2015; Zermoglio et al., 2017), humidity (Burgess, 1959; Eiras and Jepson, 1994; van Breugel et al., 2015) and colors (Bidlingmayer and Hem, 1980; Browne and Bennett, 1981; Muir et al., 1992; Gibson and Torr, 1999; Bentley et al., 2009; van Breugel et al., 2015).

The compound 1-octen-3-ol elicits a positive effect to CO_2-sensing neurons (Gonzalez et al., 2015), while 1-butanal, 1-hexanol, ethyl pyruvate and methyl pyruvate inhibit olfactory receptor activities (Turner et al., 2011; Tauxe et al., 2013). Among all active odor chemicals, 2,3-butanedione causes an unusual ultra-prolonged activation of CO_2-detecting neurons and thus disrupts CO_2-mediated activation as well as source-finding behaviors in mosquitoes, even after the odor is no longer present (Turner et al., 2011). However, 2,3-butanedione is a relatively toxic compound (mammalian median lethal dose LD_{50} = 1580 mg/kg), which limits its practical usefulness. The compound 2,3-pentanedione (LD_{50} > 2.5 g/kg) has a similar chemical property but is much less toxic than 2,3-butanedione (Sawyer et al., 1991). The roles of these odor chemicals on mosquito landing were investigated in this study.

A previous study suggested that cyclopentanone mimics the electroantennogram responses induced by CO_2, and therefore can lure mosquitoes to traps in the absence of carbon dioxide (Tauxe et al., 2013). Ethyl pyruvate and methyl pyruvate, in contrast, strongly inhibit the activity of olfactory receptor neurons (Tauxe et al., 2013). The roles of CO_2 mimics (in the absence of carbon dioxide) on mosquito landing were tested in this study.

Most previous experiments used lab-reared mosquitoes that were tested in one-way or two-way enclosed tunnels (Lacey and Cardé, 2011; Lacey et al., 2014; van Breugel et al., 2015). The behavior of wild mosquitoes in natural open spaces surrounded with complex lures is less well known. Attraction of a mosquito does not necessarily mean that it will land on a trap or other surfaces. We lack understanding of the importance ranking of cues involved in mosquito landing. Here we studied wild mosquitoes and used a sticky pad/trap catches to infer landing preferences, but not behavioral preferences or near-source behaviors. Besides, both lab-reared and wild mosquitoes are prone to exploring dark objects and moist heat sources (Burgess, 1959; Bidlingmayer and Hem, 1980; Browne and Bennett, 1981; Muir et al., 1992; Gibson and Torr, 1999; McMeniman et al., 2014; van Breugel et al., 2015). The influences of heat, humidity and black color on mosquito landing were also investigated in this field study.

MATERIALS AND METHODS

Animals

The behaviors of wild mosquitoes (mostly *Anopheles sinensis*, *Anopheles lesteri*, *Culex fatigans*, *Culex tritaeniorhymchus* and *Aedes albopictus*) were studied. *Aedes albopictus* only appeared in late summer and early autumn, and therefore was not counted. We counted insect bodies, no matter if their legs were missing. The experiments were performed from June 1st to September 1st in 2015, 2016 and 2017, when the mosquitoes were most active. Usually 9–17 *Anopheles* and 30–56 *Culex* were caught on each sticky plate per night and the numbers of *Anopheles* and *Culex* did not significantly change from June 1st to September 1st each year (Supplementary Figure S1).

Field Trapping

The field experiment was performed at the Wenjiang Campus of Sichuan Agricultural University in Chengdu (30°41'N, 103°49'E at an altitude of 558 m). The mosquito traps were placed in a ventilated corridor near the third teaching building (0.3 m to the wall of the building; 0.5–0.6 m to the bushes adjacent next to a drainage channel; **Figure 1**) from 8:00 p.m. to 8:00 a.m. of the next day. The light intensity during the trapping times ranged from about 10–50 μmol photons m^{-2} s^{-1} and the temperature ranged from 18°C to 25°C. A transparent umbrella prevented the trap from getting wet during rainfall.

The Basic Trap

Four metal blocks were heated to 37.0°C, a petri dish of water (without a lid) was placed directly above the blocks, and then a faint-yellow 15 cm × 15 cm sticky plate (designed for house flies) with four 1 cm diameter holes was placed above. A simple carbon-dioxide generator with sodium citrate and sodium bicarbonate was placed on the left side of the sticky plate (outlet of the hose faced the sticky plate) with a gas flow of about 500 mmol h^{-1}. A black polyethylene bag (60 cm × 60 cm) was placed adjacent to the right side of the sticky plate (**Figure 1**).

Chemical Treatments

Attractive odor compound 1-octen-3-ol, persistent CO_2-activation odor chemicals 2,3-butanedione and 2,3-pentanedione, repellents 1-butanal, 1-hexanol, ethyl pyruvate and methyl pyruvate, contact-repellents indole and DEET (N, N-diethyl-meta-toluamide), CO_2 mimics cyclopentanone and pyridine and an insecticide meperfluthrin were tested. All chemicals were dissolved at 10^{-1} or 10^{-2} in paraffin oil or water (Tauxe et al., 2013), except for indole which was dissolved in 70% ethanol (Gonzalez et al., 2015). The 50 mL chemical solutions were added to the petri dish of each trap. All chemicals were purchased from Sigma-Aldrich Company (St. Louis, MO, USA).

Variations to the Basic Trap

The metal blocks were heated to 25.0, 37.0 or 50.0°C, respectively. To achieve a dry heat plume, a petri dish without water was placed on the metal blocks with a sticky plate, without holes, above them. We used a humidifier to increase the humidity above the trap. In some experiments, the faint-yellow sticky plate (max reflectance across 250–750 nm ≈65%) was replaced by a black sticky plate (max reflectance of 32%) and the black polyethylene bag (max reflectance of 33%) was replaced by a white paper (60 cm × 60 cm; max reflectance of 67%) with or without a black square (15 cm × 15 cm) at the center, a black polyethylene bag with a white square (15 cm × 15 cm) at the center, a faint-yellow sticky plate, or a black sticky plate. These treatments are indicated in the figures. For the nearly darkness treatment, a black umbrella was placed above the trap to restrict illumination to less than 1 μmol photons m^{-2} s^{-1}. Five independent replicates (on different nights) were performed for each varied trap.

Temperature Measurements IR Thermal Imaging and Sunlight Reflectance Measurements

Infrared radiation photos of the traps were taken by a FLIR T620 thermal-imaging camera (Thermal CAM-FLIR Systems, USA; Zhang et al., 2016). The ambient temperature was set at 21.1°C. Temperatures of the thermal plume created by the heated trap at distances of 0–20 cm (away from the sticky plate) were measured with a digital thermometer (van Breugel et al., 2015). The sunlight reflectance across 250–750 nm range for the different objects was measured by using a reflectance meter (RCRM01, Rinch Industrial Company, China).

Statistical Analysis

All experiments were performed randomly across months and years. Significant differences among different traps or different treatments were analyzed according to Duncan's multiple range test at the 5% level. ANOVA was performed by using the software

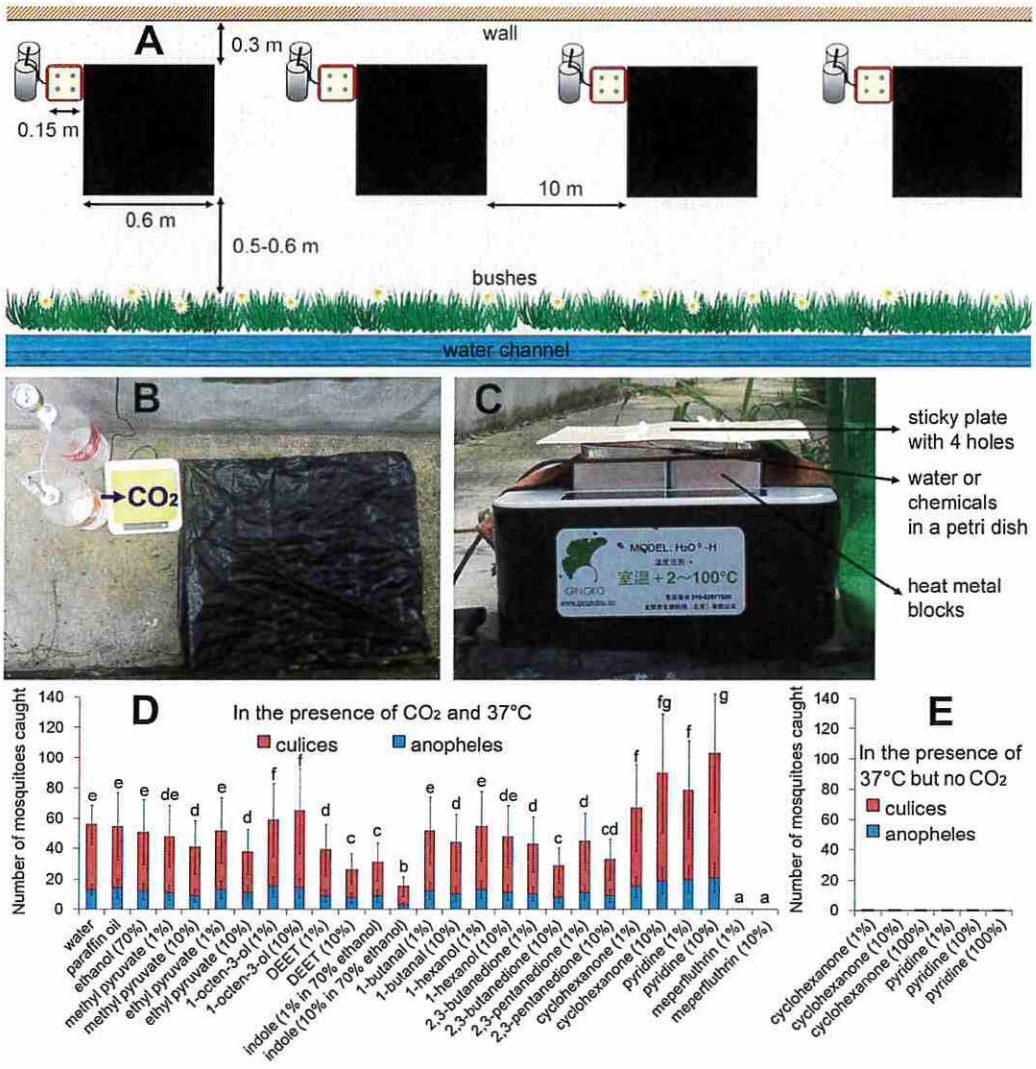

FIGURE 1 | The field trapping device with different odorant chemicals. **(A)** Four independent traps (including one basic trap) about 10 m apart were set simultaneously on 1 day. The basic trap: four metal blocks were heated to 37.0°C, a petri dish of water was placed directly above the blocks, and then a faint-yellow 15 cm × 15 cm sticky plate with four 1 cm diameter holes was placed above. A simple carbon-dioxide generator with sodium citrate and sodium bicarbonate was placed on the left side of the sticky plate. A black polyethylene bag (60 cm × 60 cm) was placed adjacent to the right side of the sticky plate. The mosquito traps were placed in a ventilated corridor near the building (0.3 m to the wall of the building; 0.5–0.6 m to the bushes adjacent next to a drainage channel). **(B)** Top view of the basic trap. **(C)** Lateral view of the basic trap. **(D)** Effects of odorant chemicals on mosquito capture rate. Water refers to the basic trap presenting in addition a Petri dish with water. **(E)** Cyclopentanone and pyridine treatments in the absence of CO_2 resulted in a zero capture rate. Error bars show standard deviations (n = 20 for the basic trap; n = 5 for the others). Significant differences are indicated by different lowercase letters.

package SPSS v22.0. The experiments were also approved by the Ethics Committee of Sichuan Agricultural University.

RESULTS

The Stability Test

The number of mosquitoes present in an outdoor area can vary greatly and it is affected by mosquito species, locations, habitats, weather and other environmental factors. Therefore, we performed a stability test with the basic trap. Based on the standard deviation of 20 independent replicates in 20 different calm and rainless days (from June 1st to July 10st in 2015), we determined that the basic trap should catch 9–17 *Anopheles* and 30–56 *Culex* per night (8:00 p.m. to 8:00 a.m.) under our specific field trapping conditions. Thus for the subsequent experiments, four independent traps about 10 m apart, including one basic trap, were set at the same time on each day (**Figure 1A**). If the basic trap caught fewer or more mosquitoes than the above thresholds, the data of that day was rejected because of heavy rain, strong wind, or human disturbances. From June 1st to September 1st in 2015, 2016 and 2017 (279 days), 207 of 214 calm and rainless days met this criterion and their month-by-month and

year-by-year differences were not significant (Supplementary Figure S1). Therefore seasonal or yearly effects could be ruled out.

Effects of Odorant Chemicals on Mosquito Landing

Attractive odor compounds or repellents resulted in higher or lower numbers of mosquitoes caught by the traps, respectively (**Figure 1D**). 2,3-butanedione or 2,3-pentanedione causes an unusual ultra-prolonged activation of CO_2-detecting neurons and thus disrupts CO_2-mediated source-finding behaviors in mosquitoes (Turner et al., 2011). Although 50% declines were observed for these two chemicals (**Figure 1D**), neither 2,3-butanedione nor 2,3-pentanedione can prevent mosquitoes from finding the CO_2 source. Indole and DEET (N,N-diethyl-meta-toluamide) are mosquito contact-repellents (Gonzalez et al., 2015). However, a large number of mosquitoes were still caught on the sticky plates in the presence of either indole or DEET (**Figure 1D**).

A previous study indicated that cyclopentanone and pyridine mimic the electroantennogram effects of CO_2, and therefore are able to attract mosquitoes in large numbers in the absence of carbon dioxide (Tauxe et al., 2013). However in our experimental system, no mosquitoes were caught by using these two chemicals at 37°C but without CO_2 (**Figure 1E**). Although they attracted mosquitoes (many mosquitoes hovered 5–20 cm over the traps), neither cyclopentanone nor pyridine could replace the role of CO_2 in mosquito landing. Among all chemicals tested, only meperfluthrin (a volatile pyrethrin insecticide) treatments resulted in a zero capture rate (**Figure 1D**).

Effects of the Black Color on Mosquito Landing

A previous report found that when mosquitoes were exposed to a CO_2 plume, they spent much of their time exploring a dark visual feature on a gray background (van Breugel et al., 2015). However it is unclear whether they were attracted to the dark color or to a black and white high-contrast graphic pattern. **Figures 2A,B** indicates that a large area of black color got the highest capture rate; a large area of white color got the lowest capture rate; while the same area of 50% gray color got a medium rate. A black square on a large area of white caught significantly fewer mosquitoes than a white square on a large area of black did. Thus the larger the area of black color near the CO_2 source is, the more mosquitoes are trapped. Mosquitoes seem to be more attracted to dark objects than to black and white patterns.

We studied how mosquitoes find dark objects. In nearly darkness (illumination <1 μmol photons m^{-2} s^{-1}), white objects and black objects had similar capture rates (**Figures 2A,B**), which implies that a visual cue is involved. Mosquitoes cannot use infrared radiation (IR) as an orientation cue (Gingl et al., 2005; Zermoglio et al., 2017). We found that white objects and black objects present similar IR thermal images (**Figures 2D,E**).

Effects of Heat on Mosquito Landing

When the mosquitoes encounter a CO_2 stimulus, it can lead to higher levels of attraction to visual and sensory objects (McMeniman et al., 2014; van Breugel et al., 2015). In the current study, we used the sticky plate as the visual cue when testing the impacts of heat. Although the visual target is faint-yellow, it might be a high-contrast visual object to the mosquito. To rule out possible visual disturbance, we tested the basic trap but removed the black polyethylene bag. Interestingly, the faint-yellow sticky plate alone [Trap (2) in **Figure 2A**] got a capture rate similar to the faint-yellow sticky plate adjacent to a white paper [Trap (3) in **Figure 2A**]. Considering that the floor of the ventilated corridor also is faint-yellow, very close to the color of the sticky plate (reflectance difference <2%; Supplementary Figure S2), the solo faint-yellow sticky plate may not be a high-contrast visual object to the mosquito. In other words, the Trap (2) in **Figure 2A** may present a heat cue alone without a visual object.

With these traps of different colors, we found that although black color caught more mosquitoes than the white color, the temperature of the heat source had a greater influence on mosquito landing. No mosquito was caught in the absence of heat, although some mosquitoes hovered 3–20 cm over the CO_2 outlet (**Figure 2C**). Temperature of the heat source appears to play a much more important role in mosquito landing than the black color.

Effects of Humidity on Mosquito Landing

Besides CO_2, heat and the black color, mosquitoes had a significantly stronger response to moist and heated objects (van Breugel et al., 2015). However, for the capture rate (mosquito landing), the wet heat source (relative humidity at 10 cm above the sticky plate ≈80%) and the dry heat source (environment relative humidity ≈30%) caught almost the same numbers of mosquitoes. A humidifier alongside a dry sticky plate increased the relative humidity to about 80% (at 10 cm above the sticky plate), but resulted in a lower capture rate (**Figure 3**), because the water vapors may disturb mosquito orientation behaviors. To determine the spatial scale over which wet/dry thermal cues could realistically be detected by a mosquito, we measured the temperatures away from the sticky plate above the heated metal blocks at an ambient temperature of 21.1°C. At a distance of 10–15 cm, the difference between the dry heat source and ambient temperature was less than 0.2°C, which is the detection threshold for *Aedes* (Davis and Sokolove, 1975). However the distance was greater than 20 cm to the wet heat source (**Figure 3**). Therefore, humidity affected the detection distance, but did not have an influence on mosquito landing.

Behavioral Responses of Mosquitoes to Separated Cues

In most cases of animal hosts, these three cues (CO_2, heat and sometimes the black color) co-exist in the same place. What happens if one cue is separated from the others? When the CO_2 and heat source were combined, while the black object was separated, most mosquitoes landed on the heat source. Only 10% of the mosquitoes landed on the black object nearby (**Figure 4**). If either the CO_2 or the heat source was separated from the others, mosquitoes still explored the heat source, irrespective of its color (**Figure 4**).

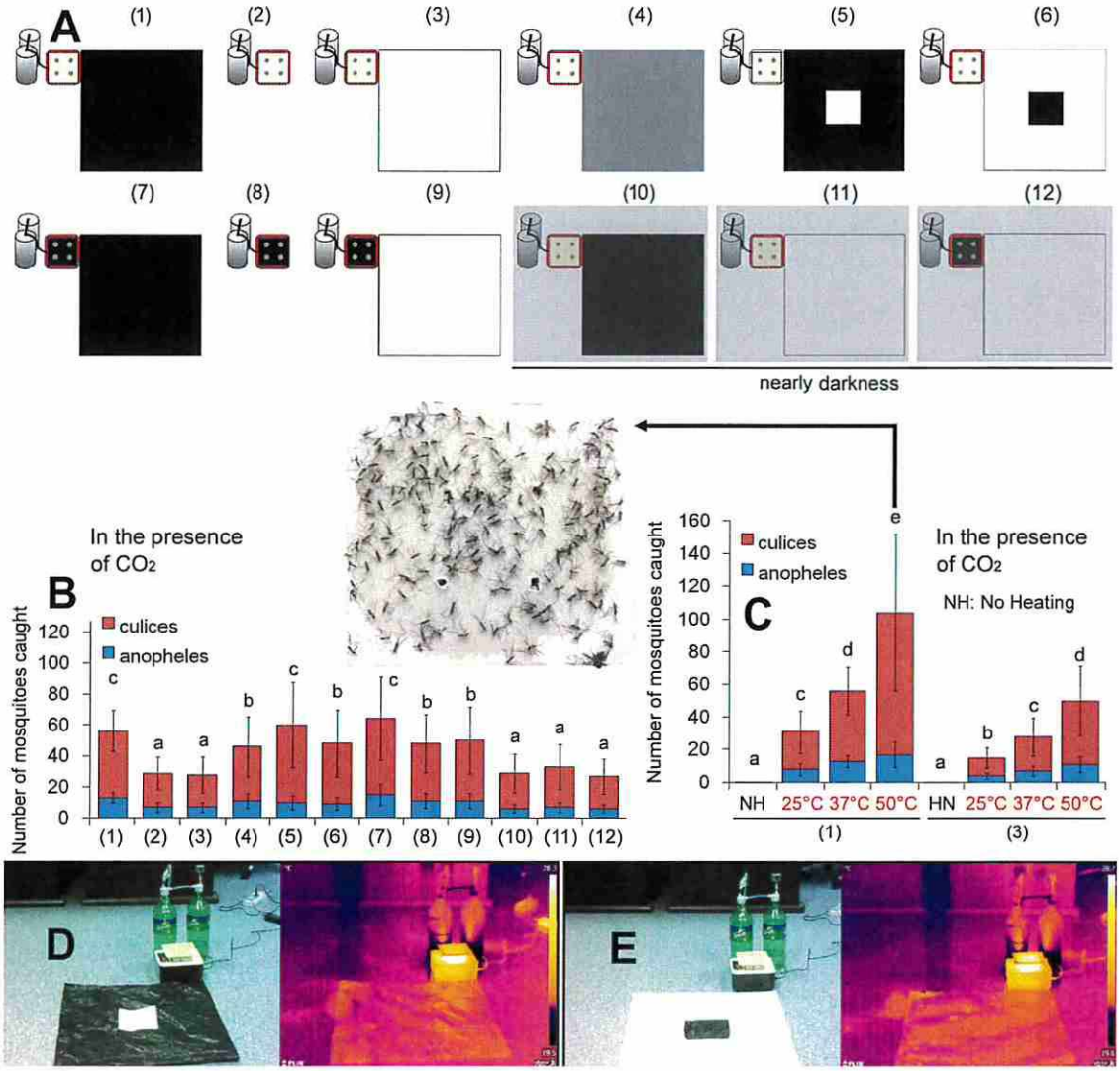

FIGURE 2 | Mosquitoes are attracted to heated black objects. **(A)** Variations to the basic trap (see "Materials and Methods" section for details). **(B)** Capture rates of 12 different traps as indicated in **(A)**. **(C)** Higher temperatures caught more mosquitoes, and no heating resulted in a zero capture rate. The Trap (1) and Trap (3) were used. A representative sticky plate of the basic trap [Trap (1)] at 50°C is shown. Error bars show standard deviations ($n = 20$ for the basic trap; $n = 5$ for the others). Significant differences are indicated by different lowercase letters. **(D)** The Trap (5) presenting a black polyethylene bag with a white square at the center. Its infrared thermal image is displayed on the right pane. **(E)** The Trap (6) presenting a white paper with a black square at the center. Its infrared thermal image is displayed on the right pane.

DISCUSSION

Indole (although it is an attractant for flies; Gonzalez et al., 2015) and DEET (Ditzen et al., 2008; Lee et al., 2010; DeGennaro et al., 2013; Stanczyk et al., 2013) are mosquito contact-repellents, which inhibit the approach of female mosquitoes to hosts. Mosquitoes are repelled within 60 ms of contact with DEET-treated skin. However, a large number of mosquitoes were still caught on the sticky plates in the presence of either indole or DEET (**Figure 1D**), which may be because the chemicals were not sprayed on the sticky plate directly, but placed 0.5 cm below the sticky plate.

Many studies have demonstrated that mosquito females use vision to actively orient towards black objects (Bidlingmayer and Hem, 1980; Browne and Bennett, 1981; Muir et al., 1992; Gibson and Torr, 1999). It is interesting that mosquitoes can discriminate not only black and white, but also maybe different colors. A field study showed that about 44% of mosquitoes were trapped on diode-equipped sticky cards fitted with green light-emitting diodes (LEDs). Significantly more females of *Aedes* and *Culex* were captured by blue LEDs compared with red or infrared LEDs. Sticky cards with blue LEDs captured significantly more *Culex* females than those with infrared LEDs (Bentley et al., 2009). However, additional work is still needed to answer

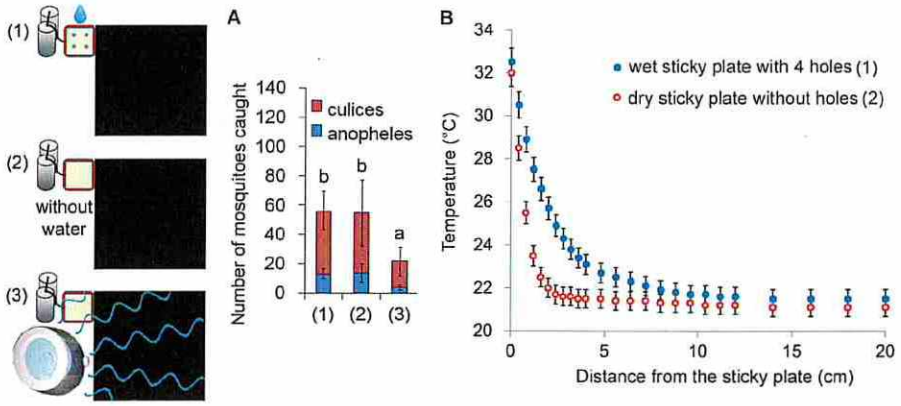

FIGURE 3 | Humidity may affect the detection distance, but not mosquito landing. **(A)** Capture rates of three different traps of a wet sticky plate with four holes [Trap (1); the basic trap], a dry sticky plate without holes and water [Trap (2)] and a humidifier alongside a dry sticky plate respectively. The humidifier was associated with a lower capture rate, because the water vapors may disturb mosquito's orientation behavior. Error bars show standard deviations (n = 20 for the basic trap; n = 5 for the others). Significant differences are indicated by different lowercase letters. **(B)** Thermal signatures of the wet heated sticky plate and the dry heated sticky plate. Temperatures away from the sticky plate (above the heated metal blocks) were measured at an ambient temperature of 21.1°C. The metal blocks were heated to 37.0°C.

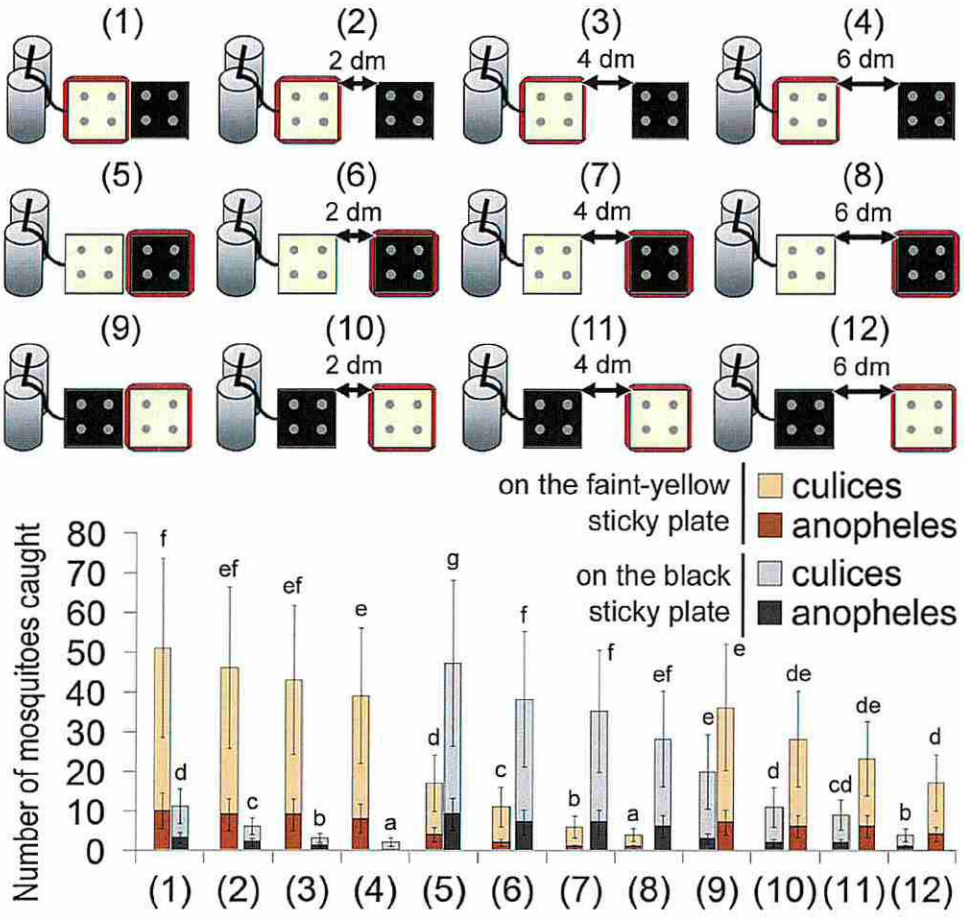

FIGURE 4 | Capture rates of 12 different traps with two sticky plates of different colors. For each trap, a faint-yellow sticky plate and a black sticky plate were placed together at an interval of 0, 2, 4 or 6 dm. One of them was heated to 37.0°C (indicated by a red square frame), and the other one was not heated. Error bars show standard deviations (n = 5). Significant differences are indicated by different lowercase letters for each color of sticky plate.

how the light wavelength composition influences mosquito behaviors.

A previous report demonstrated that mosquitoes had a significantly stronger response to moist and warm objects at distances of 6–8 cm, rather than the short 2 cm region above the floor in which mosquitoes responded to the heat plume without water (van Breugel et al., 2015). This observation suggested that the secondary effect of humidity may be an additional cue. This orientation behavior would help mosquitoes differentiate warm radiant objects (such as dark rocks heated by the sun) from animals, which increase the humidity around them when they perspire (Burgess, 1959; Eiras and Jepson, 1994). The wet heat plumes would likely disperse more slowly than the dry heat plume, however they resulted in similar capture rates. Thus, humidity may affect the detection distance, but probably not mosquito landing.

McMeniman et al. (2014) found that CO_2 evokes mosquito's heat-seeking behaviors, and van Breugel et al. (2015) showed that CO_2 influenced visual target seeking. Both of these studies suggest that CO_2 can gate responses to other sensory stimuli. Our field study also showed that, for the mosquito species tested, CO_2 is the most important cue (prerequisite) for the landing. A previous study indicated that cyclopentanone and pyridine mimic the electroantennogram effects of CO_2, and therefore are able to attract mosquitoes in large numbers in the absence of carbon dioxide (Tauxe et al., 2013). Here in this study, it is interesting to observe that cyclopentanone and pyridine lured mosquitoes but did not lead to landing. Mosquitoes may be attracted by CO_2 analogs, but they can discriminate CO_2 from other compounds and therefore do not land. There appear to be some differences in electroantennogram responses to CO_2, cyclopentanone and pyridine stimulations (Tauxe et al., 2013).

For all odorant chemicals used in this study, no one can replace or completely inhibit the role of CO_2 in mosquito landing, except for meperfluthrin. Use of meperfluthrin, permethrin or other pyrethroids (Xue et al., 2012) may be the most simple and feasible anti-mosquito strategy. Although the degree of cytotoxicity of pyrethroids on human cells is much lower than on insect cells (Yun et al., 2017), these insecticides induce significant increases in sister chromatid exchanges (therefore showing some genotoxic effects) and cause apparently oxidative stresses in cultured human lymphocytes in a dose-dependent manner (Azab et al., 2017). More efficient and safer pyrethroids should be developed (Haverinen and Vornanen, 2016).

AUTHOR CONTRIBUTIONS

SY designed the study and wrote the article. YH-Z, Z-WZ, Y-FF and G-CZ performed the research. All the authors analyzed the data, discussed the results and made comments on the manuscript.

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SUPPLEMENTARY MATERIAL

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Modelling optimum use of attractive toxic sugar bait stations for effective malaria vector control in Africa

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Abstract

Background: The development of insecticide resistance and the increased outdoor-biting behaviour of malaria vectors reduce the efficiency of indoor vector control methods. Attractive toxic sugar baits (ATSBs), a method targeting the sugar-feeding behaviours of vectors both indoors and outdoors, is a promising supplement to indoor tools. The number and configuration of these ATSB stations needed for malaria control in a community needs to be determined.

Methods: A hypothetical village, typical of those in sub-Saharan Africa, 600 × 600 m, consisting of houses, humans and essential resource requirements of *Anopheles gambiae* (sugar sources, outdoor resting sites, larval habitats) was simulated in a spatial individual-based model. Resource-rich and resource-poor environments were simulated separately. Eight types of configurations and different densities of ATSB stations were tested. *Anopheles gambiae* population size, human biting rate (HBR) and entomological inoculation rates (EIR) were compared between different ATSB configurations and densities. Each simulated scenario was run 50 times.

Results: Compared to the outcomes not altered by ATSB treatment in the control scenario, in resource-rich and resource-poor environments, respectively, the optimum ATSB treatment reduced female abundance by 98.22 and 91.80 %, reduced HBR by 99.52 and 98.15 %, and reduced EIR by 99.99 and 100 %. In resource-rich environments, n × n grid design, stations at sugar sources, resting sites, larval habitats, and random locations worked better in reducing vector population and HBRs than other configurations ($P < 0.0001$). However, there was no significant difference of EIR reductions between all ATSB configurations ($P > 0.05$). In resource-poor environments, there was no significant difference of female abundances, HBRs and EIRs between all ATSB configurations ($P > 0.05$). The optimum number of ATSB stations was about 25 for resource-rich environments and nine for resource-poor environments.

Conclusions: ATSB treatment reduced *An. gambiae* population substantially and reduced EIR to near zero regardless of environmental resource availability. In resource-rich environments, dispersive configurations worked better in reducing vector population, and stations at or around houses worked better in preventing biting and parasite transmission. In resource-poor environments, all configurations worked similarly. Optimum numbers of bait stations should be adjusted according to seasonality when resource availability changes.

Keywords: Malaria, *Anopheles gambiae*, Attractive toxic sugar bait, ATSB, EIR, Individual-based model, Agent-based model

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Background

Indoor residual spraying (IRS) and insecticide-treated nets (ITNs)/long-lasting insecticidal nets (LLINs) have been widely used as malaria vector control tools and have achieved significant reduction of malaria transmission globally [1–5]. However, the increased use of pyrethroids for the treatment of nets has resulted in increased pyrethroid resistance in anopheline mosquitoes, reducing the efficacy of both IRS and ITNs/LLINs [6–8]. The use of these two indoor tools has also caused behavioural changes in anopheline mosquitoes in multiple locations over the world: the indoor host-seeking behaviour has shifted to a more exophilic (outdoors) behaviour [9–12]. It is questionable whether IRS and ITNs/LLINs alone will achieve malaria elimination [13].

Residual malaria transmission [14] has been consistently demonstrated even in areas with good coverage of IRS and LLINs; the World Health Organization has declared an urgent need for new vector control tools, one type of which is sugar baits [15]. Attractive toxic sugar bait (ATSB) is a new method that uses attractive plant substances in the bait; contact with the sugar elicits a lethal effect on the mosquito after feeding on the sugar and toxin mixture [16–18]. Several field trials have proved the effectiveness of ATSB in controlling anopheline mosquitoes [16–20]. In addition, the risk of developing resistance is low since several different oral toxins can be used with ATSB, and it could be a promising simple and low-cost tool to supplement IRS and ITNs/LLINs combating ongoing residual malaria transmission [19]. ATSB can be either sprayed on vegetation or used in bait stations, can be applied outdoors or indoors [21], and can be applied in different configurations [16–20, 22, 23]. Accordingly, it is necessary to determine the number of ATSB stations and configuration of these bait stations needed for optimal malaria control in a village. ATSB has been effective even in sugar-rich environments [18], where natural sugar sources compete with ATSB, but such variations in environmental resource availability impact the survival and human-biting behaviour of *Anopheles gambiae* [24], and may alter the selection of optimum distribution and frequency of ATSB applications. While several field studies have been performed to evaluate the effectiveness of ATSB, all of these, except for one that also used vector capacity as an outcome [18], have been more focused on the decrease of vector abundance as the outcome [16, 17, 19, 25]. Evaluating the impact of ATSB on malaria transmission is a step forward toward the assessment of the method.

While field studies and community trials may be the best methods for answering these questions, they are expensive and time consuming. In addition, it can be very difficult to identify several villages that are comparable in both environmental structure and human demographics, such that sufficient controls and replicates are available. Using the same village through several periods of time to compare different ATSB configurations is also problematic, because the trials have to be carried sequentially, and additional washout periods between trials are needed to control the carryover effects. In addition, seasonality can affect the comparability of trials. It is therefore reasonable to carry out this spatial individual-based modelling (IBM) study simulating several comparable environments to evaluate the impact of different ATSB configurations on malaria transmission, and it can be useful in guiding experimental field designs. Recently, a spatial IBM was developed to simulate the interactions of *An. gambiae* mosquitoes and their environment, such as sugar feeding, blood feeding, resting, and oviposition and the study demonstrated a great impact of environmental sugar sources and outdoor resting sites on survival of *An. gambiae* and malaria parasite transmission [24]. By adding a few new features regarding ATSB, such as the interaction between mosquitoes and ATSB, the model can be used to simulate and evaluate the outcome of ATSB applications in different densities and configurations particularly in habitats that are optimally or marginally suitable for *An. gambiae*.

The objective of this study is to determine the optimum configuration and number of ATSB stations for *An. gambiae* and malaria control in resource-rich and resource-poor environments. Employing the modified model, different ATSB configurations were simulated and their impacts on the survival of *An. gambiae* and malaria transmission were evaluated and compared. Entomological inoculation rate (EIR), the rate at which people are bitten by infectious mosquitoes, has a log-linear relationship with malaria prevalence [26] and provides a direct measure of mosquito-to-human malaria parasite transmission intensity [27–29].

Methods

Model design

The basic model design was described in detail in Zhu et al. [24] according to ODD (overview, design concepts, and details) protocol on IBMs [30, 31]. With a few adjustments, this model was used for the purpose of this study. JAVA 7 (Oracle Co, Redwood, CA, USA) and Mason package v17 [32] were used to develop this model.

For the evaluations, the size of the hypothetical simulated village was 600 × 600 m in the continuous space, and 25 houses were randomly located in a 100 × 100 m area in the centre of the village. Two environmental resource availability levels were simulated: in resource-rich environment, 50 natural sugar sources, outdoor resting sites, and larval habitats were randomly located in the

whole village; whereas in resource-poor environment, 25 of each were randomly located. One-hundred humans were living in the village, and they moved around in the daytime and went back and stayed in their homes at night. Protection by LLINs or IRS was not simulated.

One-thousand male *An. gambiae* mosquitoes and 1000 female *An. gambiae* mosquitoes were simulated at the beginning, with age, location and gravid status randomly assigned to them. The maximum lifespan of *An. gambiae* was 10 days for male and 30 days for female [33–35]. If the mosquitoes could not find sugar or blood meals at night and remained hungry by morning, they would die of starvation. They could also die from reaching the life spans or feeding on ATSB. At night, male *An. gambiae* could sugar feed, rest and fly around, whereas female *An. gambiae* could also blood feed and oviposit.

In the model, males, if they were hungry, would begin to seek sugar sources; if they were fed, then they would seek resting sites to rest. Females need blood to develop eggs and need sugar to survive and fly [36]; females in the model, if they were in need of blood meal, would begin to seek a human host, but if they became too hungry before they could find a human, they would begin also to seek a sugar meal to provide energy for further activities. Because of the inhibition of host-seeking response in *An. gambiae* following blood feeding as recorded [37], in the model if the females were blood-fed and gravid but the eggs were not ready, they usually did not search for a blood meal; when they were hungry, they would seek sugar sources. But if they were very hungry, they would start to seek hosts as well, but sugar seeking would still be a priority. If they were fed and not hungry, they would begin to seek resting sites. If the females were ready to oviposit, they would seek a larval habitat, but if they became hungry, they would seek a sugar source first. Every blood meal would be kept track of in the model, and the probability of infection with malaria parasite for an uninfected female *An. gambiae* by biting a human was considered as 20 % [38–45]. After an extrinsic incubation period of 10 days [38, 46], the female would become infectious, and afterwards biting of a human would be counted as potential malaria infection and used to calculate EIR.

In the model, when seeking either sugar, blood, resting sites, or larval habitats, the mosquitoes were able to sense the targets in the circle around them, the radius of which was the maximum attractive distance of the target. If they could find one, they would fly toward the target directly, but if they could not, they would fly in a random direction for the current step, and begin seeking again in the next step. Two-thousand random movements, which represented 2000 m of flight, would result in an additional hunger level [47].

Density dependence was applied in the development in the larval stage. Eggs hatching rate was 70 % of eggs oviposited as recorded [48, 49]. Number of larvae in each larval habitat on each day was calculated as the number of hatched eggs on that day, plus number of larvae that had survived from the previous day, minus number of larvae that had pupated on that day [50]. Independent mortality 'm' (without impact of larvae density) of larvae was 0.1 per day [51]. The overall mortality of larvae on day t 'M_t', considering the density-dependent mortality, was calculated as $M_t = m \times (1 + L_t/K)$, where L_t is the number of larvae on day t, and K is the larval habitat capacity. K was assumed to be 300, deducting from the formula above, it means when the number of larvae in one habitat reached nine times of K (2700), the overall mortality would become one per day. Emerging rate from pupae to adult was 70 % of mature pupae [52]. Table 1 summarizes the parameters and assumptions used.

Simulations

Eight types of ATSB station configurations and two density levels of each were simulated in resource-rich and resource-poor villages. The eight types of ATSB configurations were: n × n (7 × 7 or 5 × 5) stations placed in grid design over the entire area, 48 or 24 stations placed evenly at the periphery of the house area, 50 or 25 stations placed evenly in a transact across the village, 50 or 25 stations located within the natural sugar sources, 50 or 25 stations at houses, 50 or 25 stations within the resting sites, 50 or 25 stations at the larval habitats, and 50 or 25 stations randomly placed in the entire area. A scenario in which no ATSB station was placed out in the village was the configuration used as the control scenario. When two ATSB stations were put at each house, they were put 5 m apart in both the x and y horizontal directions. When two ATSB stations were put at each sugar source/resting site/larval habitat, they were put 1 m apart in both the x and y horizontal directions. After selecting an optimum configuration design, additional densities of ATSB stations placed in that design were simulated to determine the optimum number (a minimum number that is needed to achieve effective vector control that drives EIR to near zero) of stations needed. Figure 1 demonstrates the simulated map of the village and the locations of different objects. All ATSB stations were assumed to be working continuously for the whole period, because in reality, regular stations last for 1 month and bio-filmed stations work for 6 months, and they can be replaced easily.

Pilot simulations showed that in the control scenario, mosquito population equilibrated after day 40, with mild fluctuations. So, only the population dynamics after day 40 are shown in Fig. 2. ATSB stations were placed at the beginning of day 61, and the simulation continued to day

Table 1 Parameters and assumptions used in the model

Parameters/inputs	Values	References
Village size	600 × 600 m	Assumption
House distribution	100 × 100 m	Assumption
No. houses	25	Assumption
No. humans	100	Assumption
Initial no. male *An. gambiae*	1000	Assumption
Initial no. female *An. gambiae*	1000	Assumption
Human moving outdoors	07:00–20:00	Assumption
Active time of *An. gambiae*	19:00–05:00	Assumption
Max life span of male *An. gambiae*	10 days	[33–35]
Max life span of female *An. gambiae*	30 days	[33–35]
Hunger level threshold of sugar-seeking females switching to accepting blood	2	[37, 55–57] and assumption
Hunger level threshold of blood-seeking females switching to sugar-seeking	2	[37, 55–57] and assumption
No. random movements leading to an additional need for sugar meal	2000 steps	[47] and assumption
Extrinsic incubation period	10 days	[38, 46]
Minimum number of sugar meal of male *An. gambiae* per night	2	[53]
Minimum number of sugar/blood meal of female *An. gambiae* per night	1	[53]
Days needed to develop eggs after blood-feeding	2–3 days	[58]
Average size of egg batches	100	[59]
Attractive distance of sugar source	5 m	Unpublished data
Attractive distance of human	40 m	Unpublished data
Sensing distance of larval habitat site	5 m	Unpublished data
Sensing distance of resting site	5 m	Unpublished data
Duration of aquatic stage	12 days	[52, 60]
Larval habitat site capacity	300	Assumption
Egg hatch rate	0.7	[48, 49]
Independent mortality of larvae	0.1	[51]
Emerging rate of pupae	0.7	[52]

120, allowing time for the mosquito population to equilibrate again. Each scenario was simulated 50 times, each with a different pseudo-random initiator.

Statistical analysis

Human biting rate (HBR) was defined as the total number of bites per day divided by the number of humans. Daily abundance was defined as the number of *An. gambiae* mosquitoes left at the end of each day before recruits of newly emerged adults. EIR was defined as the total number of infectious bites per day divided by the number of humans.

Only data after the population equilibrated (from day 101 to 120) were used for the comparison analysis. To provide the distributions of mosquito survival as a baseline information of the model, the means of daily survival rates for day 101–120 were calculated, and the average ages of all the mosquitoes at the time point of midnight of day 111 were calculated, both for the control scenario and the 7 × 7 design scenario in sugar-rich environments. The means of daily abundance, HBRs and EIR, and the percentages of decreases from control scenario were calculated. ANOVA and Tukey post hoc tests were used to compare outcomes of simulations of different ATSB configurations. SAS 9.3 (SAS Institute, Inc., Cary, NC, USA) was used for the analyses.

Results

Table 2 shows the distribution of *An. gambiae* survival of ATSB-treated and untreated scenarios. ATSB treatment had greater impact on age composition in females than in males. At the time point of age recording (midnight of day 111), there was no female beyond extrinsic incubation period (EIP) left. ATSB treatment also substantially reduced daily survival rates in both males and females.

Figure 2 shows the daily changes of male and female *An. gambiae* abundance with different ATSB configurations during the two-month period. To make the graphs more concise, only high densities of ATSB stations placed in resource-rich environments and low densities of ATSB stations placed in resource-poor environments are shown.

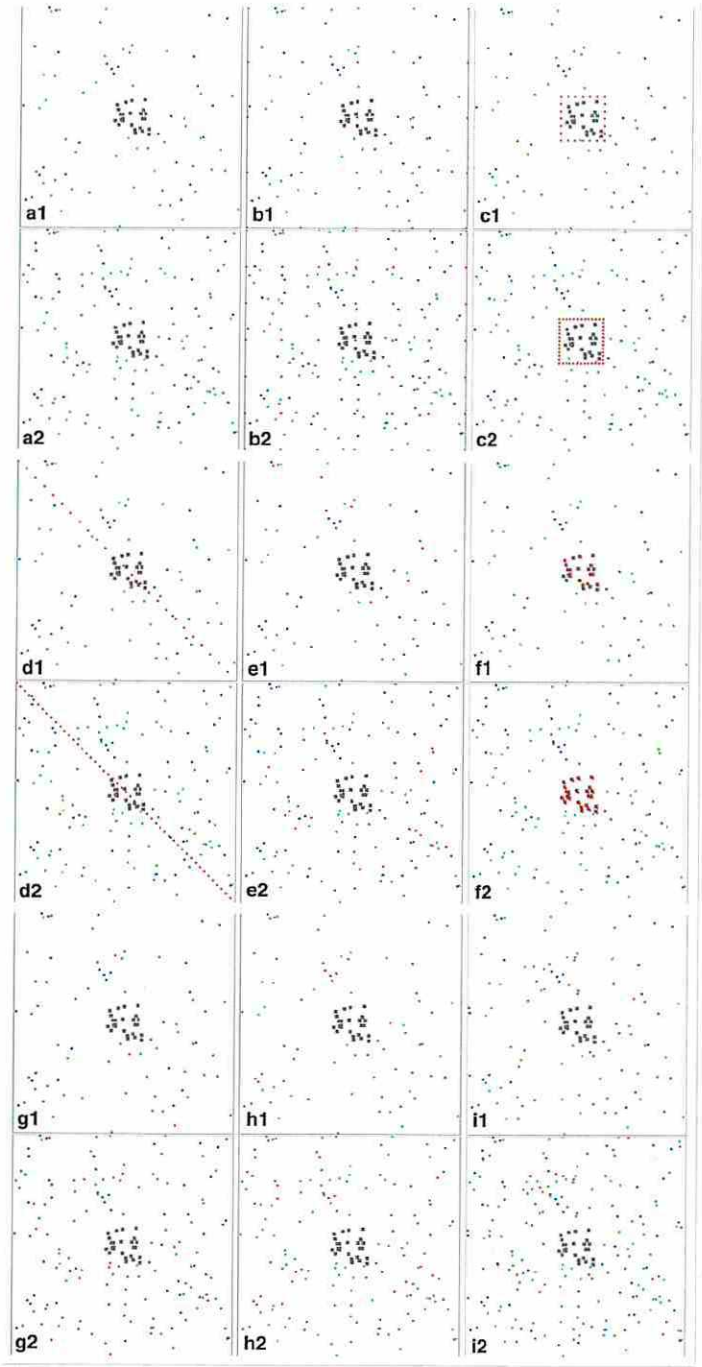

Fig. 1 Configurations of ATSB stations in resource-rich and resource-poor environments. To be concise, the Figure contains only low-density (25) stations for resource-poor environments and high-density (50) stations for resource-rich environments. In each sub-figure, the *grey dots* represent houses, the *green dots* represent sugar sources, the *light blue* dots represent outdoor resting sites, the *dark blue dots* represent larval habitats, and the *red dots* represent ATSB stations. Sub-figures **a1**–**i1** are control, 5 × 5 grid design, house periphery design, transect design, stations at sugar sources, stations at houses, stations at resting sites, stations at larval habitats, and stations at random locations in resource-poor environments; sub-figures **a2**–**i2** are the same order of designs in resource-rich environments. In designs where stations were placed at resources (e.g., sugar sources), the *dots* representing the resources are hidden behind *red dots* and not shown. The series of n × n grid design are the same designs as **b1** and **b2**, except that the numbers in *each row* and *column* are 0, 2, 3, 4, 5, 6, 7, 8, 9

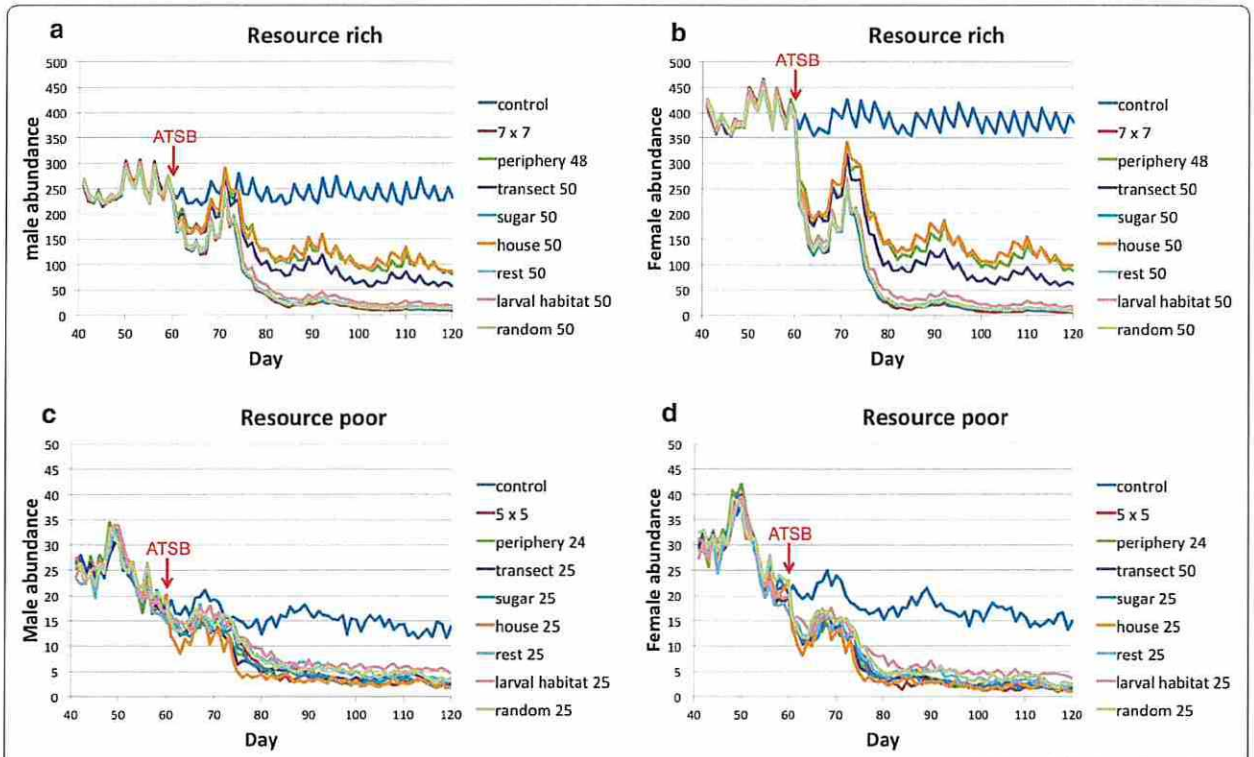

Fig. 2 Average daily abundances for male and female *Anopheles gambiae* mosquitoes along time in simulations with different ATSB configurations. Sub-figures **a** and **b** are average daily abundances of male and female *An. gambiae* mosquitoes in resource-rich environments with different ATSB configurations. Sub-figures **c** and **d** are average daily abundances of male and female *An. gambiae* mosquitoes in resource-poor environments with different ATSB configurations. In each sub-figure, *x-axis* is the time in days; *y-axis* is the abundance (number of mosquitoes). Each *line* represents an ATSB configuration according to the legend on the *right*. The *red text* 'ATSB' and the *arrow below mark* the timing of ATSB treatment, which is at the beginning of day 61

Table 2 Survival distribution of *Anopheles gambiae* in ATSB-treated and control environments

Sex	Treatment	Age					Daily survival rate	
		Mean	SD	Median	Min	Max	Mean	SD
Female	Control	4.2	4.6	2.2	0.2	29.2	0.81	0.05
	7 × 7 ATSB	1.4	1.7	0.2	0.2	9.2	0.32	0.39
Male	Control	2.3	2.4	1.2	0.2	9.2	0.72	0.05
	7 × 7 ATSB	2.2	2.4	1.2	0.2	9.2	0.41	0.43

In resource-rich environments, male populations equilibrated at about 240 mosquitoes, and female populations equilibrated at about 380 mosquitoes. After the placement of ATSB stations at day 61, male and female populations in all ATSB-treated scenarios began to drop. Population sizes included adults newly emerged from pupae, which were resting for the first night and, therefore, did not approach the ATSB stations. Mosquitoes at this stage, that developed from eggs laid before ATSB placement caused a slight increase in the populations. However, with the beginning of ATSB effect on the rate of reproduction, the populations crashed again. Then both male and female population sizes equilibrated at about 60 (from about ten to 110 for males and from ten to 120 for females in different configurations). The optimum configurations to control mosquito populations were in the 7 × 7 grid design, 50 stations either at sugar sources, resting sites, larval habitat sites, or random locations. Stations placed at periphery of house area, at houses, or along a transect were less effective.

In resource-poor environments, the equilibrated number of males was about 14, and females equilibrated at about 16. After the placement of ATSB stations at day 61, all populations except the controls began to drop. A small increase in populations was also observed due to the delayed effect of pre-imaginal stages and thus on mosquito emergence. The equilibration level of both male and female populations was reduced to around or below five. Stations at houses and 5 × 5 grid design were most effective, and the differences between different ATSB configurations were negligible.

Table 3 shows the daily mean of mosquito abundance, HBR and EIR of scenarios with different ATSB configurations. In resource-rich environments, compared to the outcomes not altered by ATSB treatment in the control scenario, male abundance was reduced by 95.80 %, female abundance was reduced by 98.22 %, HBR were reduced by 99.52 %, and EIR was reduced by 99.99 %, with the optimum ATSB station configuration. In resource-poor environments, compared to the outcomes not altered by ATSB treatment in the control scenario, male abundance was reduced by 82.45 %, female abundance was reduced by 91.80 %, HBR were reduced by 98.15 %, and EIR was reduced by 100 %, with the optimum ATSB station configuration. The decreases in abundance, HBR and EIR were all significant in both resource-rich and resource-poor environments ($P < 0.0001$).

Results of post hoc analysis showed that the differences of abundances, HBRs and EIRs between control and all the other ATSB configurations were significant ($P < 0.0001$). In resource-rich environments, control of female *An. gambiae* population was most effective with n × n grid design, designs of bait stations at sugar sources, resting sites, larval habitats, and random locations ($P < 0.0001$). There was no significant difference between the results within these configuration patterns ($P > 0.05$). Except for bait stations near larval habitats ($P = 0.0322$), there were no significant differences in the effects of high and low concentrations of ATSB stations on female abundance ($P > 0.05$). The estimated reduction of HBRs was similar to the results of female abundance. However, there was no significant difference of EIR reduction between all ATSB configurations ($P > 0.05$). In resource-poor environments, there was no significant difference in female abundance, HBRs and EIR between all ATSB configuration designs ($P > 0.05$).

Based on the results that an n × n grid design was an optimum configuration in both resource-rich and resource-poor environments, it was selected for testing the impact of different numbers of ATSB station on vector control and EIR. Figure 3 shows the means of male abundance, female abundance, and EIR with 0, 2 × 2, 3 × 3, 4 × 4, 5 × 5, 6 × 6, 7 × 7, 8 × 8, and 9 × 9 ATSB stations in grid design in both resource-rich and resource-poor environments. An exponential trend line of female abundance was added for each plot. In resource-rich environments, mosquito population and EIR decreased rapidly when total number of stations increased from 0 to 25; after that, further increase of stations did not significantly improve the outcomes. In resource-poor environments, absolute numbers of mosquito populations and EIRs did not significantly decrease as the number of stations increased. However, the results demonstrate that after nine stations, the decrease was even slower.

Discussion

Based on the spatial IBM, the study showed that ATSB application effectively reduced the density of *An. gambiae*, HBR and EIR in both resource-rich and resource-poor environments. Configurations of dispersed ATSB stations were significantly more effective for vector control in resource-rich environments; but in resource-poor environments, all configurations worked similarly. No significant difference in EIR reduction was found among all configurations in both resource-rich and resource-poor environments. Reduction of *An. gambiae* density and EIR increased with the increase in numbers of ATSB stations, but it reached a point at which further increase was ineffective.

Without ATSB treatment, female mosquitoes survived better than males in both resource-rich and resource-poor environments, but the difference in survival in resource-poor environments was negligible. However, after the ATSB treatment, male and female populations were reduced to very similar sizes, with the decrease in female numbers being greater than that of males. This finding was consistent with the observation for *Anopheles claviger* reduction in an earlier field study in Israel, where average daily female catches per trap decreased from about 25 to under five, and average daily male catches per trap decreased from about 15 to under five [16]. The trend of population dynamics was also similar to the field trial in Mali showing that daily female catches decreased from approximately 180 to 25, and male catches decreased from approximately 90 to ten, where ATSB spray was used around larval habitats, and *An. gambiae* were collected by CDC light traps [17]. Similar population reduction trends were also observed in field trial of *Anopheles sergentii* [18]. The observed longer survival of females in untreated environments increases the risk of malaria transmission. It is, therefore, important to note that ATSB treatment effects on females are even stronger than on males.

In both resource-rich and resource-poor environments and with all ATSB configurations, percentages of

Table 3 Comparison of daily mean of abundance, human biting rate (HBR) and entomological inoculation rate (EIR) between different ATSB configurations and control

Environment	ATSB configuration	Male abundance		Female abundance		HBR		EIR	
		Means	% decrease	Means	% decrease	Means	% decrease	Means	% decrease
Resource rich	Control	239.375	0.00	380.781	0.00	6.69513	0.00	0.50085	0.00
	7 × 7	10.06	95.80	6.791	98.22	0.03203	99.52	0.00007	99.99
	5 × 5	31.285	86.93	28.168	92.60	0.18999	97.16	0.00088	99.82
	Periphery 48	96.289	59.77	108.078	71.62	0.66992	89.99	0.00152	99.70
	Periphery 24	99.447	58.46	111.905	70.61	0.69635	89.60	0.00176	99.65
	Transect 50	66.742	72.12	71.676	81.18	0.50966	92.39	0.00101	99.80
	Transect 25	70.795	70.43	75.996	80.04	0.56473	91.57	0.00089	99.82
	Sugar 50	12.723	94.68	9.375	97.54	0.04629	99.31	0.00011	99.98
	Sugar 25	29.25	87.78	26.644	93.00	0.17223	97.43	0.00042	99.92
	House 50	103.22	56.88	118.499	68.88	0.79739	88.09	0.00157	99.69
	House 25	104.922	56.17	121.827	68.01	0.83236	87.57	0.00185	99.63
	Rest 50	16.056	93.29	12.249	96.78	0.0697	98.96	0.00029	99.94
	Rest 25	36.883	84.59	35.806	90.60	0.27758	95.85	0.00198	99.60
	Larval 50	22.01	90.81	20.23	94.69	0.15867	97.63	0.0018	99.64
	Larval 25	49.956	79.13	53.629	85.92	0.51431	92.32	0.00763	98.48
	Random 50	14.545	93.92	11.493	96.98	0.07178	98.93	0.00009	99.98
	Random 25	42.344	82.31	43.48	88.58	0.37372	94.42	0.00643	98.72
	F	94.61		185.46		474.6		42.86	
	P	<0.0001		<0.0001		<0.0001		<0.0001	
Resource poor	Control	13.716	0.00	15.748	0.00	0.32559	0.00	0.01364	0.00
	7 × 7	2.544	81.45	1.292	91.80	0.00603	98.15	0.00003	99.78
	5 × 5	2.873	79.05	1.65	89.52	0.01071	96.71	0.00003	99.78
	Periphery 48	2.809	79.52	1.974	87.47	0.00898	97.24	0.00015	98.90
	Periphery 24	3.341	75.64	2.371	84.94	0.0113	96.53	0.00006	99.56
	Transect 50	2.601	81.04	1.765	88.79	0.0091	97.21	0.00012	99.12
	Transect 25	3.364	75.47	2.208	85.98	0.0123	96.22	0.0001	99.27
	Sugar 50	3.183	76.79	1.985	87.40	0.01208	96.29	0.00006	99.56
	Sugar 25	3.013	78.03	1.985	87.40	0.01162	96.43	0.00006	99.56
	House 50	2.407	82.45	1.957	87.57	0.00985	96.97	0.00032	97.65
	House 25	2.491	81.84	1.863	88.17	0.01057	96.75	0.00016	98.83
	Rest 50	3.316	75.82	2.215	85.93	0.01626	95.01	0.00009	99.34
	Rest 25	3.967	71.08	2.8	82.22	0.02411	92.59	0	100.00
	Larval 50	5.133	62.58	4.245	73.04	0.04778	85.33	0.0014	89.74
	Larval 25	5.609	59.11	4.48	71.55	0.04869	85.05	0.00099	92.74
	Random 50	2.745	79.99	1.873	88.11	0.01463	95.51	0.0001	99.27
	Random 25	4.234	69.13	3.316	78.94	0.03447	89.41	0.00004	99.71
	F	4.17		9.46		19.2		6.46	
	P	<0.0001		<0.0001		<0.0001		<0.0001	

reduction in HBRs were always greater than percentages of reduction in female populations, and percentages of reduction in EIRs were also always greater than percentages of reduction in HBRs. In other words, ATSB stations are more effective against females that are more likely to bite a human host, and females that carry malaria parasites. When comparing the increases from percentage reduction of female abundance to percentage reduction of EIR between different configuration designs, the intensity of increase was greatest in two configuration designs: bait stations at houses (e.g., percentage reduction increased from female abundance reduction of 68.88 % to EIR reduction of 99.69 % in resource-rich environments with 50 stations) and bait stations at the periphery of the

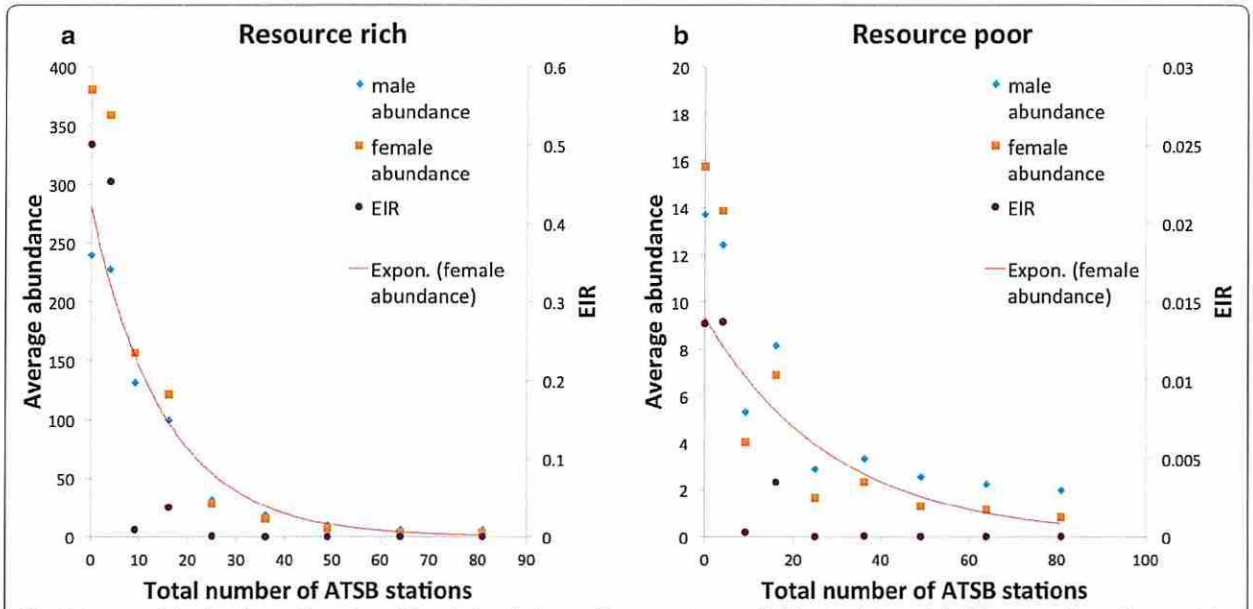

Fig. 3 Average daily abundances for male and female *Anopheles gambiae* mosquitoes and EIR in simulations with different numbers of n × n grid design ATSB stations. This figure shows the average daily abundances for male and female *An. gambiae* mosquitoes and average EIRs calculated from the period from day 101 to 121 (after population equilibrium). Sub-figure **a** shows results in resource-rich environments, sub-figure **b** shows results in resource-poor environments. In each sub-figure, *x-axis* is the total number of ATSB stations; *y-axis* on the *left* is the abundance (number of mosquitoes) for the males and females; *y-axis* on the *right* is the EIR value. *Blue dots* represent male abundance, *orange dots* represent female abundance, and *purple dots* represent EIR. The 10 dots in each *colour*, from *left* to *right*, represent results with grid designs of 0, 2 × 2, 3 × 3, 4 × 4, 5 × 5, 6 × 6, 7 × 7, 8 × 8, 9 × 9

house area (e.g., percentage reduction increased from female abundance reduction of 71.62 % to EIR reduction of 99.70 % in resource-rich environments with 50 stations). Although these two designs are not as good at reducing vector population, these results demonstrate that they have a better ability to target human-biting and parasite-transmitting mosquitoes. This can be explained by the results shown in Table 2: ATSB treatment killed most of the older mosquitoes and lowered the average age of the whole population. As the malaria parasite undergoes an EIP in the mosquito to become infectious, a female *An. gambiae* has to live longer than the EIP to transmit the parasite. During this time period the mosquito will need several sugar meals [36, 53], increasing the probability of the mosquito becoming attracted to and killed by ATSB before it becomes infectious. This is consistent with findings from a field study conducted in three oases in Sahara-Arabian phyto-geographical zone that ATSB treatment reduced the proportion of older more epidemiologically dangerous mosquitoes [18].

ATSB treatment is effective in both resource-rich and -poor environments. This finding is supported by the results of a previous field study concluding that ATSB decimate populations of *Anopheles* mosquitoes regardless of the local availability of sugar sources [18]. In resource-rich environments the mosquito population is higher than in resource-poor environments but the impact of ATSB application causes a similarly low population density in the treated areas whether the environment offers optimal conditions for mosquitoes or not. Thus, the difference of the treated areas from their respective controls is caused by differences in population size in the controls, which are not accompanied by a parallel variation in the treated areas. In other words, regardless of the availability of environmental resources and the initial similarity of the population size, ATSB treatment can reduce the population to a similar very low level. Moreover, with optimum ATSB station configuration, EIR can be reduced to below 0.0001 in both environments. Achieving this low level of EIR is important for malaria elimination strategies [28].

In resource-rich environments, configurations of dispersed ATSB stations were more effective in reducing mosquito populations than concentrated application configurations. However, EIR can be reduced by more than 99 % with 50 stations placed in any configuration. Placing the stations at the randomly distributed sugar sources, resting sites or larval habitats did not have any extra benefit over randomly placed stations at other locations. Placing stations at sugar sources, resting sites or

larval habitats is not recommended. Additional benefits of this observation are the lower effort and expertise that are required to place bait stations in exactly identified locations that suit the mosquito biology. In resource-poor environments, all configurations of ATSB stations had similar effect as indicated by the lack of statistically significant difference. However, if fewer than 25 stations are used in the field in a resource-poor environment, as the one simulated in this study, the difference of impact between different configurations may become visible, and there might be an advantage of one configuration over others.

Use of bait stations at all houses in villages should be considered in field trials. Although placement of ATSB stations at houses was not the most effective configuration in resource-rich environments, it reduced the EIR by over 99 %. Operationally, placing ATSB stations at houses may be the most feasible and least expensive strategy. Location of ATSB stations at houses saves the labour of having to cover the entire village and periphery area. In addition, the ATSB stations at houses are more protected against damage and there is less need for replacement. Based on the current findings of this study, it is also likely that ATSB stations placed at each house to directly target human biting and parasite transmission may be most effective when houses are scattered rather than highly concentrated.

Placing an additional ATSB station at each house did not significantly increase mosquito mortality or reduce EIR. This finding was related to the assumption in the model that each ATSB could attract mosquitoes from all directions, so two stations close to each other did not work more effectively than a single station. In many areas in Africa, houses have a gap between the roof and the walls for ventilation, which accords with the assumption in this model that the odour of ATSB can distribute across the house. However, in other areas, where there are no gaps under house roofs, an additional ATSB station on the other side may be beneficial to optimize ATSB attraction.

A series of increasing numbers of ATSB stations in n × n grid design was tested to further explore the selection of optimum number of stations. For resource-rich environments simulated in this study (50 of each type of resources), increases in effectiveness were minor after the number of stations reached 25. For resource-poor environments simulated in this study (25 of each type), even ten stations can achieve satisfactory results; further increase of stations did not alter the outcome significantly. Therefore, using additional stations beyond the optimum number is not recommended. Seasonality was not simulated in this model; however, as seasons change, resource availability in the same village can change. The optimum number of stations should be adjusted according to the changes to ensure effectiveness. The highest number of ATSB stations are needed in rainy seasons when there is abundant sugar sources and resting sites available.

Extinction is not an issue in this study. Although a few repetitions ended up with zero mosquitoes at the end, as the average abundances over the last 20 days were calculated, it reduced the proportion of repetitions with the outcome of extinction. In addition, extinction only happened in the control/untreated scenario in resource-poor environments or scenarios with ATSB treatment, so it is a result of the given condition. Because of the stochasticity in the simulations, some repetitions had lower abundances, including extinction, and others had higher abundances; it evened out and the mean of the outcome values from the 50 repetitions was presented. Also, as the 'highs' and 'lows' happened randomly/equally in both the control and the treatment scenarios, it would not affect the comparison results.

Although the model simulated a hypothetical village in Africa, it can be generalized to other types of communities that need vector-borne disease control, with a few adjustments of parameters and modelling assumptions. There are some simplifications in the model that could influence the results. First, in the model, female *An. gambiae* had a constant rate of 20 % of becoming infected with malaria after biting a human. This was assumed to avoid the complexity in human malaria infections such as immunity. However, as EIR decreases, malaria prevalence could decrease too. So the assumption of a constant rate could result in an under-estimation of the impact of ATSB applications. Second, humans did not kill mosquitoes in the model, and IRS and LLINs were not simulated. This could result in an over-estimation of the abundance and EIR in all scenarios. An analysis of the synergistic impact of ATSB with LLINs and IRS on mosquito density is already modelled by Marshall et al. [54]. Third, successful mating was assumed in all females, so the decrease in male populations did not interact with or show any effect on female outcomes. However, if mating behaviour was incorporated in the model, the decrease in male populations may cause females to have to fly longer for successful mating, resulting in an increased need of energy sources. In addition, those females not mated will not produce eggs, which might further reduce the whole mosquito population. Thus, the impact of ATSB could have been under-estimated.

Conclusions

In this model, application of ATSB stations significantly reduces *An. gambiae* abundance, HBR, and EIR in both resource-rich and resource-poor environments. All

configurations of ATSB stations led to significant reduction of EIR to near zero, demonstrating a promising strategy for malaria elimination. In resource-rich environments, configurations of ATSB stations dispersed over the whole village achieved better control of mosquito vectors; among the dispersed ATSB station configurations, both the n × n grid design and the random location design are suggested. Stations at or around houses are less efficient in reducing vector population, but they work better in preventing biting and parasite transmission. As all ATSB station arrangements are similarly effective in resource-poor environments, any can be used. Modelling indicates one bait station at each house is an effective and feasible application strategy but in the field if it does not achieve satisfactory vector reduction, other dispersed configurations can be combined in parallel. Optimum number of stations should be adjusted according to seasonal changes in environmental resources, with highest numbers of bait stations placed during and after rainy seasons when there is an abundant of microhabitats where mosquitoes sugar feed and rest outdoors.

Authors' contributions

LZ developed the model. JMM helped with the population dynamics modelling. WAQ, JCB, YS, SFT, SD, and GCM provided consulting on vector biology assumptions in the model design. JWM provided suggestions on individual-based modelling. WMH provided suggestions on epidemiological study design. KLA helped with the data analysis. LZ wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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