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Assessment Of Floral-Derived Aromatic Compounds And Sugar Lures To Capture Male And Female Aedes Aegypti (diptera: Culicidae)

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Assessment of floral-derived aromatic compounds and sugar lures to capture male and female *Aedes aegypti* (Diptera: Culicidae)

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Abstract

The viruses transmitted by *Aedes aegypti,* including dengue and Zika viruses, are rapidly expanding in geographic range and as a threat to public health. In response, control programs are increasingly turning to the use of sterile insect techniques resulting in a need to trap male *Ae. aegypti* in order to monitor the efficacy of the intervention. However, there is a lack of effective and cheap methods for trapping males. We attempted to exploit the male physiological need to obtain energy from sugar feeding in order lure the mosquitoes into a passive trap. We tested promising aromatic and sugar lures identified in the literature in order to determine whether small-scale attraction is indicative of success in larger scale trapping. First, all five lures were compared against a water control in an attraction assay using males and females (nulliparous and gravid). Guava mango was indicated to be the most promising lure among males, although it did not perform statistically significantly better than the water control (P=0.08). Next, the number of mosquitoes captured by a Gravid *Aedes* Trap (GAT) treated with guava mango was compared to the number captured by a control GAT. No statistical difference in the number of mosquitoes captured was detected among males (P=0.45), nulliparous females (P=0.67), or gravid females (P=0.47). Our findings suggest that the use of the floral-derived aromatic compounds and sugar mixtures that have been identified in the literature is not an effective lure by which to capture *Ae. aegypti* in the GAT. Future trapping efforts would likely be more successful if focused on more promising methods for male capture.

Keywords: *Aedes aegypti*, entomological surveillance, mosquito trap, floral lures, sugar lures, Zika, dengue

Introduction

The resurgence of once geographically limited vector-borne diseases, particularly those viruses transmitted by mosquitoes such as dengue, Zika and chikungunya viruses, have become an increasingly serious threat to public health in recent years. The expansion of these diseases is largely spurred by anthropogenic activities including increased mobility of human populations, habitat modification, and climate change (Patz and Reisen 2001, Adams and Kapan 2009). In the wake of these changes, vector-borne diseases are moving from one place to another at unprecedented rates, causing progressively more people to be at risk of contracting these diseases (Patz and Reisen 2001, Adams and Kapan 2009). In particular, diseases transmitted by *Aedes aegypti*, the yellow fever mosquito, have experienced a notable resurgence and expansion in recent years, especially Zika and dengue viruses (World Health Organization 2014, Kindhauser et al. 2016). In the past year, Zika virus has spread through much of the Western Hemisphere, resulting in a public health emergency due to its likely association with microcephaly, a serious congenital malformation (World Health Organization 2016, Messina et al. 2016, Teixeira et al. 2016). Furthermore, the incidence of dengue has increased 30-fold in the past 50 years. As a result, 2.5 billion people currently live at risk of contracting this disease, and there are 50 million infections and 22,000 deaths per year (World Health Organization 2014). The cost of dengue on public health is substantial, including direct costs to local and global health organizations and immense economic and social costs (Shepard et al. 2011, Gubler 2012).

In response to the threat of dengue in endemic countries (and impending threat in non-endemic countries), scientists have increasingly turned to population level manipulations that rely upon males for optimal efficiency and successful dissemination. For example, the Eliminate Dengue program releases both male and female *Ae. aegypti* infected with *Wolbachia,* which

reduces the ability of the mosquito to transmit dengue viruses (Hoffmann et al. 2011). Additionally, biotech companies, such as Oxitec, produce genetically engineered sterile male *Ae. aegypti*, which suppresses vector population levels (Lacroix et al. 2012). Other male-based sterile insect techniques (SITs), including radiation and feeding with double stranded RNA, also rely upon the release of sterile males for control of *Ae. aegypti* (Rodriguez et al. 2013, Lees et al. 2015, Whyard et al. 2015). With the advent of such technologies, it has become increasingly important to trap males, in addition to females (the traditional target of mosquito control programs) in order to monitor the efficacy of these technologies. However, the tools available to sample wild male *Ae. aegypti* are limited. Those that do exist, such as the Biogent Sentinel Trap BGS, are prohibitively expensive for large, wide-ranging studies and rely upon energy from batteries or mains power to function (Kroeckel et al. 2006). The passive trap options available for the capture of female *Ae. aegypti*, such as the Gravid *Aedes* Trap (GAT) and autocidal gravid ovitrap, are more practical and affordable (Mackay et al. 2013, Eiras et al. 2014, Ritchie et al. 2014). These traps mimic the ecological drivers of oviposition site selection, such as dark color and odor of fermented plant material, in order to lure gravid females into the trap to lay eggs. Naturally, this technique is not effective for male *Ae. aegypti*, so passive trap designs must rely upon other physiological needs pertinent to male survival, such as sugar-feeding. Both male and female *Ae. aegypti* acquire energy in the form of carbohydrates from plants. This is the only source of food for males, as opposed to females, which primarily derive energy from blood meals (Foster 1995).

Anopheles control programs have already successfully implemented strategies that exploit the sugar-feeding behavior of mosquitoes in the form of attractive toxic sugar baits (ATSB) (Müller et al. 2008, Müller and Schlein 2008, Müller et al. 2010). The ATSB uses aromatic compounds from a flower or fruit to attract the mosquitoes, sugar to induce feeding, and an oral toxin to kill the mosquitoes. The technique has resulted in substantial reductions in the mosquito populations at the sites where it has been tested (Müller et al. 2008, Müller and Schlein 2008, Müller et al. 2010, Revay et al. 2015). *Aedes albopictus* control programs have also had similar success with ATSB (Xue et al. 2006, Naranjo et al. 2013, Revay et al. 2014).

This same physiological need could be harnessed in order to trap *Ae. aegypti*. Several floral-derived aromatic compounds (Table 1) have been shown to be attractive to *Ae. aegypti* in small scale experiments (Jhumur et al. 2007, Oppen et al. 2015). In the discussion of each of these papers, the authors highlighted the potential to use the promising compounds identified as attractants in traps (Jhumur et al. 2007, Oppen et al. 2015). Additionally, the guava mango bait that was successful in the *Ae. albopictus* control program (Naranjo et al. 2013) is included in the list of potential lures due to the biological and ecological similarities between *Ae. aegypti* and *Ae. albopictus* (Kaplan et al. 2010).

We tested the promising aromatic and sugar lures identified in the literature and in preliminary experiments in order to determine whether small-scale attraction is indicative of success in larger scale trapping. The success of floral-derived aromatic compounds and sugar lures in attracting male *Ae. aegypti* into passive traps would enable the creation of a practical and economically viable trap to monitor and aid control programs, especially SIT programs.

Materials and Methods

***Aedes aegypti* colony.** Mosquitoes used in the studies were from a colony established from eggs collected in ovitraps in Cairns (QLD, Australia) and were periodically supplemented with wild collections to maintain genetic vigor. Mosquito larvae were reared on fish food powder

(TetraMin Rich Mix, Tetra Melle, Germany). Adults were fed on a 50% honey solution and were blood-fed 3 times per week using human volunteers (Human ethics approval from James Cook University H3555). Mosquitoes were starved overnight prior to use in trials (about 18 hours).

Lure Selection and Presentation. *General lure selection and presentation.* Based on the literature and preliminary comparisons, five potential lures were chosen including three aromatic compound lures, one sugar lure and one combination aromatic compound + sugar lure (Table 1). The three aromatic lures were presented on a 3 x 3 cm sponge soaked in 12 mL of distilled water in volumes of 200 µL for phenylacetaldehyde, 200 µL for acetophenone, and a combination of 50 µL each of phenylacetaldehyde, linalool oxide, phenylethyl alcohol and acetophenone. A sugar lure was created based on a recent Attractive Toxic Sugar Bait recipe (Naranjo et al. 2013). Initial sugar lure preparation involved the mixing of 0.2 liter of guava nectar (Golden Circle), 0.2 liter of mango nectar (Golden Circle), 0.2 liter of distilled water and 200g of brown sugar in an Erlenmeyer flask over heat using a heating pad and magnetic stirrer. When the sugar was fully suspended, the mixture was poured into a plastic container and allowed to cool and ferment for 24h at room temperature. This mixture is referred to as guava mango through the remainder of the paper. For each trial, 12 mL of guava mango was pipetted onto a sponge for presentation. A combination lure of guava mango and phenylacetaldehyde was also created by soaking the sponge with 12 mL of guava mango and 200 µL of phenylacetaldehyde. All lures were displayed on a piece of sponge that was previously soaked in a 1% sodium hypochlorite (Chlorox®, Oakland, CA, USA) solution to dispel the chemicals used for packaging, after which the sponge was thoroughly rinsed and dried. The same procedure is used to sugar-feed the mosquitoes with 50% honey solution for lab-rearing.

Development and validation of blue dye and fipronil to assess sugar-feeding. To assess sugar-feeding we incorporated blue food dye and the insecticide fipronil (0.06% by volume, Termidor®, Victoria, AU) (Pridgeon et al. 2008, Xue et al. 2008, Ritchie et al. 2013) in our control (distilled water) and treatment solutions (aromatic and sugar lures) to knock down mosquitoes that ingested the lure and provide a visible blue dye in the abdomen (Fig. 1) to indicate that death was caused by ingestion and not natural causes. The rationale was that a larger number of *Ae. aegypti* dead after 24h would mean that a larger number of mosquitoes ingested the lure from the sponge, which would suggest that the mosquitoes were more attracted to that lure.

The time to death or incapacitation after ingesting fipronil was determined by aspirating 13 male *Ae. aegypti* into a Bug Dorm insect cage (30 x 30 x 30 cm) with 12 mL of guava mango treated with blue dye and fipronil and observing the number of landings on the sponge and the number of knock-down mosquitoes over two hours. We tested if mosquitoes readily fed on fipronil-treated lures by aspirating 20 male *Ae. aegypti* into 2 buckets with mesh covering. The treatment bucket contained a sponge with 12 mL of guava mango treated with blue dye and fipronil while the control bucket contained a sponge with 12 mL of guava mango treated only with blue dye. The number of mosquitoes in the treatment bucket that were killed and had visible blue in their abdomen was counted. The number of live mosquitoes in the control bucket was counted and then frozen so that the number with visible blue in the abdomen could be counted. The number that ingested the guava mango was compared between treatment and control to see if there was any aversion to the fipronil treatment. The same procedure was repeated with 13 females.

Are *Ae. aegypti* attracted to aromatic and sugar lures? For the attraction assay, we set up six tents (3.24 m^3, Wild Country, AU) in a temperature and humidity controlled semi-field cage. The

temperature and humidity in the semi-field cage track those of the outdoors, reflecting normal conditions in Cairns, Australia between June and August. The mean daily high temperature for June, July and August was 26.8°C, 25.9°C, and 27.1°C respectively and the mean daily low temperature was 20.1°C, 17.4°C, and 16.6°C respectively (Meteorology 2015) . The floors of the tents were covered with white tarp so that the dead mosquitoes would be easily spotted for counting. An overturned black plastic bucket from a Gravid *Aedes* Trap (GAT) (Ritchie et al. 2014) was placed at the middle of each tent to attract and induce swarming by male mosquitoes. A small dish with the lure-treated sponges was placed on top of each GAT bottom. This feature was added after preliminary trials showed very little interaction with the sponge in order to maximize the chance that the male *Ae. aegypti* would smell the lures by inducing swarming behavior around the dark GAT bottom.

We released 20 male *Ae. aegypti* into each tent after starving them overnight (about 18h). They were left in the tent for 24h, after which the dead mosquitoes on the ground of the tent were counted and inspected under a stereo microscope for blue dye in the abdomen or crop. The number with visible blue dye was noted, as this was considered the number definitively killed by the fipronil-treated attractant or control. The remaining live mosquitoes were cleaned from the tent using a Prokopak aspirator (Vazquez-Prokopec et al. 2009). This procedure was repeated five times, for a total of five replications. The tent in which each of the five different treatments and the control were placed for each replication was randomized using the random number generator random.org. The same procedure was used with 20 nulliparous females as well as with 20 gravid female *Ae. aegypti.* The gravid females were blood fed six to seven days prior to use. Both the nulliparous and gravid females were starved overnight before use in the trials.

Can the guava mango lure be used to trap *Ae. aegypti* in the GAT? *Choice test between guava mango lure and water control.* The attraction assay indicated that guava mango was the most attractive lure for male *Ae. aegypti,* so a secondary experiment was conducted to assess the potential to use this attraction to capture mosquitoes in the GAT. The guava mango lure was prepared in the same way as in the attraction assay preparation, however the blue dye and fipronil were not added. The same six tents described for the attraction assay were used for the choice test. Each tent had two GATs set up at opposite corners across a diagonal (Fig. 2). The GATs were ~1/5 filled with tap water and five alfalfa pellets. Insecticide-treated bed net (5% alphacypermethrin) was placed over the screens of the GAT heads in order to knock down any mosquitoes that entered the traps. Each GAT was placed on a circular tray covered with talcum powder to prevent ants from entering the trap. In each tent, one GAT was the control, with a sponge soaked in 12 mL of distilled water in a plastic dish. The other GAT was the treatment, with a sponge soaked in 12 mL of the guava mango lure in a plastic dish. In order to control for placement bias, the placement of the control and treatment GATs was switched in every other tent. Therefore, three of the tents had the guava mango GAT in the far left corner and three had the guava mango GAT in the near right corner, while the control GAT was at the other side of the diagonal in each tent. Twenty male *Ae. aegypti* were released into each tent and left for three nights (about 72 hours). Thereafter, we removed the GATs from the tents and counted the number of mosquitoes in each. The same procedure was repeated with four tents over a separate 72 hours for a total of 10 replicates. The procedure was also repeated with 20 nulliparous females and with 20 gravid female *Ae. aegypti.* Six replicates were conducted for each of these experiments using the six tents over one 72-hour period in both cases.

Larger scale choice test. In a separate experiment, we increased the amount of liquid presented in each GAT. We scaled it up ten-fold from 12 mL to 120 mL in order to see if larger

quantities would improve results. A full sponge was placed in a larger dish in the treatment and control GATs in order to absorb the increased quantity of guava mango and water respectively. This was conducted in the six tents over a 72-hour period for a total of six replicates. The same procedure was followed as described for the 12 mL paired test. However, this larger scale version was only conducted with male mosquitoes (20 male *Ae. aegypti* per tent).

Statistical Analysis. Bonferroni analysis of variance (ANOVA) was used to compare the number of mosquitoes killed among the treatments and controls in the attraction assays. Paired t test was used to compare the number of mosquitoes captured by the sugar lure (guava mango) and control GATs in the choice test.

Results

Validation of blue dye and fipronil to assess sugar-feeding. The observation of 13 male *Ae. aegypti* in a Bug Dorm with fipronil-treated guava mango lure showed that those males that ingested the lure were knocked down within two hours of exposure. This shows the quick lethal action of the insecticide in *Ae. aegypti.* The comparison of 18 males fed with fipronil-treated guava mango juice versus 19 males fed with insecticide-free guava mango juice demonstrated that there is no aversion to fipronil. All 18 of the mosquitoes in the fipronil-treated bucket died, whereas none of the 17 mosquitoes in the untreated control died. Additionally, 15 of the 18 males in the fipronil-treated bucket had a visibly blue abdomen, which indicated substantial consumption of the guava mango juice due to the blue food dye. In the untreated bucket, all 19 males had visibly blue abdomen. The same comparison was repeated with 13 females in the bucket with fipronil-treated guava mango juice and 13 females in the bucket with untreated guava mango juice. The fipronil-treated bucket resulted in 13 dead mosquitoes, all with a blue abdomen. The untreated bucket resulted in 13 live mosquitoes, all with blue abdomen.

Attraction assays. *Male response to attraction assay.* In order to measure the efficacy of each lure, we measured the number of mosquitoes dead and the number of mosquitoes with blue coloration in the abdomen after 18 hours (Fig. 3A, Table 2). Bonferroni analysis of variance (ANOVA) showed that none of the lures performed significantly better than the control ($F_{5, 5}$=2.28, P=0.08). However, the guava mango lure attracted and killed the highest mean number of male *Ae. aegypti* and performed particularly well in a couple of replicates with 21.0±11.3% released males observed dead with indication of ingestion of the lure (Table 1, Fig. 3A).

Female response to attraction assay. No significant difference in attractiveness was observed among the treatments and controls as indicated by the number of nulliparous ($F_{5,5}$=0.80, P=0.56) and gravid ($F_{5,5}$=2.13, P=0.10) females with blue-colored abdomens (Table 2) or the percentage of released mosquitoes observed dead with and without blue abdomens, but with blue on the body indicating ingestion (Fig. 3A).

Choice test between guava mango lure and water control. *Male response.* When compared directly to a water control, the guava mango lure was not successful in attracting male *Ae. aegypti* into the GAT (Table 3, Fig. 3B). Overall, a mean (±SD) percentage of released male *Ae. aegypti* that entered and died in the GAT baited with guava mango lure was 19.5 ± 4.4% after 10 replicates, whereas a mean of 32.0 ± 5.2% of released males entered and died in the GAT baited with water control (Fig. 3B). Analysis with a paired t test showed that the preference for the control over the guava mango lure was approaching significance (t=-1.51, df=9, P=0.0833).

Nulliparous Females. The guava mango lure was not successful in attracting nulliparous female *Ae. aegypti* into the GAT (Table 3, Fig. 3B). The mean (±SD) percentage of female *Ae. aegypti* that entered and died in the GAT baited with guava mango or the control lure was 26.7 ±

8.8% and 32.5 ± 9.9%, respectively. There was no significant difference between the guava mango and the control (t=0.47, df= 5, P=0.67).

Gravid Females. The guava mango lure was not successful in attracting gravid female *Ae. aegypti* into the GAT (Table 3, Fig. 3B). The mean percentage of gravid female *Ae. aegypti* that entered and died was 38.3 ± 7.5% in the GAT treated with guava mango lure and 45.8 ± 6.5% in the control GAT. The paired t test showed that there was no significant difference between guava mango and control (t=-0.087 df= 5, P=0.467).

Larger Scale Lure. When the amount of guava mango lure was increased by ten times the volume, it was still not successful in attracting male *Ae. aegypti* into the GAT (Table 3, Fig. 3B). The mean percentage of released male *Ae. aegypti* that entered and died was 35.8 ± 9.1% in the GAT treated with guava mango lure and 27.5 ± 5.6% in the control GAT. Analysis with a paired t test showed that there is no significant difference between the capture of the guava mango and control GATs (t=0.13, df= 5, P=0.45).

Discussion

The aromatic compounds and sugar lures reported as attractive to *Aedes* mosquitoes did not attract male and female *Ae. aegypti* regardless of physiological status (i.e. gravid or nulliparous) when presented in larger enclosures (tents) more reflective of field conditions. In the attraction assay, the guava mango lure attracted slightly more males than the other four lures and the control, however the difference was not significant. Nonetheless, the guava mango lure performed particularly well in a couple of replicates. Since the main interest of the study was to find a lure that could successfully trap males for control and monitoring purposes, we proceeded with the most promising lure among the male trials. The next step was to bait a GAT with the guava mango lure and pair it with a water control to compare the number of *Ae. aegypti* captured in each. Consistent with the findings of the attraction assay, the guava mango GAT did not capture significantly more male, nulliparous or gravid female *Ae. aegypti* than the control GAT.

These results were unexpected because the bulk of the pre-existing literature suggested that male *Ae. aegypti* rely upon plant sugars in order to survive. Additionally, previous studies identified specific aromatic compounds that are particularly attractive to *Ae. aegypti* in small-scale enclosures under laboratory conditions. Furthermore, a sugar lure was identified as a successful attractant for *Ae. albopictus* in attractive toxic sugar bait field studies. The existing evidence therefore suggested that these floral-derived aromatic compound and sugar lures would be attractive to *Ae. aegypti* on a larger spatial scale and would facilitate passive trapping of males in addition to females. However, once tested, this was not the case.

Despite the widespread acceptance that *Ae. aegypti,* especially males, derive energy from flower nectar (Foster 1995), there is a growing body of evidence that the extent of this behavior is limited, especially among females (Edman et al. 1992, Spencer et al. 2005). A mark-release-recapture study in Thailand showed that females in the field did not consume sugar over a two to three day period. In the same study, only one third of male *Ae. aegypti* consumed sugar (Edman et al. 1992). Furthermore, there is evidence that in females, sugar feeding is actually detrimental to survival when compared to blood feeding alone (Harrington et al. 2001). The conclusions reached in the aforementioned papers support the conclusion of our study, that the impetus to sugar feed is not strong enough to merit the basis of a lure for passive trapping.

Our findings suggest that it is ineffective to use floral-derived aromatic compounds and sugar lures that target sugar-feeding behavior in order to trap *Ae. aegypti* in a GAT. In contrast, recent research has shown that sound lures mimicking the frequency of the female wing beat provide a highly effective mechanism to trap male *Ae. aegypti* using a GAT (Johnson and Ritchie 2015). This

may be a promising alternative to the lures investigated in this study for the capture of male *Ae. aegypti*.

In conclusion, the attraction assay showed that the floral-derived aromatic compounds and sugar mixtures previously identified in the literature as potential trap lures were not more attractive than water to male or female (nulliparous and gravid) *Ae. aegypti*. The most promising of these lures for males, a combination of guava and mango nectars, did not facilitate passive trapping of the mosquitoes. These data suggests that the use of the floral-derived aromatic compounds and sugar mixtures that have been identified in the literature is not an effective lure to passively capture male or female *Ae. aegypti* in the GAT or at ATSB stations. Future efforts to trap male *Ae. aegypti* would likely have improved success if focused on more promising methods.

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Appendix

Table 1. Floral-derived aromatic compounds and sugar lures assessed in this study for the passive collection of *Ae. aegypti*

Treatment	Study Design	Authors
Phenylacetaldehyde	electroantennography, wind tunnel bioassays	Jhumur et al. (2007)
Phenylacetaldehyde + linalool oxide + phenylethyl alcohol + acetophenone (PLPA)	electroantennography, wind tunnel bioassays	Jhumur et al. (2007)
Acetophenone	Y tube olfactometer	Von Oppen et al.(2015)
Guava mango	small screen cage studies, semi-field and field evaluations	Naranjo et al. (2013)
Guava mango + phenylacetaldehyde	semi-field cage evaluation	Fikrig et al. unpublished data

Table 2. The mean number (± SE) of dead *Ae. aegypti* observed in the tent attraction assays with blue abdomens indicating ingestion of the lure. Twenty mosquitoes released for 18 hours (N=5).

Treatment	Males		Nulliparous		Gravid	
	Mean (SE)	*P**=0.08	Mean (SE)	*P**=0.56	Mean (SE)	*P**=0.10
Control	0.2 (0.2)		0.2 (0.2)		0.4 (0.2)	
Acetophenone	0.0 (0.0)		0.0 (0.0)		0.4 (0.2)	
Guava mango	2.6 (1.6)		0.2 (0.2)		0.0 (0.0)	
Phenylacetaldehyde	0.0 (0.0)		0.0 (0.0)		0.0 (0.0)	
Guava mango + phenylacetaldehyde	0.4 (0.2)		0.0 (0.0)		0.0 (0.0)	
PLPA	0.0 (0.0)		0.0 (0.0)		0.0 (0.0)	

*P value from Bonferroni analysis of variance

Table 3. The mean number (± SE) of dead *Ae. aegypti* observed in the guava mango GAT choice test with blue abdomens indicating ingestion of the lure. Twenty mosquitoes released for 72 hours.

Group	N	Mean (SE) in guava mango	Mean (SE) in control	*P-value**
Male	10	3.9 (0.9)	6.4 (1.0)	0.08
Male (120 ml)	6	7.2 (1.8)	5.5 (1.1)	0.45
Nulliparous	6	5.3 (1.8)	6.5 (2.0)	0.67
Gravid	6	7.7 (1.5)	9.2 (1.3)	0.47

*P-value from paired t test

Figure 1. Example of blue honey solution visible in the abdomens of female *Aedes aegypti.*

Figure 2. (A) Picture of tent with treatment and control GAT. **(B)** Diagram of experimental set up in the semi field cage. The grey squares represent the floor of the tents, the black circles represents the GATs treated with guava mango and the white circles represent the control GATs.

A.

B.

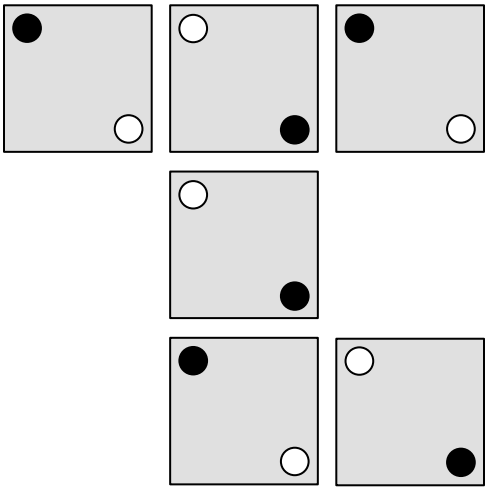

Figure 3. (A) The percent of total number of mosquitoes released found dead with and without blue abdomens, but with blue on the body indicating ingestion, for each treatment in the attraction assays (N=5). **(B)** The percent of total number of mosquitoes released caught in GATs baited with the guava mango lure or water controls over a 72-hour period. All experiments used 12 mL of guava mango and control solution except one in which 120 mL of solution was used to assess the effect of dosage. For both experiments 20 mosquitoes were released during each replicate with a minimum of five replicates being completed for each of the lures and control. The "a" and associated black bars indicate no significant differences among the treatments and controls.