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LRH: Dutton, Shore, and Frederickson

RRH: Extrafloral Nectar Increases Ant Seed Removal

Extrafloral Nectar Increases Seed Removal by Ants in *Turnera ulmifolia*

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1 **ABSTRACT**

2 To investigate whether extrafloral nectar (EFN) increases seed dispersal in *Turnera ulmifolia* we
3 measured seed removal on plants with and without EFN. Plants producing EFN had more seeds
4 removed than control plants, suggesting that EFN does play a role in seed dispersal. This is a
5 novel function of EFN.

6

7 **Keywords:** ant defense; extrafloral nectar; Florida; seed dispersal; mutualism; myrmecochory

Extrafloral nectaries, sugar-secreting glands on plant parts other than flowers (Elias *et al.* 1975), occur on more than 4000 plant species (Weber and Keeler 2013). They are almost always involved in ant defense (Weber & Agrawal 2014), and occasionally attract pollinators (Knox *et al.* 1985). Cuautle *et al.* (2005) found that *Turnera ulmifolia* attracts ants for both defense and seed dispersal, and many ant species collecting EFN from *T. ulmifolia* also collect elaiosome-bearing seeds. They hypothesized that EFN increases seed dispersal on *T. ulmifolia*. Here, we test this hypothesis by manipulating EFN production and measuring seed removal, providing the first experimental evidence that EFN increases seed dispersal. We also investigate phenological changes in EFN production as a possible mechanism by which plants alter ant visitation.

We conducted our field experiment from 21 to 25 September 2014 at the edge of a tropical hardwood hammock located in John Pennekamp Coral Reef State Park (25°07'32" N, 80°24'22" W, elevation ~ 1 m asl), Key Largo, Florida, U.S.A. The climate is warm and humid, with ten-year average September temperature and precipitation of 28°C and 200 mm respectively (NOAA National Climatic Data Centre, Florida Keys Time Series, 2014). *Turnera ulmifolia* plants were located in a large patch (~ 60 plants) dominated by poisonwood (*Metopium toxiferum*) and sea grape (*Coccoloba uvifera*).

Turnera ulmifolia L. is a weedy, perennial shrub native throughout South America and the West Indies, and naturalized in Florida (Barrett 1978). Each leaf has a pair of extrafloral nectaries on the petiole (Elias *et al.* 1975, Gonzalez 1996). Flowers are ephemeral, opening once for three to five hours, and located on the distal portion of the petiole, directly above extrafloral nectaries (Elias *et al.* 1975). In most regions, including Florida, plants flower and set fruit year

1 round with a peak in the rainy season (Cuautle *et al.* 2005). Thus, many individuals display all
2 phenological stages simultaneously throughout the year. Fruits are axial, small, round, usually
3 green, dehiscent capsules that take about 21 d to develop (Cuautle *et al.* 2005, Salazar-Rojas *et*
4 *al.* 2012). Seeds are about 3 mm long and bear large elaiosomes (Barrett 1978, Cuautle *et al.*
5 2005).

6 To determine if EFN plays a role in seed dispersal in *T. ulmifolia*, we manipulated EFN
7 secretion and measured the effect on ant abundance and seed removal. The week before the
8 experiment, seeds were collected daily from 28 *T. ulmifolia* plants and stored in an air-tight
9 container. We then randomly assigned each plant to one of two treatments: an EFN exclusion
10 treatment in which we applied clear nail polish on all extrafloral nectaries on the plant to prevent
11 nectar secretion, and a control treatment in which we applied clear nail polish near, but not on,
12 extrafloral nectaries. The nail polish did not appear to damage plant tissues (E. Dutton, pers.
13 obs.) and similar methods have been used previously (Rudgers & Gardener 2004); furthermore,
14 any effects of nail polish were consistent across treatments. Starting the day after we imposed the
15 treatments, each morning between 0900 h and 1100 h for three days, we took a snapshot count of
16 all ants on each plant and identified them to species. Immediately following, we taped a
17 microcentrifuge tube cap to an upper leaf on each plant (where fruits are normally found) and
18 added 10 *T. ulmifolia* seeds (the number of seeds in a typical fruit in this population). We put
19 seeds in these “depots” instead of using dehiscing fruits to standardize the number, quality, and
20 presentation of the seeds. After an hour, we counted the number of seeds remaining. We
21 measured ant presence and seed removal in the morning because this is when seeds were

normally most available. We analyzed the effect of treatment on the three-day average of ant abundance and number of seeds removed using a generalized linear model (GLM) with quasi-poisson errors in R v. 3.0.2 (R Core Team 2013).

One caveat of our experimental design is that we measured removal, not dispersal. However, seed removal is often used as a proxy for seed dispersal, and is thought to closely relate to seed dispersal (Carney *et al.* 2003, Zelikova *et al.* 2008, Türke *et al.* 2012). Furthermore, ant composition was similar between treatments, suggesting any difference between removal and dispersal was consistent between treatments, although we did not formally test this.

EFN secretion increased ant abundance and seed removal. Control plants with active extrafloral nectaries had 7.8 ± 1.7 (mean $\pm$ 1SE) ants per plant, whereas EFN-excluded plants had 3.0 ± 0.4 ants per plant, a significant decrease in ant abundance ($F_{1,26} = 10.14$, $P < 0.01$). Similarly, control plants had 5.0 ± 0.8 seeds removed, whereas EFN-excluded plants had 2.6 ± 0.5 seeds removed, a decrease of almost 50 percent ($F_{1,26} = 6.21$, $P = 0.019$).

Our results suggest that EFN increases seed removal (and likely seed dispersal) in *T. ulmifolia*. Given that only ~1% of seeds successfully germinate (Salazar-Rojas *et al.* 2012), such an increase probably affects reproductive success. Thus, our study provides the first experimental evidence of a novel function of EFN and supports the hypothesis suggested by Cuautle *et al.* (2005). The continuous presence of ants at extrafloral nectaries may decrease the time it takes ants to discover and remove seeds, especially given their close physical proximity (Elias *et al.* 1975), thereby reducing the risk of falling to the ground where predation by red land crabs

(*Gecarcinus lateralis*) is high (Salazar-Rojas *et al.* 2012). Additionally, seeds may be more likely to be removed by omnivorous, scavenging ants that typically act as high quality dispersers (Cuautle *et al.* 2005, Prior *et al.* 2015). Interestingly, many myrmecochorous plants have prostrate fruits, but *T. ulmifolia* seeds stay attached to the valves of fruits during dehiscence, meaning most seeds (~92%) are removed from on (rather than beneath) the plant, where they remain close to extrafloral nectaries (Cuautle *et al.* 2005, Salazar-Rojas *et al.* 2012).

The most common ants on *T. ulmifolia* were *Wasmannia auropunctata* (75% of plants), *Paratrechina longicornis* (43% of plants), *Camponotus floridanus* (18% of plants), *Tapinoma melanocephalum* (4% of plants), and *Brachymyrmex obscurior* (4% of plants), all of which were observed collecting EFN. There were no clear differences in ant composition between treatments. *Wasmannia auropunctata* is often considered a poor disperser because it can remove elaiosomes *in situ* (Rico-Gray & Oliveira 2007); however, we repeatedly observed *W. auropunctata* removing seeds with elaiosomes attached and carrying them to the forest floor, suggesting effective dispersal. Furthermore, seeds that remained at the end of the experiment always had intact elaiosomes, suggesting that *W. auropunctata* does not “rob” *T. ulmifolia* elaiosomes. *Paratrechina longicornis* was also observed removing entire seeds with elaiosomes, and can effectively disperse seeds of many myrmecochorous plants (Rico-Gray & Oliveira 2007). Little is known about the seed-dispersing efficacy of the other three ant species. Only ant species that also visited extrafloral nectaries were observed collecting seeds.

Knowing that EFN increases seed removal, a possible mechanism by which plants may enhance seed dispersal is by increasing EFN production at fruit dehiscence. To explore this, we

1 studied a population of *T. ulmifolia* plants collected in Ewarton, Jamaica in 1994 and propagated
 2 vegetatively in glasshouses (23-32°C) at York University, Toronto, Canada (43°46'29" N,
 3 79°30'18" W). Ants frequently visit extrafloral nectaries on *T. ulmifolia* in Jamaica (Barrett
 4 1978), much as they do in Florida, but we do not know whether these populations differ in EFN
 5 production, nor how EFN production is affected by glasshouse conditions. On glasshouse plants
 6 in 2015, we simultaneously measured EFN production at extrafloral nectaries on bare leaves and
 7 on leaves subtending floral buds, open flowers, wilted flowers (one day after flowering), and
 8 fruits before (1 day to maturation), during, and after dehiscence. Fruits were produced by hand-
 9 pollinating flowers the required number of days before taking measurements. Thus, all 10 plants
 10 had pairs of extrafloral nectaries at every stage, which were measured simultaneously. We
 11 removed EFN 24 h prior to taking measurements to ensure the same amount of time for nectar
 12 accumulation across stages and to mimic field conditions where ants regularly remove nectar.
 13 We collected nectar from both extrafloral nectaries on one leaf at each phenological stage on
 14 each plant in 5 µL microcapillary tubes (Drummond Scientific Company, Broomall, USA) and
 15 determined volume by measuring the height of nectar with microcalipers. We diluted samples
 16 with known volumes of 0.5-3.0 µL of distilled water and measured concentration (°Brix) with a
 17 low-volume hand-held refractometer (Bellingham and Stanley, Kent, UK). We estimated sugar
 18 mass per mL following Búrquez and Corbet (1991) and multiplied this by nectar volume to
 19 obtain sugar mass. We analyzed the data in R v. 3.0.2 using a linear mixed model with sugar
 20 mass (square-root transformed) as the response variable, stage as a fixed effect, and plant ID

1 treated as a random blocking factor, followed by a Tukey-adjusted least-squares mean test to
 2 compare phenological stages.

3 EFN production peaked when the flower bloomed and just before the fruit dehisced ($F_{6,54}$
 4 = 21.2, $P < 0.0001$; Fig. 1). The peak at flowering is common to most species with extrafloral
 5 nectaries (Marazzi *et al.* 2013). The peak immediately before dehiscence may attract seed-
 6 dispersing ants. Although the peak in nectar production occurred the day before fruit dehiscence,
 7 it can take some ant species a long time to update their foraging locations in response to shifting
 8 food availability (Beckers *et al.* 1990, Grüter *et al.* 2012), and of course there is food available
 9 close by in the form of elaiosomes once fruits dehisce. Because different phenological stages are
 10 present on the same plant simultaneously, the peak in EFN production at fruit dehiscence
 11 probably shifts the distribution of EFN (and ants) within the plant, more than it changes a plant's
 12 total EFN production. Alternatively, the peak near dehiscence may attract ants to defend against
 13 seed predators.

14 Our field and glasshouse results suggest *T. ulmifolia* may have experienced stronger
 15 selection for EFN production during fruit development than other extrafloral nectary-bearing
 16 plants because of the additional selective advantage of attracting seed-dispersing ants. Of course,
 17 this requires further investigation as we worked on non-native populations and we know little
 18 about the evolutionary history of this ant-plant interaction. Our previous work in the same
 19 population demonstrated that ants collecting EFN decrease florivory (Dutton *et al.*, in press),
 20 perhaps explaining the peak in production at flowering. This also suggests the intriguing
 21 possibility that in *T. ulmifolia* EFN may be an “ecologically pleiotropic” trait (*sensu* Strauss &

Irwin 2004). Ecological pleiotropy has previously been documented between antagonists and mutualists; however is rarely considered between two mutualists (Brody 1992, Galen & Cuba 2001, Strauss & Irwin 2004). Future studies could determine if there are additive or synergistic effects of defense and dispersal that could increase the strength of positive selection (compared to the effect of only one mutualism) on EFN (Afkhami *et al.* 2014).

In this study, we demonstrated for the first time that EFN can play a role in seed dispersal. While beneficial for seed dispersal, the effects on plant defense are unknown. In many taxa, the nutrient composition of elaiosomes has converged with that of insects. Consumption of elaiosomes could decrease the appetite of ants for insect herbivores (Hughes *et al.* 1994, Rico-Gray & Oliveira 2007), negatively impacting defense. However, this would have to be tested explicitly. Ants that visit extrafloral nectaries can also harass or deter pollinators, with mixed effects on seed set (Ness 2006, González *et al.* 2013), suggesting that attracting ants with EFN to increase seed dispersal could come at the cost of seed production; however this would also require further investigation. It is unknown how many myrmecochorous plants also produce EFN, although similar relationships are likely throughout the genus *Turnera*, and possibly in *Acacia* where ants have been observed dispersing seeds and collecting EFN (Rudgers and Gardener 2004).

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1 **Figure Legends**

2 **FIGURE 1.** Mass of extrafloral nectar sugar (mean $\pm$ 1SE) produced by one nectary pair over 24
3 hours at various phenological stages (depicted in line drawings). Arrows indicate the locations of
4 extrafloral nectaries. Letters indicate statistically different stages based on a Tukey-adjusted
5 least-squares means test ($P < 0.05$).

1 **Figure 1**

2