Parasitol Res (2014) 113:73–79
DOI 10.1007/s00436-013-3628-4

ORIGINAL PAPER

Control of *Aedes albopictus* with attractive toxic sugar baits (ATSB) and potential impact on non-target organisms in St. Augustine, Florida

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Received: 16 June 2013 / Accepted: 26 September 2013 / Published online: 12 October 2013

Abstract The purpose of this study was to test the efficacy of bait stations and foliar applications containing attractive toxic sugar baits (ATSB) and eugenol to control *Aedes albopictus*. At the same time, the potential impact of these control methods was evaluated on non-target organisms. The study was conducted at five tire sites in St. Augustine, Florida. *A. albopictus* populations were significantly reduced with ATSB–eugenol applications applied directly to non-flowering vegetation and as bait stations compared with non-attractive sugar baits and control. The application of ATSB made to non-flowering vegetation resulted in more significant reductions of mosquito populations compared to the application of ATSB presented in a bait station. Over 5.5 % of the non-targets were stained in the flowering vegetation application site. However, when the attractive sugar bait application was made to non-flowering vegetation or presented in bait stations, the impact on non-target insects was very low for all non-target orders as only 0.6 % of the individual insects were stained with the dye from the sugar solutions, respectively. There were no significant differences between the staining of mosquitoes collected in flowering vegetation (206/1000) or non-flowering vegetation (242/1000) sites during the non-target evaluation. Our field studies support the use of eugenol as an active ingredient for controlling the dengue vector *A. albopictus* when used as an ATSB toxin and demonstrates potential use in sub-tropical and tropical environments for dengue control.

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Introduction

Aedes albopictus (Skuse) is a major public health concern because this species is considered a main vector in the global resurgence of dengue (Lambrechts et al. 2010; Gubler 1998). This mosquito species exhibits opportunistic host-seeking and oviposition behaviors, and thrives in heavily vegetated habitats; as a result, control efforts have fallen short (Hawley 1988; Braks et al. 2003). In addition to vector control problems, re-emergence of locally acquired dengue cases in Florida (Radke et al. 2010) has served as an impetus for the development and implementation of new control strategies geared to better protect general public health.

The novel method, attractive toxic sugar baits (ATSB), targets the sugar feeding behavior of mosquitoes. Male and female mosquitoes require carbohydrates for energy production and survival. They can often meet this need from natural sources such as plant tissues, flowers, extrafloral nectaries, and honeydew (Yuval 1992; Foster 1995). Furthermore, laboratory and field studies have demonstrated that *A. albopictus* needs regular sugar meals for nutrition and energy (Xue et al. 2008; Xue et al. 2010; Braks et al. 2006). Exploiting this physiological requirement, Xue et al. (2006) and Naranjo et al. (2013) reported that foliar application of a sugar bait containing boric acid was successful in controlling this mosquito species in residential communities in St. Augustine, FL, USA.

The purpose of this study was to test the field efficacy of foliar spray and bait stations containing an attractive sugar bait combined with the US Environmental Protection Agency (USEPA) exempt toxic active ingredient, eugenol, to reduce populations of *A. albopictus*. At the same time, the potential impact of this novel control method on indigenous non-target organisms was evaluated.

Materials and methods

Experimental site

Field experiments were conducted from mid-September to late November 2012 in sub-urban and rural tire dump sites in northern Florida (St. Augustine). Five tire dumps were used as follows: tire site one was located at the edge of an oak forest with approximately 100 tires (tire pile size 1,200 m^2). Tire site two was located at an auto repair shop with approximately 100 tires (1,200 m^2). Tire site three was located in an industrial area on the property of Anastasia Mosquito Control District, St. Augustine, FL, USA. This site was surrounded by open grassland with approximately 100 tires clustered on less than half a hectare. Tire site four (1,200 m^2) was located on another auto repair shop with approximately 100 tires. Tire site five was located in an agricultural area surrounded by farmland. This site contained approximately 200 tires (2,500 m^2). Bait stations were placed along the perimeter of the tire sites.

Equipment and materials used

Foliar applications were carried out using a manual backpack pressure sprayer (Pestro 2000 Back pack sprayer, B&G, GA, USA). The bait stations consisted of opaque ethylene vinyl acetate panels fashioned into a hollow box, 23 cm×23 cm×15 cm (Fig. 1) mounted on a plastic pole. The treatments were applied to the bait stations with a paint brush. Care was taken to completely cover the surface of each station with a thin film of liquid and allowed to dry.

Bait mixture and application

Attractive sugar bait used in our study was prepared from industrial-grade sugar concentrate (Westham Ltd., Tel Aviv, Israel) by diluting concentrate 1:4 in regular tap water. Eugenol was (Terminix ® AllClear®) added at 0.8 % *w/w* of bait concentrate for bait stations or diluted (1:4) for foliar application. Eugenol was used as the toxic portion of ATSB because it is a minimum-risk pesticide not subject to USEPA federal registration requirements (EPA 2013). Previous laboratory studies determined the concentration to be used in the field trials (W.A. Qualls, unpublished data). The bait that contained 0.8 % eugenol only without the sugar additive for spraying or painting was prepared by mixing 1:1 white refined sugar with tap water for bait station application (non-attractive toxic sugar bait). For spraying onto vegetation, this solution was further diluted 1:2.

At tire site two, the non-attractive toxic sugar bait (500 g of refined sugar in 0.5 l water) and at tire site four, ATSB were strictly applied only on non-flowering vegetation (Table 1).

Fig. 1 Bait stations constructed from ethylene vinyl acetate panels made into a hollow box (23 cm×23 cm×15 cm) mounted on a plastic pole at tire dump 5

Table 1 Description of the treatment type, application method, and mixture of the ATSB or non-attractive sugar bait at the different tire sites

Treatment site	Treatment type	Application	Mixture
Tire site 1	Control	N/A	N/A
Tire site 2	Non-attractive toxic sugar bait	10 % of non-flowering vegetation	0.8 % eugenol diluted in 500 g of refined sugar and 0.5 l of water
Tire site 3	Non-attractive toxic sugar bait STATION	Three bait stations	0.8 % eugenol diluted in 500 g of refined sugar and 0.5 l of water
Tire site 4	Attractive toxic sugar bait	10 % of non-flowering vegetation	0.8 % eugenol *w*/*w* diluted 1:4 in water
Tire site 5	Attractive toxic sugar bait station	Six bait stations	0.8 % eugenol *w*/*w* diluted 1:1 in water

Both were applied in the same manner by spraying strips (0.5 m×0.5 m; 0.5 m×several meters) of vegetation with non-attractive toxic sugar bait or ATSB (1:4 ratio of concentrate/water) from a backpack sprayer while moving the nozzle up- and downwards to cover both the under and upper side of the foliage. A total of up to 10 % of vegetation surrounding the tires (0.013 ha) was sprayed wet with bait just before runoff.

At the other tire sites, the surface of the bait stations was painted with either ATSB concentrate or toxic non-attractive sugar bait. The bait stations were placed around the tires at a rate of 24 U/ha.

Monitoring

Mosquito populations were monitored before and during treatment using human bait. Two of the participating authors attracted *A. albopictus* during daytime with their bare feet. The mosquitoes trying to land were collected using a backpack aspirator in intervals of 5 min. Before ATSB treatment, the mosquitoes were monitored within 1 week for 3 days (two times per day), and during the test for 4 weeks, twice per week (two times per day). At each site, two samples were taken from 0700 to 1100 and 1400 to 1800. The participants were fully informed of the nature, objective, and procedures of the test including any physical and mental health consequences that are reasonably foreseeable.

Percent reduction between treatment site and control was calculated using the formula $(P+C)-T/(P+C)$, where P stands for populations before treatment, C stands for populations at the control site, and T stands for populations at the treatment site (Mulla et al. 1971).

Non-target evaluation

Non-target field studies evaluating the feeding by insects from the following selected six orders on vegetation treated with ASB were conducted by dissecting and examining guts for food dye under a dissecting microscope. The insect orders included: Hymenoptera (with focus on Aculeata including honey bee (*Apis mellifera*), wild bees, and wasps), Lepidoptera (Rhopalocera, families of Macroheterocera and Microlepidoptera), Coleoptera (Carabidae, Tenebrionidae, Scarabaeidae, Cerambycidae, and Chrysomelidae), Diptera (Brachycera only), Hemiptera (Cicadomorpha and Heteroptera), and Orthoptera (Caelifera and Ensifera).

One and a half hectares, near one of the tire sites, was treated with either the blue- or red-stained ASB solution using a backpack pressure sprayer (Pestro 2000 Backpack sprayer, B&G). Non-flowering vegetation and flowering vegetation were treated with either the (1:200) blue (Blue Food Dye No. 1) or red (Azorubine food dye (Stern, Natanya, Israel) ASB solution to differentiate non-target feeding (Schlein and Müller 2008). Another three acres were selected near tire site five for evaluation of bait stations and non-target arthropods. Sixteen bait stations were placed 10 m apart with a mixed of flowering and non-flowering vegetation alongside the road leading to the fifth tire site. The EPA guidelines were followed to ensure that the testing conditions resembled the conditions likely to be encountered under actual use of the product. Specifically, the test substance should be applied to the site at the rate, frequency, and method specified on the label [EPA 712-C-017] (EPA 2012a, b, c). The food dye colors, at least for 24 h, stayed in the guts of the insects that fed on the bait (Müller and Schlein 2008). The percentage of the stained insects after the first day of ASB application can, therefore, be seen as a potential maximal daily feeding/killing rate (Müller and Schlein 2008).

Non-target insects were monitored 1 day/night after the ASB application at the treated site with 50 yellow plates (yellow disposable plastic plates 25-cm diameter filled with water and a drop of Triton X-100 as detergent), four Malaise traps (2 and 6 m; Model 2875D, BioQuip, Rancho Dominguez, CA, USA), two ultraviolet light traps (generator powered 250 ML light bulb mounted in front a white 2 m×5-m white linen sheet), six ultraviolet tray traps (Müller et al. 2011), 50 pitfall traps (500-ml plastic cups buried to the rim in the ground, baited with 10-ml vinegar) (Leather 2005), sweep nets (BioQuip) (two collectors), and aerial hand nets (BioQuip). For a more detailed description of the sampling methods, see Müller et al. (2005, 2006). The collected insects were stored at −20 °C in a freezer before being processed. Traps were kept at a distance of at least 5 m to treated patches of vegetation while manual collecting was conducted randomly over the treatment site.

Because of the large number of non-targets that were collected, aliquots from each collecting method were used to determine the percentage of stained insects. Identification was based on the characteristics distinct to each taxa group based on gross morphological characteristics as opposed to identifying each specimen to species level.

Statistical analysis

Mosquito landing count data was averaged for each week by treatment and bait station where applicable, then transformed into percent change from baseline (i.e., zero). A generalized linear mixed model was used to perform a repeated measure analysis of variance utilizing the percent change from baseline as the dependent variable, and the fixed effects was used for the treatment, week, and treatment by week. The random effect was used for the trap nested within the treatment. An unstructured covariance matrix was used to represent the correlated data structure. Planned comparisons were made for each group at each week and for weeks averaged.

The counts of the stained insects from the non-target study were analyzed with a generalized linear model for an outcome with a negative binomial distribution. The negative binomial analysis fits a Poisson distribution with an extra parameter to control for overdispersion. Separate analyses were done for ATSB and bait stations. Both analyses used an offset of the total number of insect species to yield a percent and also used the count of stained insects as the dependent variable. The bait station analysis used species as the independent variable. The ATSB analysis used species, vegetation type (flowering/non-flowering), and the interaction of species and vegetation type as independent variables. Mean percent and standard error were reported. Planned comparisons were made among the species or species within vegetation type.

SAS (SAS Institute 2011) was used for all analyses. Differences in all mean data were considered significant at $P \leq 0.05$.

Results

ATSB field experiments

There was a significant interaction of treatment by week ($F=14.1$, $df_{1,2}=12,25$, $P<0.001$) on *A. albopictus* populations. The populations at the control tire site did not change significantly over the 4-week study compared with the pre-treatment population (pre-treatment 38.5±6.2, post-treatment 36.3±5.9) but significantly increased from baseline at week 3 and decreased similarly at weeks 1 and 4 (Table 2). The mosquito density significantly declined over the 4-week treatment period (84.9±7.3 %, $P<0.001$) after exposure to the ATSB application on non-flowering vegetation (Table 3).

ATSB applied to vegetation was significantly better than non-attractive sugar bait application for 3 of the first 4 weeks post-application (pre-treatment numbers 64.7±8.1, Table 3). While ATSB applied to vegetation was overall a better application than ATSB presented in bait stations, reductions of *A. albopictus* populations varied by week, and reductions were only significant at week 1.

At the tire site that received the ATSB station application, *A. albopictus* densities significantly declined over the 4-week post-treatment period (62.3±7.3, $P<0.001$). Reductions in the mosquito populations were significant at all weeks post-treatment compared with pre-treatment numbers (150.9±12.2). For all weeks post-application except for week 3, ATSB presented on bait stations was significantly better than non-attractive sugar bait station. When comparing ATSB applied as bait stations with non-attractive sugar bait applied on vegetation, control of *A. albopictus* was significantly better at weeks 2 and 3 post-application (Table 3).

For the tire site that received non-attractive sugar baits applied on vegetation, *A. albopictus* densities significantly declined over the 4-week post-treatment period (23.9±7.3 %, $P=003$). The percent reduction was significant for weeks 1, 2, and 4 post-evaluation compared to the pre-treatment numbers (30.1±2.1); however, there was a significant increase from pre-treatment counts at week 3 (Table 2). Comparing the non-

Table 2 Mean±SE reduction post-application of the different treatment methods compared to baseline pre-treatment numbers

	Non-attractive sugar bait		Non-attractive sugar bait–vegetation		Attractive toxic sugar bait		Attractive toxic sugar bait–vegetation		Control	
Week post-treatment	Mean±SE	P^a	Mean±SE	P^a	Mean±SE	P^a	Mean±SE	P^a	Mean±SE	P^a
1	−31.8±9.6	0.003	51.7±9.6	<0.001	48.2±9.6	<0.001	82.8±9.6	<0.001	19.9±9.6	0.048
2	24.4±9.6	0.018	30.0±9.6	0.005	63.2±9.6	<0.001	81.7±9.6	<0.001	−10.6±9.6	0.281
3	53.9±11.6	<0.001	−39.2±11.6	0.003	63.1±11.6	<0.001	93.7±11.6	<0.001	−31.5±11.6	0.012
4	−0.4±10.4	0.971	53.1±10.4	<0.001	74.8±10.4	<0.001	81.3±10.4	<0.001	40.9±10.4	0.001
Average	11.5±7.3	0.126	23.9±7.3	0.003	62.3±7.3	<0.001	84.9±7.3	<0.001	4.7±7.3	0.525

[a] P value for a test of the percent change vs. zero

Table 3 Between-group comparisons of the different treatments at weeks post-treatment

Week post-treatment	NSB vs. NSV	NSB vs. ATSB	NSB vs. ATSV	NSB vs. C	NSV vs. ATSB	NSV vs. ATSV	NSV vs. C	ATSB vs. ATSV	ATSB vs. C	ATSV vs. C
1	<0.001	<0.001	<0.001	0.001	0.799	0.031	0.028	0.017	0.048	<0.001
2	0.009	0.009	<0.001	0.016	0.022	0.001	<0.001	0.185	<0.001	<0.001
3	0.581	0.531	0.023	<0.001	<0.001	<0.001	0.646	0.074	<0.001	<0.001
4	<0.001	<0.001	<0.001	0.009	0.152	0.066	0.415	0.660	0.030	<0.001
Average	0.241	<0.001	<0.001	0.513	0.001	<0.001	0.074	0.038	<0.001	<0.001

NSB non-attractive sugar bait, *NSV* non-attractive sugar bait applied to vegetation, *ATSB* attractive toxic sugar bait, *ATSV* attractive toxic sugar bait applied to vegetation, *C* control

attractive sugar bait applied to vegetation with the non-attractive sugar bait station, the control was significantly better at weeks 1, 2, and 4 for the non-attractive sugar bait on vegetation (Table 3).

The populations of mosquitoes at the tire site that received the non-attractive sugar bait station did not significantly decline over the 4-week post-treatment period (pre-treatment number 18.2±3.0, 11.5±7.3 %, $P=0.126$). The percent change was significant at weeks 2 and 3; there was a significant increase at week 1 (Table 2).

Non-target evaluation

The potential impact on non-target insects of ATSB applied on flowering vegetation was greater for higher Diptera, Hymenoptera, and Hemiptera compared with that on mosquitoes (Table 4). However, when ATSB was applied to non-flowering vegetation, the impact on non-target insects was low for all non-target orders. There were three mosquito species collected stained, *A. albopictus*, *Culex quinquefasciatus*, and *Uranotaenia sapphirina*. There were no significant differences between the numbers of the three collected mosquito species in sites that the ASB was applied to flowering vegetation (206/1000) compared with that to the non-flowering vegetation (242/1000).

When the ASB was presented in bait stations, significantly more mosquitoes (129/1,000; 12.9 %) and higher dipterans were stained compared to the other non-target orders (Table 5). Eight mosquito species were collected at this tire site: *A. albopictus* (12/1,000), *Aedes infirmatus* (493/1.000), *Aedes taeniorhynchus* (25/1,000), *Aedes vexans* (197/1,000), *Anopheles crucians* (4/1,000), *Coquillettidia perturbans* (2/1,000), *Culex nigripalpus* (260/1,000), and *Psorophora columbiae* (3/1,000).

Table 4 Mean (±SE) percentage of individuals from various insect orders stained and collected after exposure to attractive sugar bait applications to flowering and non-flowering vegetation

Species	Flowering vegetation (F) % Stained±SE[a]	Non-flowering vegetation (NF) % Stained±SE[a]	F vs. NF P^{b}
Mosquitoes	18.5±9.8 a	38.9±19.9 a	0.322
Coleoptera	3.5±1.2 b	0.5±0.2 b	0.001
Diptera[c]	11.0±8.5 a	2.1±1.7 b	0.141
Hemiptera	7.6±4.4 a	0.0	
Hymenoptera	9.6±4.2 a	0.4±0.2 b	<0.001
Lepidoptera	2.5±0.8 b	0.6±0.3 b	0.018

[a] Columns with different letters indicate significant differences in staining rate compared with mosquitoes

[b] Comparison of stained orders of flowering vegetation vs. non-flowering vegetation

[c] Without mosquitoes

Discussion

Significant reduction in *A. albopictus* populations was demonstrated up to 28 days after ATSB application. Overall, ATSB applied on vegetation is significantly better at reducing mosquito populations compared with the bait stations at an application rate of 24 U/ha. The greater reduction achieved by ATSB applied to vegetation could be explained by diurnal resting and sugar feeding behavior of this species. In our previous work, we found that *A. albopictus* possessed greater energy reserve accumulation in vegetational zones that they

Table 5 Mean (±SE) percentage of individuals from various insect orders stained and collected after exposure to attractive sugar bait stations

Species	Bait station % Stained±SE	P^{a}
Mosquitoes	13.2±2.3	
Coleoptera	0.1±0.0	<0.001
Diptera[b]	4.3±1.6	0.013
Hymenoptera	0.3±0.1	<0.001
Lepidoptera	0.3±0.1	<0.001
Neuroptera	0.4±0.3	<0.001
Orthoptera	0.3±0.4	0.002

[a] Order compared with mosquitoes

[b] Order compared without mosquitoes in raw data set

frequently were collected or found resting (Samson et al. 2013). Because mosquitoes may rest and sugar feed within the same vegetation, seeking out a sugar meal presented in a bait station may have less of an impact in sub-tropical environments where sugar meals are readily available. Bait stations have been successful in decimating important malaria vectors in arid and sub-arid environments (Müller and Schlein 2008; Müller et al. 2008). These findings highlight the impact of spatial and temporal conditions necessary to the success of ATSB application in tropical and sub-tropical environments.

In a previous study (W.A. Qualls, unpublished data), ATSB with eugenol applied as a barrier application to non-flowering vegetation in Florida demonstrated effective control of nuisance and vector mosquito populations. Field tests resulted in >88 % reductions of mosquito populations after exposure to eugenol applications of ATSB. Though the mode of action is unclear, mortality in our previous and current study demonstrated significant mosquito mortality after ingesting the 0.8 % eugenol sugar bait. The addition of the industrial-grade ASB concentrate increased the efficacy of the ATSB application as seen in the significant differences in control between the ATSB and the non-attractive toxic bait methods. The successful control of mosquito populations using active ingredients like eugenol, boric acid (Xue et al. 2006; Naranjo et al. 2013), and spinosad (Müller et al. 2008) continues to identify the role of ATSB in integrated vector management programs.

This study demonstrated that ATSB applied to non-flowering vegetation, or to bait stations in sub-tropical environments, would have very little impact on non-targets while still controlling mosquito populations. When the ASB was applied to flowering vegetation, non-target populations were significantly stained, suggesting that some non-target populations may suffer unacceptable losses. However, when the ASB was applied to non-flowering vegetation or in bait stations, non-target insect populations were not attracted and did not feed on sugar solution. The development of bait stations further enhances the ATSB strategy to reduce non-target affects. Furthermore, with an addition of protective grids covering the bait, only small biting flies would be able to feed while other insects like honey bees would be excluded (G.C. Müller, unpublished data). Most likely, the ASB-treated green vegetation and bait stations do not provide a visual attractive target for pollinators, while mosquitoes may be attracted to the scent of the sugar source; the exact mechanism remains to be proven. The findings of this study continue to support previous non-target work (Khallaayoune et al. 2013) that highlights the development of guidelines for appropriate use and adaptation of the new ATSB control methods into integrated vector management programs.

Acknowledgments We would like to thank the staff and commissioners of the Anastasia Mosquito Control District for supporting this research. The research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI100968. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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