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Effect of caffeine and γ-aminobutyric acid on preference for sugar solutions in two ant species

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ABSTRACT. Artificial sugar solutions can be used to disrupt the mutualism between aphids and ants. This provides natural enemies with increased access to aphid populations, thus enhancing biocontrol of hemipteran pests. However, research suggests that artificial solutions should be tailored to specific species in order to maximize the desired result. In this study, we test the effect of an alkaloid, caffeine, and a non-protein amino acid, γ-aminobutyric acid (GABA), on preference for sucrose solutions in two ant species, *Lasius niger* and *Oecophylla smaragdina*. These secondary metabolites have been shown to have attractive capabilities in other species of insects, who favor nectars from plants containing these compounds. In our first experiment, both species significantly preferred solutions containing GABA over sucrose-only controls, albeit *L. niger* favored higher concentrations of the amino acid. Caffeine did not significantly increase preference for sugar solutions in either of the tested species, and ants significantly dispreferred the highest concentration of caffeine offered. When the two metabolites were tested simultaneously against sucrose only, *L. niger* fed equally from all solutions, while *O. smaragdina* dispreferred caffeine. Thus, while GABA seems to be a promising ant attractant, caffeine is not an adequate choice, at least at the concentrations tested in this experiment.

Keywords *Lasius niger*, *Oecophylla smaragdina*, GABA, secondary metabolites, Formicidae, alkaloid

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INTRODUCTION

Numerous species of ants exploit honeydew-producing hemipterans as a source of carbohydrates, providing them with protection against their natural enemies in exchange (Hölldobler & Wilson 1990). These mutualistic interactions established between ants and hemipterans can thus interfere with the activity of aphid predators (Buckley 1987, Wyss et al. 1999, Herbert & Horn 2008) and parasitoids (Itioka & Inoue 1996, Stechmann et al. 1996, Beltrà et al. 2015), consequently hindering biological control of these important pests. Artificial sugar solutions offered in feeders have been used to disrupt the aforementioned ant-aphid mutualism to some degree, as when ants are given alternative sugar they no longer need to attend their hemipterans (Offenberg 2001, Nagy et al. 2013, 2015, Wäckers et al. 2017). Sometimes, however, ants continue to prefer aphid honeydew over the artificial feeders, suggesting the need to develop more complex formulations of sugar-based solutions. Previous studies found that amino acid mixtures and trisaccharides can be used to increase the attractiveness of sucrose solutions to ants (Madsen et al. 2017). However, potential additives that can induce an even stronger feeding preference remain to be identified.

Research shows that some species of insects are attracted to plant-produced secondary metabolites found in nectar, like γ-aminobutyric acid (subsequently referred to as GABA) and caffeine (Singaravelan et al. 2005, Petanidou et al. 2006, Nepi 2014). It is hypothesized that, in the Mediterranean area, where sweetness alone might not be such an important discriminatory factor, GABA (combined with NaCl) represents an important nectar phagostimulant for various pollinating guilds, e.g. beetles, bees and flies (Petanidou 2007). Other studies suggest that GABA may increase feeding behaviour by stimulating those taste chemoreceptors sensitive to sugars (Nepi 2014). Regarding caffeine, bumblebees have shown preference for artificial flowers with nectar containing low doses of this alkaloid (Thomson et al. 2015). Caffeine content in floral nectar can also elicit feeding preference in honeybees (Singaravelan et al. 2005, Wright et al. 2013) and cause them to increase foraging frequency and persistency to the forage location (Couvillon et al. 2015). If similar processes take place in ants, these metabolites could potentially be used to increase ant attraction and fidelity towards artificial sugar feeders and in this way facilitate biological control of aphid pests.

The two ant species *Oecophylla smaragdina* and *Lasius niger* both attend hemipterans on crop plants to obtain their honeydew (Offenberg et al. 2013, Nagy et al. 2013, 2015) and have both been observed to ignore artificially offered sugars during periods with abundant hemipteran trophobionts in their host trees (Offenberg, unpublished data). *Oecophylla smaragdina* is distributed throughout tropical Australasia (Wetterer 2017) where it is important as a highly effective biocontrol agent in a number of plantation crops (Way & Khoo 1992, Van Mele 2007, Offenberg 2015) - a control agent that may fertilize its host plants with nitrogen (Pinkalski et al. 2015, 2018). In Indonesia and Thailand it is used to produce insect protein for human consumption (entomophagy) and for feed (Césard 2004, Sribandit et al. 2008, Huis et al. 2013) and it is also used to produce various pharmaceuticals (Rastogi 2011). *Lasius niger* is a Eurasian ant species that can cause problems in plantation crops by attending aphid pests, e.g. on apple and broad bean (Banks & Macaulay 1967, Nagy et al. 2013, 2015). It may, however, also be manipulated to neglect its aphid partners when offered artificial sugar (Offenberg 2001, Nagy et al. 2013, 2015).

The present study investigated the effects of small added amounts of caffeine and GABA on ant preference for a sucrose solution in *O. smaragdina* and *L. niger*. If these secondary metabolites increase the attractiveness of plain sucrose, they could be used as additives in an artificial sugar-based solution aimed at disrupting ant-aphid mutualisms.

MATERIALS AND METHODS

Ant cultures

Eight queenright colonies of *L. niger* (started from founding queens collected after their nuptial flight in Denmark) were kept in plastic boxes of 21 x 17 x 15 cm filled two-thirds with a blend of mineral soil and Sphagnum (2:1). Aeration was provided by a netted hole on the lid (9cmØ), and a 10cm plastic test tube filled with water and plugged with cotton served as an *ad libitum* water source. Ant nests were kept in a temperature-controlled chamber at 25°C, where experiments were also performed. Colony sizes ranged from approximately 50 to 200 imago ants per colony.

Similarly, experiments with weaver ants, were carried out on eight different queenright colonies, each containing approximately 1000-5000 workers and a single queen. Weaver ant colonies were each established from single queens collected in Darwin (Northern Territory, Australia), and subsequently transported to Denmark by plane and maintained on coffee plants (that serve as host plants in the field and additionally thrive well under the artificial light used in the greenhouse) placed on elevated pots in a tub full of water, i.e. ant colonies were surrounded by water and thus confined to a given plant. Plants with ants were kept in a greenhouse cell whose ceiling was covered by an interior climate screen. The chamber was not temperature-controlled except that temperature was kept above 18 °C and that windows in the chamber would automatically open to let air in when the temperature exceeded 24°C. Ants and plants were provided with artificial light at a regime of 16L:8D.

In between experiments, all colonies were fed with a 20% sucrose solution and alternating 1% peptone or casein solutions, house flies, cockroaches (*Periplaneta americana)* and red midge larvae (Chironomidae) (fish food, Akvarieteknik). Weaver ant colonies were, at the time of the experiment, much larger than those of *L. niger*.

Experimental solutions

Four different concentrations of GABA were added to sucrose in the first experiment: 0.001%, 0.002%, 0.005% and 0.01% (g per 100mL of water). Since the maximum GABA concentration recorded in nectar is 0.7mM or 0.007% (Nepi 2014), the highest of the concentrations tested in our study purposely falls outside the range of what is found in nature.

In the case of caffeine, the literature shows a considerable amount of variability when it comes to acceptance of this alkaloid across insect taxa, with studies using a wide array of concentrations (Singaravelan et al. 2005, Miyashira et al. 2012, Mustard 2014, Cammaerts et al. 2014). The only studies found on ants tested caffeine at 0.1% (Cammaerts et al. 2014) and a range from 0.01% to 0.5% (of total diet weight) (Miyashira et al. 2012). Thus, the range of concentrations chosen for our experiment was larger and slightly broader than that of GABA, i.e. the highest value was twenty times greater than the lowest: 0.005%, 0.01%, 0.05% and 0.1% (g per 100mL of water). For reference, the amount of caffeine found on flower nectar of *Citrus paradisi* corresponded to a concentration of 0.005% (Kretschmar & Baumann 1999).

Experimental procedure

Ant colonies were starved before each experiment began, by removing all food sources except for water. *Lasius niger* were starved for three days and *O. smaragdina* for 24h, as the latter were kept in a greenhouse where a higher temperature led to higher respiration rates and food intake. Sugar solutions were offered to ant colonies in a cafeteria setting, in three different experiments: two where different concentrations of either GABA or caffeine added to sucrose were tested against a sucrose control, and one where two sucrose solutions, one with GABA and one with caffeine, were simultaneously tested against sucrose alone. The solution with 0.005% caffeine was chosen for the third experiment, as it had one of the highest probabilities of being chosen in both species of ants and corresponds with caffeine content of flower nectar in *Citrus spp* (Kretschmar & Baumann 1999). With regards to GABA, the two species significantly preferred different concentrations, and thus we offered 0.005% GABA to *Lasius* and 0.001% GABA to *Oecophylla*. The concentration of sucrose was always 20% (w/v), and each of the experiments was performed twice with *L. niger* and four times with *O. smaragdina.*

Following the simultaneous offering of all sugar solutions (to avoid differences in discovery time), ants were given ten minutes to forage and get familiar with the experimental setting. Then, ant visits to every sugar source were counted for an hour at: (i) five minutes intervals for *L. niger*, whose maximum drinking time is under 160 seconds (Portha et al. 2004) and (ii) three minutes intervals for *O. smaragdina*, for whom maximum drinking time was recorded in pilot experiments at 90 seconds (experimental results not shown). This guaranteed that each visit recorded at subsequent intervals was a different event. A visit was defined as an instance in which an ant was observed drinking from a given food source. Sugar solutions were placed directly on the soil, less than 5cm from a nest entrance opening in *L. niger* nests, while in the case of *O. smaragdina* they were positioned on a Petri dish elevated above water and connected to the plant by several wooden sticks (approximately 20-40 cm from their leaf nests).

After confirming there were no conflicting results among the different repetitions of the same experiment (two for *L. niger* and four for *O. smaragdina*), i.e. that ants did not show opposing preferences in the same experiment performed on different days, data was pooled for overall analysis.

Statistical analysis

Because records of visits of an ant to different choices presented simultaneously are not independent, i.e. the selection of one source will affect the number of visits to the alternatives, we used a Bayesian approach as described below. The total number of ant visits in experiments with *Lasius* was generally much lower than in those with *Oecophylla*, but the conservative nature of the analysis ensures that results are reliable for both species (Peter B. Sørensen, personal communication), i.e. that the significant relationships detected are not artifacts.

To estimate the most likely probability (that in contrast to frequency take potential asymmetries of the Dirichlet distributions into account) for a forager to choose each of the offered solutions, we used the Dirichlet distribution (with k=1), and the following formula:

$$\overline{p_i} = \frac{v_i + 1}{V + n}$$

where V is the total number of visits in the whole experiment (repetitions within each of the three experiments were pooled and total number of visits are provided in Figures 1-2), n is the number of available choices, v_i is the number of visits to source i and p_i is the probability for a visit to source i.

To calculate the probability for one solution to be preferred over another, a resampling process randomly drew $_i$ values from the probability distributions for each solution (estimated by the Dirichlet model), 10^6 times. An R code for this calculation is provided in *Supplementary material 1*. Significant relationships between different solutions were obtained by determining how many times one solution was preferred over another out of 10^6 times, resulting in probability matrices (*Supplementary material 2*) that allowed us to assign superscript letters to each solution that represent significant differences in preference. The model is thoroughly described in Madsen et al. (2017) and was adapted from a similar model used in pollination studies (Sorensen et al. 2011).

RESULTS

GABA concentration experiment

The estimated probabilities for an ant to choose each of the solutions offered in the experiment, for each of the species, can be found in Figure 1. In *L. niger,* the solution containing GABA at a concentration of 0.005% was significantly preferred over all others. Weaver ants also preferred solutions containing GABA over the sucrose control, albeit it favored lower concentrations of GABA than *L. niger*, i.e. 0.001%.

Caffeine concentration experiment

In the caffeine experiment, the solution with the highest probability of being chosen by *L. niger* was 0.01% caffeine, albeit this probability did not significantly differ from that of the sucrose control (Figure 1). In *Oecophylla* ants, the sucrose-only solution had the highest probability of being chosen, although it did not differ significantly from the lowest concentrations of caffeine. In both species, the mixture containing 0.1% caffeine was significantly dispreferred compared to sucrose only.

Preference for GABA and Caffeine

Here, we tested the preferred GABA and caffeine solutions against a sucrose-only control. *L. niger* did not discriminate between the solutions offered, while *Oecophylla* ants showed a significant dispreference for the solution containing caffeine (Figure 2).

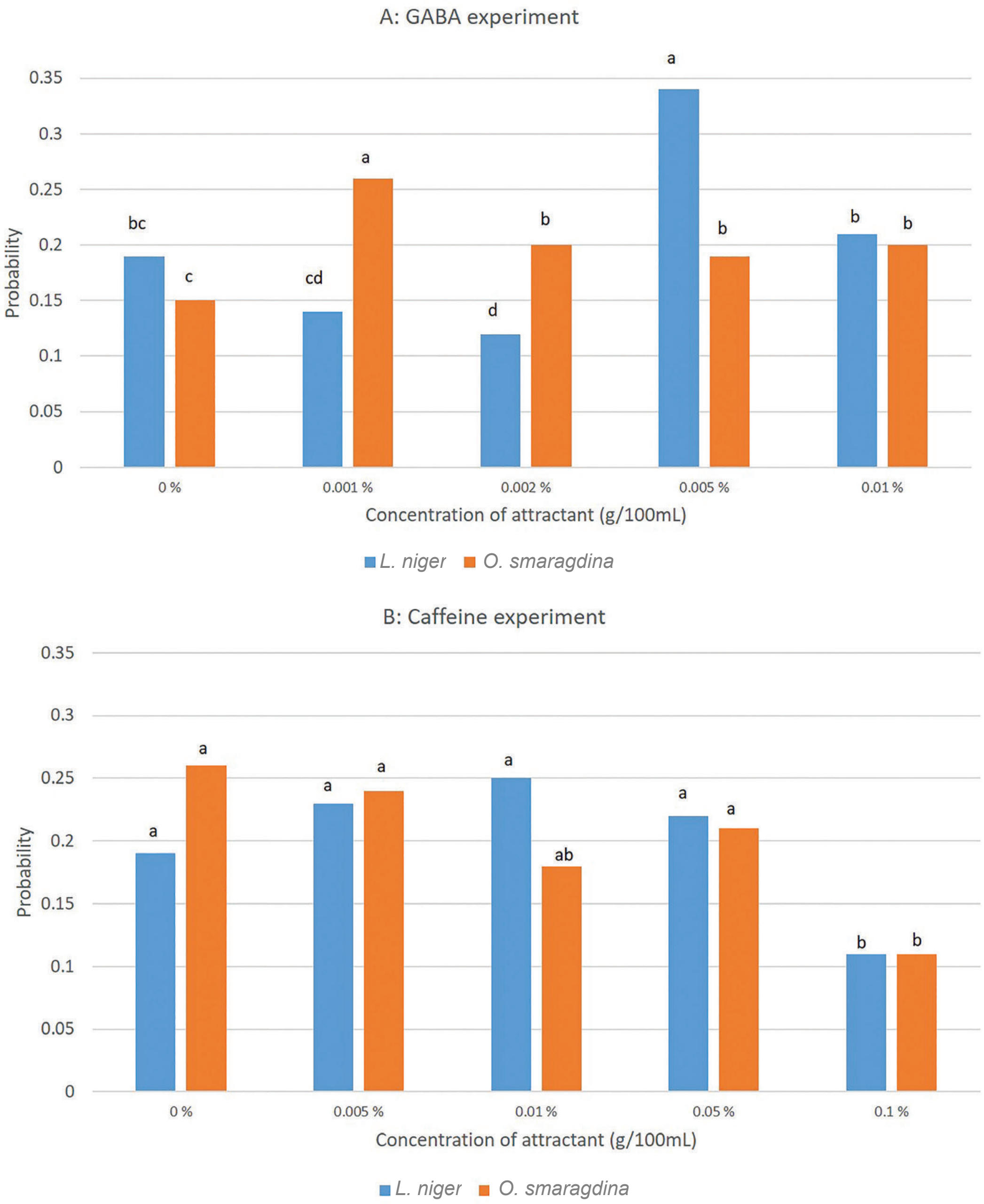

Fig. 1. Estimated mean value for the probability of a foraging ant to choose each of the presented sucrose-based solutions. Within each ant species, values with different letters are significantly different at a significance level of 0.05. A show probabilities at different GABA concentrations (with 199 and 4821 total visits of *L. niger* and *O. smaragdina*, respectively) whereas B show probabilities at different caffeine concentrations (with 314 and 136 total visits of *L. niger* and *O. smaragdina*, respectively).

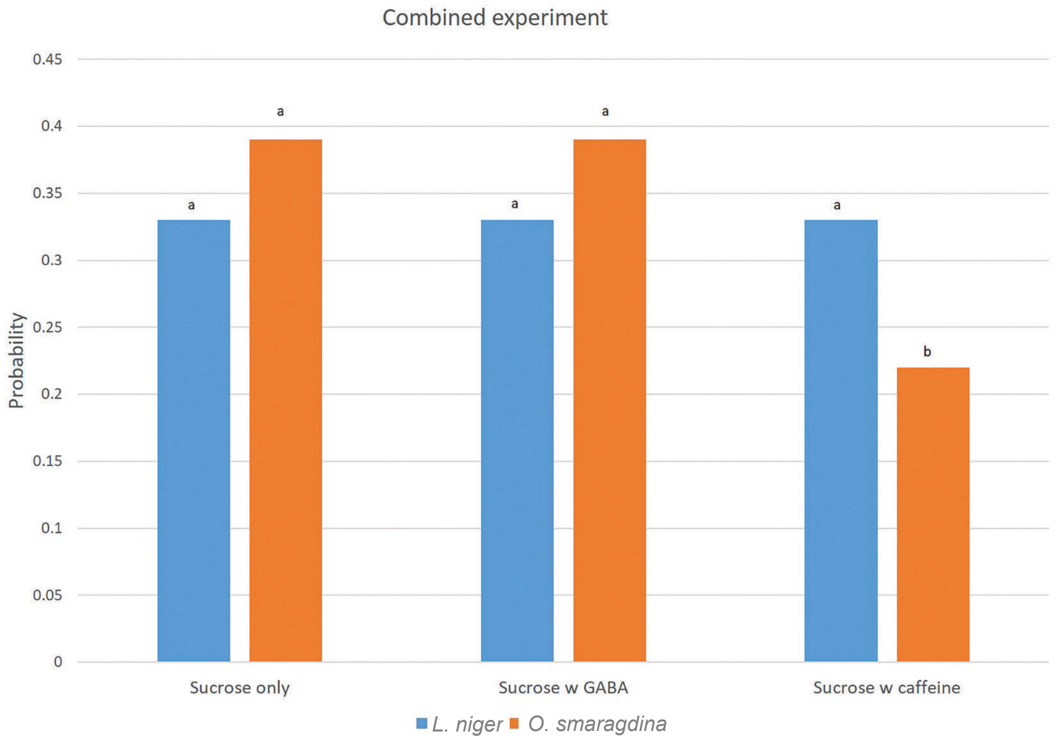

Fig. 2. Estimated mean value for the probability of a foraging ant to choose each of the presented sucrose-based solutions. Within each ant species, values with different letters are significantly different at a significance level of 0.05 (with 185 and 6278 total visits of *L. niger* and *O. smaragdina*, respectively). The caffeine concentration was 0.005 % for both ant species whereas the GABA concentration was 0.005 % in the *L. niger* and 0.001 % in the *O. smaragdina* experiment (we used the concentrations that elicited most visits in the former experiments).

DISCUSSION

Overall, our study revealed that, caffeine is unlikely to work as an attractant for *L. niger* and *O. smaragdina* ants, whereas results on GABA were inconclusive and need further work.

Results from our first experiment showed that both species of ants preferred solutions containing GABA over sucrose controls. A reason for this could be that GABA has the capacity to stimulate taste neurons that are sensitive to sugar, thus causing phagostimulation (Schoonhoven et al. 2005). However, *L. niger* favored higher concentrations of GABA than *O. smaragdina*, speaking to ant preference being species-specific to some degree and they seemingly dispreferred the solution with a GABA concentration of 0.002% (0.19mM). This dispreference, however, was only marginal significant ($p = 0.03$, see Supplementary 2) and could have been a false positive. On the other hand, similar odd choice patterns have been reported in other studies. Singaravelan et al. 2005 observed that honey bees significantly preferred caffeine concentrations of 0.0025% and 0.01% but without showing preference for intermediate concentrations of 0.005%. Similarly, Tiedeken et al. 2014 found that bumblebees dispreferred nicotine concentration of 0.0016 and 0.016% but not higher concentrations of 0.16%. Other insects are known to be attracted to nectars containing GABA including long-tongued bees (Anthophoridae, Andrenidae) and hover flies (Syrphidae)(Petanidou et al. 2006, Petanidou 2007).

In our second experiment, caffeine did not increase the ants' preference for a sucrose solution, in contrast both species significantly dispreferred the highest concentration offered, i.e. 0.1% (5mM). There is a high degree of variability regarding acceptance of caffeine across insect taxa. Studies by Singaravelan et al. (2005) reported honeybees being deterred by caffeine concentrations of 0.015% (0.77 mM) or higher, which lie above the range of caffeine content found in nature (Kretschmar & Baumann 1999, Singaravelan et al. 2005). On the other hand, they found increased attractiveness at the lower concentration of 0.0025% (0.13mM) compared to sucrose-only. Experiments on fruit flies or larvae of gypsy moth *Lymantria dispar* reported deterrence thresholds for caffeine at 0.002% (0.1mM) (Shields et al. 2008, Sellier et al. 2011), a level almost 100 times lower than bumblebees that was only deterred at concentrations of 0.19 % (10mM) (Tiedeken et al. 2014). Our experiments detected aversion only at the highest concentration used, i.e. 0.1% (5mM).

Our study did not test learning and memory, but only gustatory effects/attraction. It should be noted though, that caffeine can affect learning, memory and other behavioral traits in insects. In the ant *Myrmica sabuleti*, 0.1% caffeine has been shown to increase memory and conditioning capability in foragers, who also moved faster and less sinuously after having consumed the alkaloid (Cammaerts et al. 2014). However, because *M. sabuleti* had no alternative to caffeine in that experiment, it is unknown whether this ant would have shown any preference or dispreference for that concentration. Caffeine can also improve cognitive performance in honeybees (Mustard 2014, Strachecka et al. 2014, Stevenson et al. 2017). For example, honeybees rewarded with nectar with caffeine in naturally occurring concentrations (10^{-6} to 10^{-4} M) were three times more likely to remember learned associations of scent and nectar compared to bees that received nectar without caffeine (Wright et al 2013). Due to our experimental design in the present study we are unable to test such potential effects of caffeine and GABA in our focal ant species. *L.* niger's preference for GABA was not confirmed when presented simultaneously with caffeine, in a multi-choice experiment versus a sucrose control. There, foragers fed equally from all available solutions. Colonies of *O. smaragdina* significantly dispreferred the solution containing caffeine but showed no difference in preference between the sucrose-only solution and the one containing GABA. These results contrast with the previous experiments where GABA was tested alone and was significantly preferred over sucrose. Ant choices therefore are inconclusive in the present experiment. One possible explanation for these contrasting patterns could be context-dependence, as also seen in some of our previous laboratory studies where trisaccharides increased the attractiveness of sucrose-only solutions, but not when an amino acid source was added to the mix (Madsen et al. 2017). More work is needed to test if ant attraction to particular formulations depend on what other choices are available simultaneously.

In conclusion, our results indicate that (i) caffeine cannot be used to increase the attractiveness of sucrose solutions in *L. niger* and *O. smaragdina*, whereas (ii) results with GABA were equivocal and suggests that more work is needed on this amino acid. If GABA or other potential gustatory stimulants can be used to attract weaver ants to sugar feeders this may be used to improve the effectiveness of *O. smaragdina* pest control in Asian plantations. *Oecophylla* spp. have a high potential as biocontrol agents in Asia and in the Old World tropics in general. Here they control a high number of insect pests in various plantation crops, often equally or even more efficiently than chemical insecticides (Van Mele 2007, Offenberg 2015). However, there also exist cases where they have proven ineffective due to their positive effects on tended trophobionts. In a Thai mango plantation, e.g., chemical pesticides led to higher yields than pest control by *O. smaragdina* as the ants continued attending honeydew producing leaf hoppers despite being offered an artificial sugar solution (Offenberg et al. 2013). Offering the ants sugar with attractants (Madsen et al. 2017) may make such solutions attractive enough to outcompete hemipteran honeydew. In that case ants may stop attending their trophobionts partners and may even start to prey on them instead, using them as a protein source (Way 1954, Offenberg 2001). This could lead to more effective biocontrol programs in Asia. Similarly, the addition of effective attractants may improve European crops that are plagued by *L. ni-*

ger attended pest aphids. Offering *L. niger* more attractive artificial sugar solutions may reduce the density of their aphid partners (Nagy et al. 2013, 2015, Wäckers et al. 2017), but further research is needed to identify effective attractants, and test how they work in combination with other attractants.

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