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Nectar protein content and attractiveness to *Aedes aegypti* and *Culex pipiens* in plants with nectar/insect associations

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ABSTRACT

We chose five easily propagated garden plants previously shown to be attractive to mosquitoes, ants or other insects and tested them for attractiveness to *Culex pipiens* and *Aedes aegypti*. Long term imbibition was tested by survival on each plant species. Both mosquito species survived best on *Impatiens walleriana*, the common garden impatiens, followed by *Asclepias curassavica*, *Campsis radicans* and *Passiflora edulis*, which sponsored survival as well as the 10% sucrose control. Immediate preference for imbibition was tested with nectar dyed *in situ* on each plant. In addition, competition studies were performed with one dyed plant species in the presence of five undyed plant species to simulate a garden setting. In both preference studies *I. walleriana* proved superior. Nectar from all plants was then screened for nectar protein content by SDS–PAGE, with great variability being found between species, but with *I. walleriana* producing the highest levels. The data suggest that *I. walleriana* may have value as a model plant for subsequent studies exploring nectar delivery of transgenic mosquitocidal proteins.

1. Introduction

Nectar is a metabolic expenditure borne by plants to modulate insect behavior. While floral nectar is used to attract pollinators, extrafloral nectar is produced on nectary organs located on the petioles and leaf edges of plants in order to attract aggressive insects such as ants to protect the plant from herbivores (Grasso et al., 2015). Mosquitoes capitalize on these plant/insect associations by using nectar as an energy source and imbibe both floral and extrafloral nectar (Foster, 1995). With this in mind, we searched the literature to identify plant species with nectar/insect associations that might be tested for attractiveness to mosquitoes. Our end goal is to identify a plant species which could serve as a transgenic model system to study the delivery of mosquito toxin or pathogen control proteins expressed in the nectar. A prime example of such a modulatory protein would be the Cry proteins from *Bacillus thuringiensis israelensis*, which show toxicity specific to mosquitoes and other *Nematocera* (Boisvert and Boisvert, 2000).

The success of attractive toxic sugar baits (ATSB) in the field provides the rationale for exploring nectar delivery of mosquitocidal proteins. Nectar and other sugar sources are the main source of energy for male mosquitoes, and females typically cannot live long exclusively on blood, drawing much of their energy from sugar sources (Foster, 1995). The two notable exceptions to this are females of the endophilic species *Anopheles gambiae* (Fernandes and Briegel, 2005) and *Aedes aegypti* (Mostowy and Foster, 2004; Nayar and Sauerman, 1975a,b), which can survive on blood alone, though sugar sources critically enhance egg laying and longevity. In fact, the highly blood-dependent *Anopheles sergentii* (Theobald) had 250 times the vectorial capacity for malaria transmission in females from an oasis with a rich supply of nectar from *Acacia* trees compared to mosquitoes from a sugar-poor oasis (Gu et al., 2011). Furthermore, an 86% reduction of *An. gambiae* females was achieved with toxic sugar baits at an outdoor test site (Müller et al., 2010a). At the control site without pesticide, 56% of the female mosquitoes had imbibed dyed bait (male results similar). Effective outdoor adult mosquito control using attractive toxic sugar baits has also been demonstrated for *Aedes albopictus* (Skuse) (Revay et al., 2014) and *Culex pipiens* (Müller et al., 2010b).

The selection of a plant species is a critical first step in developing a model nectar delivery system. The ideal plant would be attractive to mosquitoes, induce imbibition of nectar, have robust nectar protein expression, and be relatively easy to transform and propagate. Unfortunately, almost all plants species noted in the literature as being attractive to mosquitoes have no published transformation protocols. Conversely, nectar proteins have been studied extensively only in the *Nicotiana* (tobaccos) (Park and Thornburg, 2009), which also include many easily transformed species, but these species are not attractive to mosquitoes (e.g., note data herein for *Nicotiana benthamiana*). Thus, we broadened our selection criteria

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Table 1
Taxonomy and common names of all plants used in study.

Family	Species	Common name
Balsaminaceae	*Impatiens walleriana* (Hook.)	Common impatiens
Euphorbiaceae	*Ricinus communis* (L.)	Castor bean
Bignoniaceae	*Campsis radicans* (Seem.)	Red trumpet flower vine
Asclepiadaceae	*Asclepias curassavica* (L.)	Tropical milkweed
Passifloraceae	*Passiflora edulis* (Sims)	Passion flower
Solanaceae	*Nicotiana benthamiana* (Domin)	Muntju tobacco
Amaranthaceae	*Beta vulgaris* (L.)	Beet

to choose plant species with known plant/insect associations. From these, we selected those which also had published tissue culture and/or transformation protocols and were easily propagated.

In this study, we determined attractiveness of the candidate plant species for both *Cx. pipiens* and *A. aegypti*. We first determined long term survival of the mosquitoes in cages with a single potted plant to ascertain if the mosquitoes would imbibe nectar from these plant species. We next used plants with dyed nectar to test the immediate preference for imbibition. Competition studies were then performed to test the preference for that plant species over competitive nectar plant species in a large-cage setting. Finally, nectar protein content was examined by SDS–PAGE. All results pointed toward *Impatiens walleriana*, the common garden impatiens, as an excellent species from which to develop a growth-room model system for the study of transgenic nectar-protein delivery to mosquitoes.

2. Materials and methods

2.1. Mosquitoes

Eggs of *Ae. aegypti* were supplied by Margaret Wirth (University of California Riverside, CA), and egg rafts of *Cx. pipiens* were from Cheolho Sim (Baylor University, TX). Both colonies were maintained at 27 ± 1 °C, 80 ± 5% RH, and 13:11 (L:D). Adults were maintained in standard 33 cm × 33 cm × 33 cm mesh-covered cages and offered sugar cubes and water. Female *Ae. aegypti* were allowed to feed on mice (Baylor IACUC permit #395291-4) for 1 h for egg laying. Larvae from the collected *Ae. aegypti* eggs were raised in plastic trays (25 cm × 20 cm × 14 cm) with 1 L aged tap water and liver powder. Female *Cx. pipiens* were allowed to feed on 1- to 3-day-old chicks overnight (permit as above) for egg laying. *Cx. pipiens* larvae were raised using the same protocols as for *Ae. aegypti* but with the substitution of minced fish food (Tetramin®). For plant-attraction experiments, pupae were collected individually into plastic test tubes, and adults were used 0–12 h after emergence.

2.2. Plants

Five candidate plant species were selected for study (Fig. 1; Table 1). These plants were chosen because they, or their close relative, produce nectar foraged by insects and have published tissue culture protocols. *Ricinus communis* is readily fed upon by mosquitoes in cages (Gary and Foster, 2004; Impoinvil et al., 2004) and *Asclepias syriaca* frequently hosts mosquitoes in the field (Foster, 2008). *Passiflora* spp. (Xu and Chen, 2010), *Campsis radicans* (Elias and Gelband, 1975) and *I. walleriana* (syn. *Impatiens sultani*) (Lanza et al., 1993) have been reported to have extrafloral nectar used as food sources by ants. The candidate plants *I. walleriana* (Dan et al., 2010), *R. communis* (Malathi et al., 2006), and *Passiflora edulis* (Monteiro-Hara et al., 2011) have published transformation protocols utilizing *Agrobacterium tumefaciens*, as do two species in *Bignoniaceae* (Aslam et al., 2009; Shukla et al., 2009), the family to which *C. radicans* belongs. A tissue culture protocol has been published for *Asclepias curassavica* (Pramanik and Datta, 1986). An additional two plants were selected as negative controls: *Beta vulgaris* (preflowering), which presented only leaves lacking extrafloral nectar, and *N. benthamiana*, which has long tubular flowers with inaccessible floral nectar and no extrafloral nectar.

All plants were propagated from seeds, which were germinated in soil pots in a dedicated plant growth-room with a temperature range of 22–24 °C, under plant-spectrum fluorescent or metal halide bulbs and with automated watering. All plants were grown to a height of 20–30 cm, appropriate for the 33-cm-high mosquito cages, except for *P. edulis*, which was grown as a vine to a biomass similar to the other plants and then wrapped up to fit into the cage, and *A. curassavica*, which was grown to flowering (1 m tall) and had a cage fitted onto the flowering head.

2.3. Survival assay

Batches of 10 male and 10 female mosquitoes were placed into cages with a single potted plant, and surviving mosquitoes were counted each day for 20 days. For *A. curassavica* flower heads, a watered soil pot was included in the suspended cage to allow the mosquitoes access to water in the soil as they had with the other plant species. Cages (33 cm cube or 36 cm × 36 cm × 61 cm for *P. edulis* vines to avoid stem damage) were of mesh (Bioquip, Rancho Dominguez, CA, USA) and pots were drip-irrigated daily. For a positive control group, mosquitoes were allowed continuous access to 10% (wt/vol) sucrose in a 1.5 ml tube stuffed with cotton. The negative controls included a drip irrigated soil pot, a cotton-stuffed tube of water or an empty cage. Sucrose and water were changed every 2 days. All tests were replicated five to six times.

2.4. Dyed sugar source attraction assays

For solo and competitive plant-attraction studies, 0.2 μl of red food dye (FD&C Red 40) was applied to nectar drops on all nectaries. A single potted plant with dyed nectar was contained in a 30-cm mesh cube for the solo plant attraction study. For the competition study, a single dyed nectar plant was contained with five undyed competing plants plus a tube of undyed 10% sucrose in a 36 cm × 36 cm × 61 cm cage. For the sucrose model studies, the same dye was added as one drop in 1 ml of 10% sucrose, and the "competing" tubes contained 1 ml of undyed sucrose, all in a 30-cm-mesh cube cage. All experiments were conducted with 10 male and 10 female mosquitoes per cage, and dyed mosquitoes were counted every day over a 3-day period, with three replicate cages per treatment.

2.5. Statistical analyses

Survival analysis techniques (JMP version 10.0.0), including Log-Rank and Wilcoxon, were used to compare survival curves and to test whether the survival rate differed between different nutritional regimes. The data were confirmed by implementing R 3.0.2 (R Core Team, 2013). The significant difference of each individual survival curve was calculated by Bonferroni correction (multiple comparisons) (Hochberg and Tamhane, 1987). For this test, the day of death for both female and male adult mosquitoes was recorded. Differences among replicates of experiments were also analyzed individually and were found to be trivial, so the data sets were combined. The data from the solo plant assay, sucrose bioassay and competition assay, including the average of replicates and standard deviation (error bar), were analyzed in Excel 2010. Contingency analysis (Pearson test) was carried out to test the significance ($p < 0.001$) in JMP.

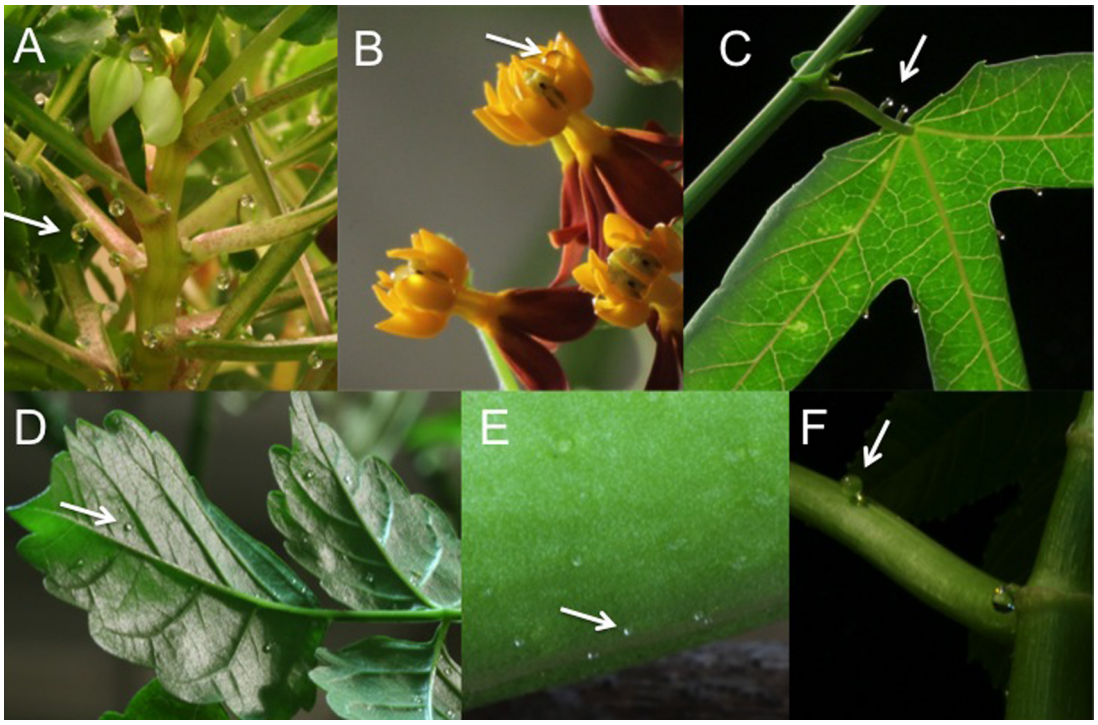

Fig. 1. Nectar location on candidate nectar plants analyzed in study. Arrows denote nectar droplets. (A) *Impatiens walleriana*, (B) *Asclepias curassavica*, (C) *Passiflora edulis*, (D) *Campsis radicans* leaf, (E) *C. radicans* green seed-pod, (F) *Ricinus communis*.

2.6. Nectar collection and SDS–PAGE

Extrafloral nectar from *I. walleriana*, *R. communis* and *C. radicans* and floral nectar from *A. curassavica* were collected in microcentrifuge tubes individually. When collecting nectar from any plant, the nectar was immediately diluted 1:3 with water and stored at −20 °C. To concentrate the nectar, 100 μl of diluted nectar was combined with 900 μl of cold 100% ethanol, iced for 15 min and then centrifuged at room temperature at 16,000 × *g* in a microcentrifuge. This deviated from Thornburg's protocol (Carter et al., 1999), which used 1 ml of pure nectar mixed with 9 ml of cold ethanol centrifuged at 65,000 × *g* for 20 min, presumably at 4 °C. The nectar was re-suspended in 10 μl of 10 mM sodium phosphate buffer (pH 7.4).

3. Results

3.1. Mosquito survival on candidate nectar plants

For each plant species, mosquitoes were tested for their ability to survive on nectar to determine if consistent nectar imbibition was occurring (Fig. 2, Table 2). In general, there were few differences seen between *Cx. pipiens* (Fig. 2A) and *Ae. aegypti* (Fig. 2B). The survival data segregated into statistically different groups. For *Ae. aegypti*, survival on *I. walleriana* was significantly higher than any other group, followed by the other four nectar plants and the 10% sucrose positive control, followed by *R. communis*. Mosquito populations declined rapidly with the negative control treatments, comprising the negative control plants (*B. vulgaris* and *N. benthamiana*), which provided no accessible nectar, and negative controls with no plants (irrigated soil pot, a single water tube, or an empty cage). For *Cx. pipiens*, *I. walleriana* provided the best survival along with *C. radicans* and *A. curassavica* and the sucrose control. In a separate experiment with *I. walleriana* and *Cx. pipiens*, over half of the mosquito populations survived in three replicate cages after 42 days (data not shown).

3.2. Solo plant attraction study

To measure readiness to imbibe, red food dye was added to the nectar of all nectaries of a single plant and the uptake of red dye by mosquitoes was counted over a 3-day span (Fig. 3). Within 1 day of imbibition, 80–95% of the mosquitoes had imbibed dyed nectar from *I. walleriana* plants, while only 10–50% had done so for *R. communis* and 50% for *C. radicans* (Fig. 3). This significantly higher imbibition level ($p < 0.0001$) was maintained by *I. walleriana* over the other nectar plants over the entire 3-day period. No significant difference was seen between days within one plant species in any test.

3.3. Competitive plant attraction studies

As a control experiment for the competitive plant attraction studies, 10% sucrose tubes were used to determine the effects of the red dye on imbibition choice and the effects of multiple sampling on the acquisition of dye in mosquitoes. A single tube of dyed sucrose was offered in the presence of an increasing number of tubes with undyed sucrose. The proportion of dyed mosquitoes fairly closely followed the proportion of dyed:undyed tubes presented to the mosquitoes, except for a slight increase in dye uptake at the midranges (1 + 1 and 1 + 3) (Fig. 4). As well, no significant difference in dyed mosquito proportions occurred between the three observation time points. We concluded that dyed nectar uptake in subsequent experiments would be expected to be an accurate measure of plant-species preference, without dye avoidance, color oversaturation or time point dependence.

In the competitive attraction tests using plants, a single dyed plant was placed in the presence of five undyed plants and a tube of undyed sucrose. Results corroborated those of the solo plant attraction assays. *I. walleriana* was imbibed significantly more ($p < 0.0001$) as the dyed target plant than *C. radicans* and *R. communis* and also significantly more ($p < 0.0001$) than the sucrose tube positive control serving as the dyed target (Fig. 5). *R. communis* was

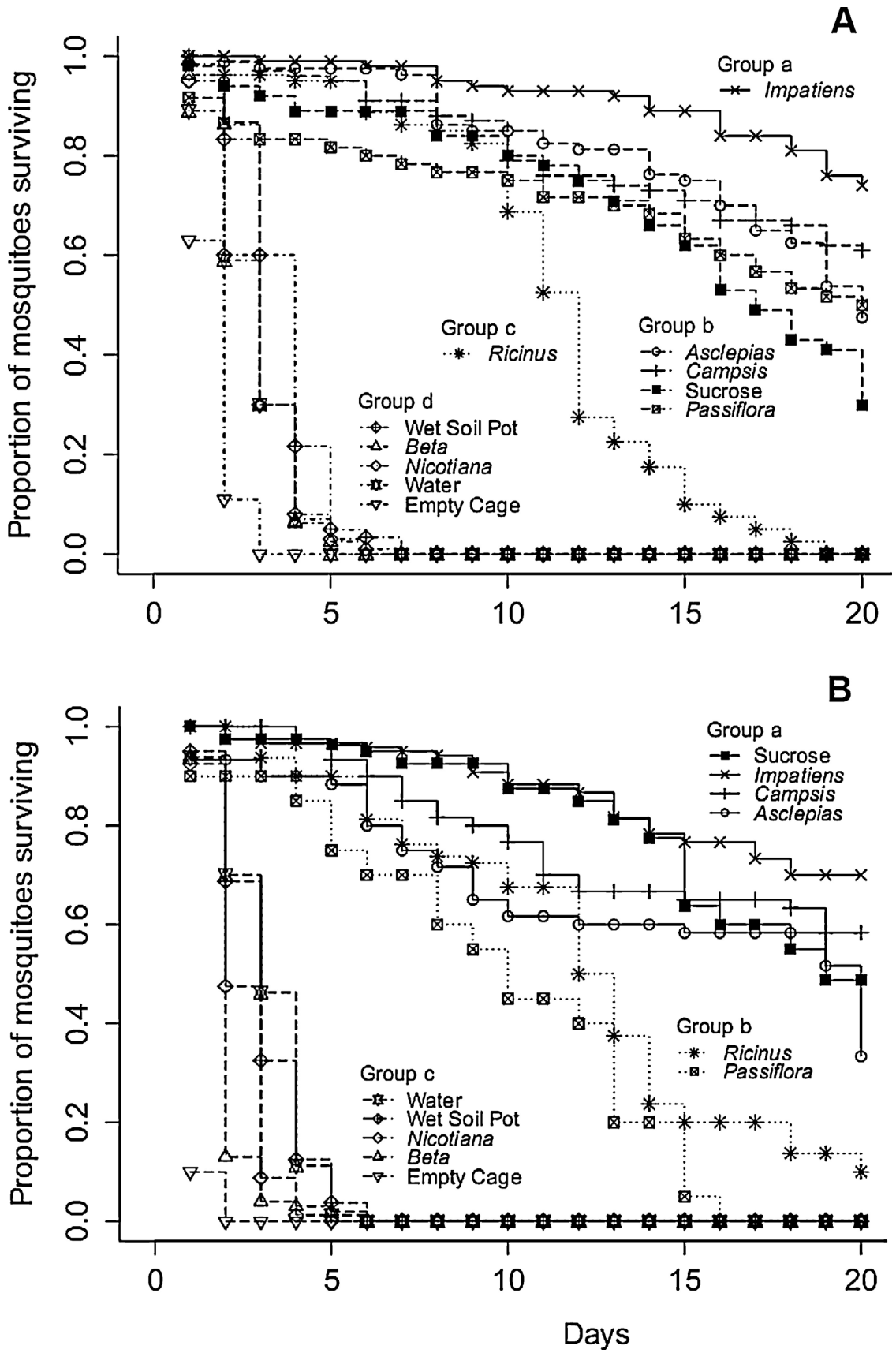

Fig. 2. Survival curves of caged mosquitoes with different nectar plants as the sole sugar source, demonstrating long term imbibition and suitability as a sugar source. *Ae. aegypti* (Panel A) and *Cx. pipiens* (Panel B). Groups (a–d) in Panel A are statistically different from each other ($p < 0.0001$), with all negative controls falling into group (d). The data formed three groups, (a–c), in Panel B. Negative controls: plants which did not produce accessible nectar (*Nicotiana benthamiana*, *Beta vulgaris*), a single watered soil pot without a plant, a tube of water, or no substrate at all (empty cage). Positive control: one tube of 10% sucrose.

Table 2
Median survival times of mosquito populations with different plant sugar sources (data from Fig. 2).

Plant species	*Ae. aegypti*			*Cx. pipiens*		
	Mean[a]	SE[b]	Sig Diff[c]	Mean[a]	SE[b]	Sig Diff[c]
I. walleriana	18.6	0.35	a	16.1	0.35	a
A. curassavica	16.9	0.54	b	14.4	0.91	a
C. radicans	16.5	0.55	b	15.3	0.70	a
Sucrose	15.3	0.58	b	16.2	0.48	a
P. edulis	14.8	0.92	b	9.6	1.05	b
R. communis	11.4	0.39	c	12.1	0.56	b
Wet soil pot	3.7	0.16	d	3.1	0.13	c
B. vulgaris	3.2	0.11	d	2.2	0.08	c
N. benthamiana	3.0	0.10	d	2.5	0.09	c
Water	2.8	0.11	d	3.2	0.13	c
Empty cage	1.7	0.06	d	1.1	0.03	c

[a] Mean number of days at which mosquito population reduced by half.
[b] SE, standard error of the mean.
[c] Groups with significant difference from each other ($p < 0.0001$) as in Fig. 2.

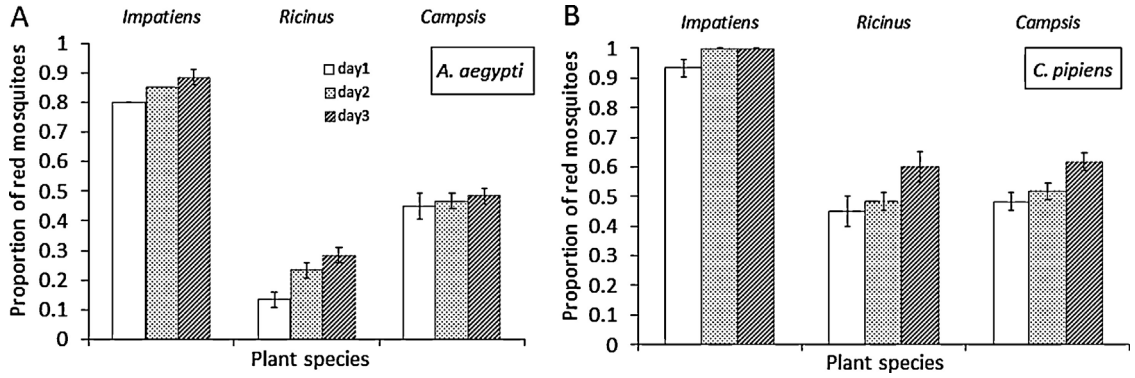

Fig. 3. Solo plant attraction assay; *Ae. aegypti* (Panel A) and *Cx. pipiens* (Panel B). The uptake of nectar dyed with red dye from plant nectaries was tallied in order to measure the readiness to imbibe nectar for each plant species. A single plant was placed in a cage with 20 mosquitoes for 3 days. *I. walleriana* was imbibed significantly more ($p < 0.0001$) than the other plant species. Bars indicate standard error.

imbibed the least out of all the plant species tested. There was no significant difference between the counts on different observation days, as was true for the control experiment with sucrose tubes (Fig. 4).

3.4. Nectar protein in the candidate plant species

The protein concentration of the nectars from each plant species varied tremendously, as determined by SDS–PAGE (Fig. 6; Table 3).

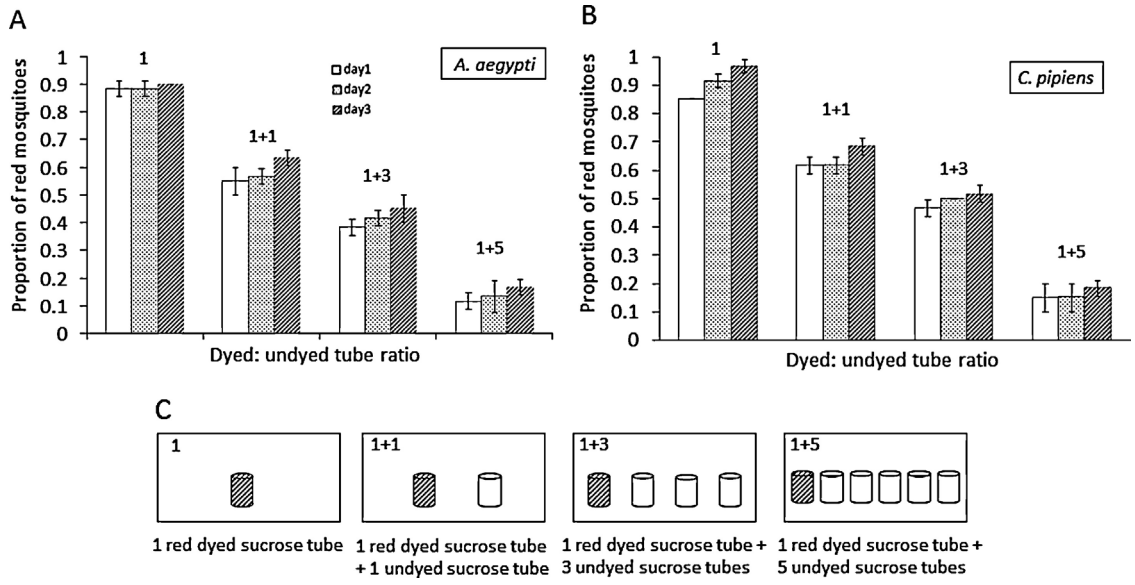

Fig. 4. Sucrose control experiment for the competitive attraction assay; *Ae. aegypti* (Panel A) and *Cx. pipiens* (Panel B). A tube of dyed 10% sucrose was tested for imbibition by mosquitoes alone (1) or in the presence of an increasing number of undyed sucrose tubes ($1 + n$). The proportion of dyed mosquitoes generally reflected the proportion of dyed:undyed tubes, indicating that multiple sampling and dye avoidance were not factors.

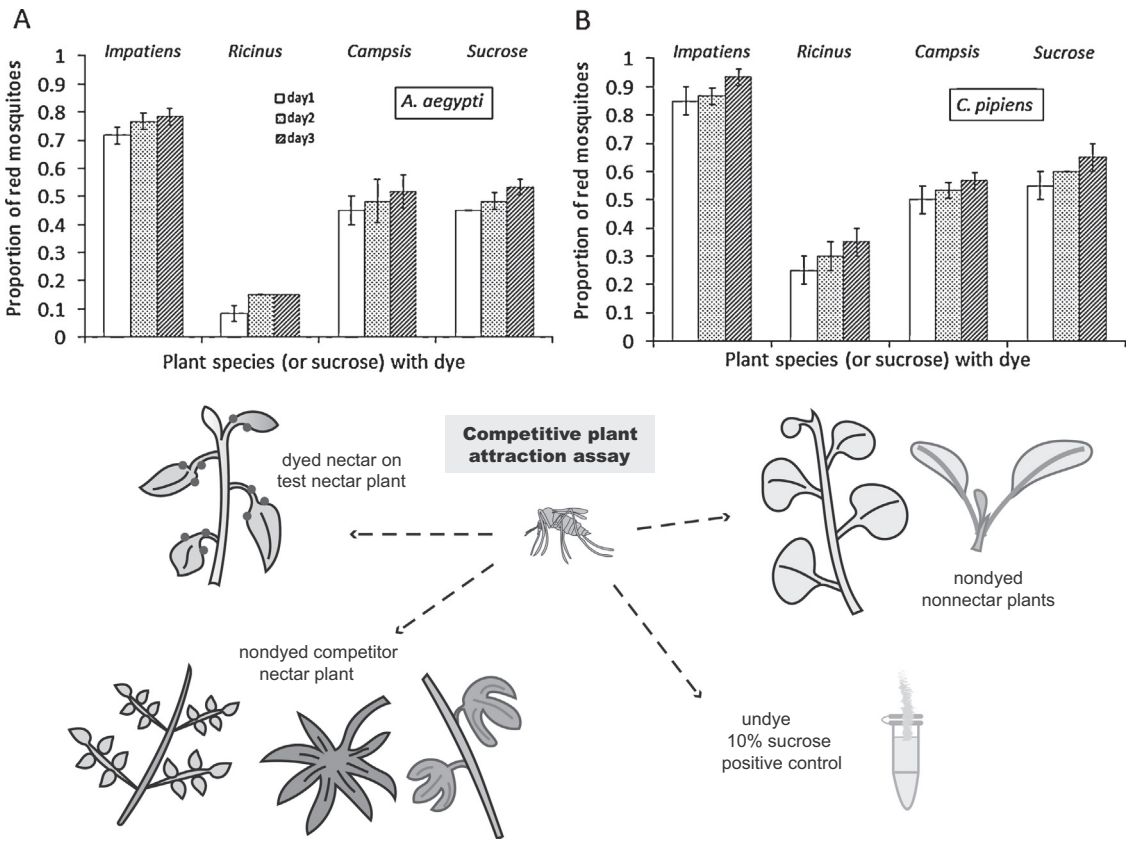

Fig. 5. Competitive attraction assay; *Ae. aegypti* (Panel A) and *Cx. pipiens* (Panel B). Plants: *I. walleriana*, *R. communis*, *C. radicans*, and *P. edulis* (nectar plants); and *N. benthamiana* and *B. vulgaris* (nonnectar plants). Sucrose test: dyed sucrose tube in the presence of six undyed plants.

Table 3
Nectar protein mass and concentration (from Fig. 6).

Plant species	Gel Lane	Protein MW (kDa)	μg protein in band	[Protein] in nectar (μg/μl)
Impatiens	# 6	80	1.29	0.43
		21	10.5	3.5
		15	2.23	0.74
Ricinus	# 2	20	0.5220	0.035
Passiflora	# 4	40	2.150	0.14
		22	6.286	0.42
Campsis	# 8	48	NC[a]	NC
(pods)		27	NC	NC
Tobacco	# 5	40	0.4325	0.029

[a] Not calculated since a wash was used to gather nectar on the pods.

The plant-nectar model system from *Nicotiana tabacum* yielded a 40 kDa protein (lane 5; presumably Nec1 (Carter et al., 1999)) at a concentration of 0.029 μg/μl, but *I. walleriana* 21 kDa protein (lane 1) accumulated in nectar to a level of 3.5 μg/μl, over 100-fold greater. Two other protein species expressing at levels more than 10-fold of Nec1 were also present in *I. walleriana* nectar. *R. communis* (lane 2) and *P. edulis* (lane 4) also produced high levels of nectar protein. To visualize individual protein bands, *I. walleriana* and *R. communis* protein extracts needed to be diluted (lanes 6 and 7) while nectar was washed from *C. radicans* seed pods collected in the field and concentrated by ethanol precipitation (lane 8).

4. Discussion

In the present study, all nectar plants tested were strongly imbibed by both species of mosquitoes tested. However, *I. walleriana* was consistently more attractive to both *Cx. pipiens* and *Ae. aegypti* than any of the other four nectar plants. *Ae. aegypti* survived significantly longer on *I. walleriana* than any other nectar plant and significantly longer than on the 10% sucrose positive control. Over half of the mosquitoes in three replicate *Cx. pipiens* populations survived after 42 days imbibing only on *I. walleriana*. In solo plant attraction studies, *I. walleriana* was imbibed significantly more than *C. radicans* and *R. communis*. In competition with three other nectar plants, two nonnectar plants and a sucrose control, *I. walleriana* was preferred significantly more than the other nectar plants. These studies clearly show *I. walleriana* as a preferred nectar host for both *Ae. aegypti* and *Cx. pipiens*.

Among the nectar plants of this study, only *R. communis* had been examined in survival studies previously, and it had supported the survival of mosquitoes fairly well (Gary and Foster, 2004; Impoinvil et al., 2004; Manda et al., 2007b). However, in our study, *R. communis* was clearly the least strong of the group. It was the only nectar plant tested which promoted mosquito survival significantly less than the sucrose positive control. It was also significantly

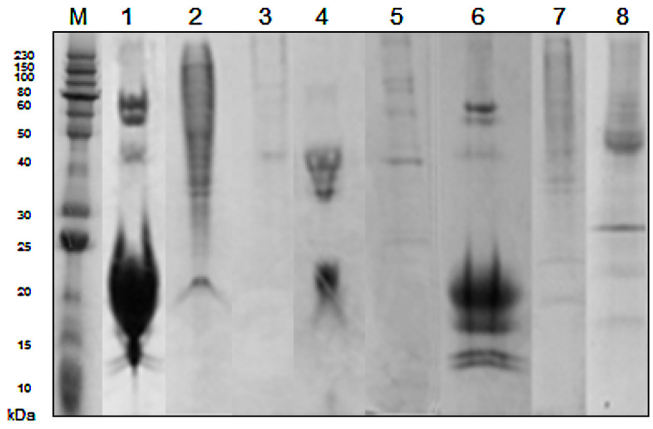

Fig. 6. SDS–PAGE of plant nectar proteins. M, marker lane; 1, *I. walleriana*; 2, *R. communis*; 3, *C. radicans* (leaf nectar); 4, *P. edulis*; 5, *Nicotiana tabacum*; 6, 7, *I. walleriana* and *R. communis* diluted nectar; 8, *C. radicans* nectar washed from green seed-pods. Nectar was ethanol precipitated to concentrate proteins; lanes 1–5 represent the equivalent of 15 μl pure nectar. Lanes 6 and 7 were diluted to reveal individual bands and each represents 3 μl pure nectar.

less attractiveness for mosquito imbibition than the other nectar plants. It should be noted that *R. communis* leaf extracts are toxic to mosquitoes (Elimam et al., 2009) and perhaps some of this toxicity is present in the nectar.

The other nectar plants of the study were generally favored by mosquitoes as much or more than the sucrose control. *A. curassavica* was another plant species selected based on published indications of attractiveness to mosquitoes. It is related to *A. syriaca*, which possesses floral scents attractive to mosquitoes (Foster, 2008; Otienoburu et al., 2012) and is commonly associated with mosquitoes in the field (W.A. Foster, personal communication). In our study, *A. curassavica* was statistically equal to the sucrose control in promoting survival and attracting mosquito imbibition. The remaining three nectar plants were selected based only on their published associations with ants. However, all three of these were strongly attractive to mosquitoes and promoted long term survival of mosquitoes. These results suggest that nectar associations with other insects can serve as an indicator of potential attraction to mosquitoes. A similar mosquito attraction study was performed with *An. gambiae* and six plant nectar hosts common to villages in western Kenya (Manda et al., 2007a). In cages with cut flowers, *An. gambiae* survival was generally less than that of the sucrose control and there was more variability in mosquito survival between the different nectar plant species tested. Mosquitoes are also capable of feeding on plant tissue when nectar is not available (Müller and Schlein, 2005; Qualls et al., 2013). The present study did not distinguish between nectar and plant tissue consumption; nectar was the assumed nutritional substrate.

There was no correlation between the amount of nectar protein produced and the attractiveness to mosquitoes among the five nectar plants examined. Although *C. radicans* was highly attractive to mosquitoes, it produced relatively low amounts of nectar protein. *R. communis* was the least attractive to mosquitoes, and yet produced a large amount of nectar protein. On the other hand, *P. edulis*, *A. curassavica* and *I. walleriana* all produced large amounts of nectar protein and were highly attractive to both species of mosquitoes tested.

However, strong production of nectar protein would be an important asset to a model nectar delivery plant species. Thornburg pioneered the study of nectar proteins, using *Nicotiana* (tobacco) species as his model system (Park and Thornburg, 2009). A limited number of proteins, mostly enzymes, are produced in nectar (Nicolson and Thornburg, 2007), but these can be produced in large amounts. A strong nectar promoter from carnation has been used to produce pharmaceutical proteins in nectar of transgenic tobacco in relatively high yield (Helsper et al., 2011). In a direct comparison in this study, the 21 kDa impatiens nectar protein was produced at levels 100-fold greater than Nec1, the most strongly expressed nectar protein in tobacco (Carter et al., 1999).

This study has identified *I. walleriana* as an excellent candidate species from which to construct a transgenic model system for the study of several aspects of nectar delivery. The robust nectar expression capacity of *I. walleriana* would facilitate the development of a strong transgenic nectar expression system. We are currently isolating the *I. walleriana* 21 kDa nectar protein promoter. The expression of both Cry protein toxins and pathogen modulatory proteins could then be examined. Any resulting mosquitocidal nectar plants would then need to be examined for their ability to control field populations of mosquitoes in variety of different locales.

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