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RAPID COMMUNICATIONS

Immediate Effects of Nectar Robbing by Palestine Sunbirds (*Nectarinia osea*) on Nectar Alkaloid Concentrations in Tree Tobacco (*Nicotiana glauca*)

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Abstract Plant secondary metabolites (PSMs), such as alkaloids, are often found in many parts of a plant, including flowers, providing protection to the plant from various types of herbivores or microbes. PSMs are also present in the floral nectar of many species, but typically at lower concentrations than in other parts of the plant. Nectar robbers often damage floral tissue to access the nectar. By doing so, these nectar robbers may initiate an increase of PSMs in the floral nectar. It is often assumed that it takes at least a few hours before the plant demonstrates an increase in PSMs. Here, we addressed the question of whether PSMs in the floral tissue are immediately being released into the floral nectar following nectar robbing. To address this research question, we investigated whether there was an immediate effect of nectar robbing by the Palestine Sunbird (*Nectarinia osea*) on the concentration of nectar alkaloids, nicotine and anabasine, in Tree Tobacco (*Nicotiana glauca*). We found that the concentration of anabasine, but not nicotine, significantly increased in floral nectar immediately following simulated nectar robbing. These findings suggest that nectar robbers could be ingesting greater amounts of PSMs than they would if they visit flowers legitimately. As a consequence, increased consumption of neurotoxic nectar alkaloids or other PSMs could have negative effects on the nectar robber.

Keywords Anabasine · Birds · Foraging · Nicotine · Pollinator · Secondary metabolites

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Introduction

Plant secondary metabolites (PSMs), such as alkaloids, can effectively defend plant tissues against many types of herbivores (Rosenthal and Berenbaum 1992; Bennett and Wallsgrove 1994; Wink 1998, 2010). These compounds may be constitutive, always present in some part of the plant, or their production or allocation to different parts of the plant may be induced upon some form of damage to the plant. According to optimal defense theory, tissues closely associated with plant fitness should have the most defenses (McKey 1974, 1979). In support of this theory, many studies have found that reproductive structures, like flowers, may have more PSMs than vegetative tissues (reviewed in Kessler and Halitschke 2009; but see McCall and Fordyce 2010). Strauss et al. (2004) found that flower petals alone had 20 % more constitutive PSMs than the leaves of *Raphanus sativus*. Moreover, Euler and Baldwin (1996) found that the concentration of nicotine, the predominant PSM in *Nicotiana attenuata*, was highest at the base of the corolla, which surrounds the valuable ovary of the flower. This location is also where floral nectar is typically found.

The assumed primary function of floral nectar is to attract and reward pollinators. However, PSMs are found in the floral nectar of many plant species (Baker 1977; Adler 2000; Irwin et al. 2004), although typically at much lower concentrations than in other plant tissues (Detzel and Wink 1993; Adler et al. 2012; Manson et al. 2012; Cook et al. 2013; Manson et al 2013). Various pollinators may discriminate against nectars containing PSMs (Detzel and Wink 1993; Tadmor-Melamed et al. 2004; Singaravelan et al. 2005; Kessler and Baldwin 2006; Kessler et al. 2008; Köhler et al. 2012), potentially reducing the number and/or duration of their floral visits and affecting plant fitness (Adler and Irwin 2005). These results may suggest that chemical defenses in nectar could be non-adaptive; perhaps simply a consequence of defending other plant parts from herbivores (Adler 2000). However, it is possible that the presence of PSMs in nectar cause pollinators to collect less nectar per flower, but visit more flowers overall, which can increase outcrossing rates or other aspects of plant fitness (Kessler and Baldwin 2006; Kessler et al. 2008). Other adaptive functions for nectar PSMs may also exist (e.g., increase fidelity of specialist pollinators, prevent fermentation of the sugar-rich nectar; see Adler 2000 for review).

PSMs have been shown to deter nectar robbers (Adler and Irwin 2005; Kessler et al. 2008); animals that remove nectar through damaged corollas without contacting reproductive structures and have the potential to reduce plant fitness (Irwin et al. 2001, 2010; Burkle et al. 2007). Primary nectar robbers actively damage the floral tissue in order to access the nectar, while secondary nectar robbers access the nectar through the holes previously made by the primary nectar robbers (Irwin et al. 2010).

The Palestine sunbird (*Nectarinia osea*) is a common pollinator of Tree tobacco (*Nicotiana glauca*) in Eastern Mediterranean regions (e.g., Israel and Sinai), but can also be found piercing the base of their corollas and robbing nectar (Tadmor-Melamed 2004). The floral nectar of *N. glauca* contains PSMs – the alkaloids nicotine and anabasine (Tadmor-Melamed et al. 2004). Sunbirds were found to discriminate against artificial nectars containing each of these alkaloids alone at the average concentrations found in *N. glauca*, in favor of alkaloid-free solutions (Tadmor-Melamed et al. 2004). Therefore, we predict that floral damage, such as that caused by nectar robbing sunbirds, may increase alkaloid levels to even less preferred concentrations, especially since sunbirds are damaging the base of the floral tissue where alkaloid concentration is likely to be highest (Euler and Baldwin 1996). This form of damage not only has the possibility of inducing greater alkaloid allocation to the flowers (Euler and Baldwin 1996), but can also potentially release the alkaloids immediately from the floral tissue into the floral nectar, as suggested by Guerrant and Fielder (1981). However, it is unclear how nectar robbers, in particular, affect PSM concentrations in floral nectar (Irwin et al. 2010).

In the present study, we investigated whether primary nectar robbing by *N. osea* immediately increases alkaloid concentrations in the floral nectar of *N. glauca*. If so, this may suggest not only that PSM levels may immediately increase following nectar robbing, but also that nectar robbers may face a cost while piercing the corolla in the form of increased PSM consumption. This, in turn, may help to explain patterns and frequencies of legitimate flower visitation and nectar robbing in nature, with some possible fitness implications for the floral visitor and the plant.

Materials and Methods

Plants were chosen from an experimental plot of approximately 120 *Nicotiana glauca* specimens growing on the Oranim campus of The University of Haifa in Kiryat Tivon, Israel. These plants were grown from seeds collected from roadside plants in Zichron Ya'acov (30 km away from Kiryat Tivon). The seeds were sown in 10 cm (diameter) pots and housed in a hoop greenhouse with a misting irrigation system until the plants were large enough to be transplanted outdoors, before they started flowering, in August 2012. Plants in the plot were planted approximately 1 m apart from each other, fertilized (with time-releasing Osmocote; Scotts Company, Maryville, Ohio, USA) shortly after transplanting, and drip-irrigated from August to November 2012.

The majority of the plants in this plot were used for a previous experiment involving different types of plant damage. Therefore, only 12 plants were suitable for this experiment when it was conducted in December 2012 because these plants had no previous damage, and also had a sufficient number of flowers. In order to avoid potential pollen contamination or tissue damage when sampling from the narrow floral opening on intact flowers, nectar was collected from excised flowers by gently separating the calyx from the corolla and collecting the nectar pool with a microcapillary tube, taking care to avoid contact with damaged floral tissue (following Kaczorowski et al. 2005; Adler et al. 2012). Previous studies on *N. attenuata*, found that floral excision does not affect nicotine concentrations in other flowers (Baldwin and Karb 1995; Euler and Baldwin 1996). Half of the open flowers on each plant were removed and sampled without damaging the corollas, while the other half of the flowers were first damaged by simulating Palestine sunbird nectar robbing, then removed and sampled immediately. Nectar robbing was simulated by using the beak of a stuffed sunbird to pierce the base of the corollas, where sunbird robbing damage typically takes place. Using a stuffed sunbird to simulate nectar robbing is more controlled since a live sunbird would likely consume, and potentially contaminate, the nectar. Sampling all flowers on a plant was performed as quickly as possible, lasting no more than 10 min per plant. Samples were weighed to determine nectar quantity and a matching amount of methanol was added to each sample for preservation. Samples were stored at −20 °C until chemical analysis.

Chemical Analysis of Alkaloid Concentrations in Nectar Samples Alkaloids were converted to their free bases by adding 50 μl of concentrated ammonia solution to the nectar sample. After saturation with sodium chloride the sample was extracted 4 times with toluol. A defined stream of nitrogen was used to reduce the volume of the combined extracts kept at 40 °C to 50 μl.

The samples were directly analyzed with a HP 5890 Series II Gas Chromatograph coupled to a Finnigan SSQ 7000 Mass Spectrometer. A Zebron ZB-1MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) was used for separation. The injector was operated in splitless mode at 250 °C and 15 kPa helium head pressure. The split valve was opened at 1.5 min after injecting 2 μl of the sample. The temperature program starts with an isothermal step at 40 °C for 2 min. The temperature was then raised to 200 °C at a rate of 8 °C/min, followed by an increased heating rate of 10 °C/min up to 300 °C.

For detection of the alkaloids, the Quadrupol mass spectrometer was operated in SIM mode at m/z=84 and 133 (the typical fragment peaks for these alkaloids) at the retention time of nicotine (15.02 min, RI=1320) and anabasine (17.20 min, RI=1445). For quantification, we compared the areas under the signals of the compounds in the ion chromatogram of m/z=84 and 133 with those of authentic standards.

Statistical Analysis Absolute quantities of nicotine and anabasine in nectar were expressed as parts per million (ppm) based on the amount of sample collected. These results were then natural log-transformed to improve normality. The immediate effects of floral damage on nectar alkaloid concentrations was analyzed by paired *t-test* (SPSS 17.0), comparing the nectar collected from control flowers to the nectar collected from "nectar robbed" flowers within plants for amounts of nicotine and anabasine (ppm, natural log-transformed). Thus, the plant was considered the unit of replication and analysis.

Results

The concentrations of nicotine found in *N. glauca* floral nectar immediately after "nectar robbing" were not significantly different from the concentrations found in the control flowers (Fig. 1a; $t_{11}=-0.499$, $P=0.628$). However, simulated nectar robbing immediately increased the amount of anabasine in the floral nectar, significantly more than in the control flowers (Fig. 1b; $t_{11}=-3.841$, $P=0.003$). Anabasine was more predominant than nicotine in floral nectar (on average, approximately 87 % anabasine and 13 % nicotine), but both alkaloids were at much lower concentrations than we previously found (Fig. 1; see Tadmor-Melamed et al. 2004).

Discussion

Nectar robbing is generally considered to be beneficial for the animal because it is assumed to increase handling efficiency or offer access to a reward that would otherwise be unreachable (Irwin et al. 2010). However, primary nectar robbing does damage floral tissue, and this could potentially induce an increase of PSMs into the flowers (Euler and Baldwin 1996). The present study, the first to our knowledge, shows that floral damage through nectar robbing can immediately increase PSM concentrations in floral nectar. We presume that the effect is probably due to leakage from damaged corolla tissue since systemic responses to damage are known to have delayed effects (Baldwin et al. 1994). These results suggest that sunbirds (or other floral visitors) that choose to rob nectar will likely ingest a greater amount of PSMs than they would if they chose to visit flowers legitimately. PSM ingestion can have negative effects on animal fitness (Manson and Thomson 2009; Köhler et al. 2012), potentially due to the neurotoxic effects of certain alkaloids, such as those found in *Nicotiana* (Wink 1998). Therefore, greater costs may be associated with a foraging strategy that involves nectar robbing.

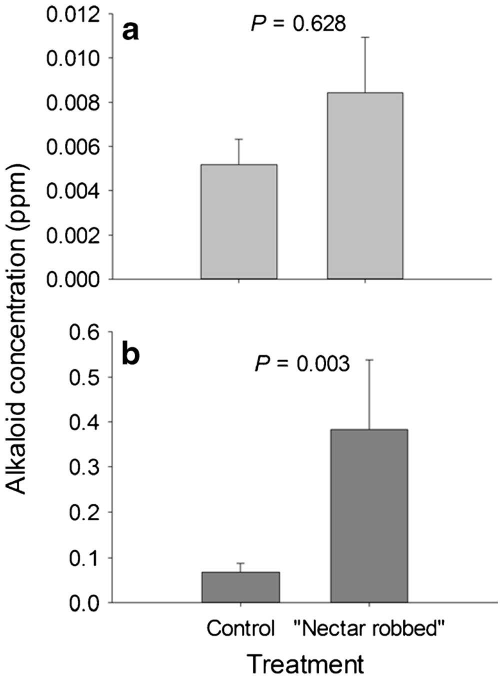

Fig. 1 Concentration (mean ± SE, in ppm) of alkaloids in the floral nectar of control flowers and "nectar robbed" (simulated with a stuffed Palestine sunbird) flowers of *Nicotiana glauca* ($n=12$ plants). **a**) Nicotine and **b**) Anabasine

Although there was no significant change in the concentration of nicotine in the nectar immediately after simulated nectar robbing, there was a significant increase in the amount of anabasine in the nectar. Anabasine is much more prevalent in *N. glauca* nectar than is nicotine (Fig. 1; Tadmor-Melamed et al. 2004). Singaravelan et al. (2005) found that sucrose solutions with naturally occurring concentrations of anabasine, but not nicotine, as found in *N. glauca*, were deterrent to honeybees (*Apis mellifera*). Both anabasine and nicotine can be strong agonists at the nicotinic acetylcholine receptor (nAChR), potentially affecting central nervous system activity (Wink et al. 1998). Nevertheless, anabasine does seem to have greater potency than nicotine (using human fetal muscle nAChR) because it has a lower half maximal effective concentration (EC_{50}; an average of 2.6 or 7.1 μM for (*R*)- or (*S*)-anabasine enantiomers, respectively, compared to approximately 26 μM for (*S*)-nicotine; Lee et al. 2006). Anabasine was also found to be the teratogenic compound responsible for causing birth defects in the offspring of large mammals that consumed *N. tabacum* or *N. glauca* plant parts (Keeler et al. 1984), while nicotine did not induce malformations (Crowe 1978; Keeler 1979).

Tadmor-Melamed et al. (2004) found average nicotine and anabasine concentrations of 0.5 and 5.0 ppm, respectively, in the floral nectar of *N. glauca*. Surprisingly, our average baseline nicotine and anabasine concentrations were considerably lower at approximately 0.005 and 0.07 ppm, respectively. We cannot fully explain this inconsistency. However, it has been shown that nicotine concentration in *N. attenuata*, a related species, can be highly variable across populations, plants, and even flowers on the same plant (Kessler et al. 2010). In addition, there may have been substantial differences in the amount of nutrients available to the plants in the different experiments since nutrient availability can have significant effects on plant alkaloid concentrations (Gondola 2002; Adler et al. 2006). Even though we found more than a 5-fold increase in nectar anabasine immediately following simulated nectar robbing, average anabasine concentration increased to only about 0.38 ppm. This is below the concentration that was found to be indiscernible from alkaloid-free nectar solutions in terms of sunbird foraging (0.6 ppm; Tadmor-Melamed et al. 2004). However, even this lower anabasine concentration (0.6 ppm) was found to reduce gut transit time and sugar assimilation efficiency in the sunbirds (Tadmor-Melamed et al. 2004). Therefore, even our relatively low anabasine concentrations found in the nectar immediately after simulated nectar robbing could have significant physiological effects. The combined effects of anabasine and nicotine could also be greater than that shown for each of the alkaloids alone. Moreover, if plant alkaloid concentrations were higher, as shown previously, a potential 5-fold increase could have considerable effects on the nectar-robber, physiologically or behaviorally. We plan to explore the potential effects of these alkaloids on sunbirds in future studies.

Numerous studies have explored the effects of different types of damage on alkaloid concentrations in different plant parts of *Nicotiana* species. These studies have revealed that leaf damage can increase the amount of alkaloids produced in a plant, increasing the concentration in the leaves (*N. sylvestris*; Baldwin et al. 1994) and flowers (*N. attenuata*; Baldwin and Karb 1995), but allocation may depend upon ontogeny (*N. sylvestris*; Ohnmeiss and Baldwin 2000). It was also found that the alkaloids in floral tissues (corollas) can increase with both leaf and flower damage (*N. attenuata*; Euler and Baldwin 1996) and nectar alkaloids can increase in response to leaf herbivory (*N. tabacum*; Adler et al. 2006). All of these studies have focused on delayed effects of damage. Although it has been suggested that the deterrent chemicals in floral tissues may contaminate nectar following floral damage (Guerrant and Fielder 1981), this has not been shown experimentally.

An extreme form of floral damage can result from florivory, where an animal consumes all or parts of the flower. Florivory has been shown to have negative effects on pollinator visitation or other aspects of plant reproductive fitness, such as reduced pollen transfer and lower fruit or seed set (Krupnick and Weis 1999; see McCall and Irwin 2006 for review). However, florivory could also increase PSMs in flowers (Euler and Baldwin 1996) that may help to deter florivores that attempt to feed on the flowers later in the season (McCall 2006). Primary nectar robbing may have similar effects on plants as florivory because it involves damaging floral tissue, although to a lesser extent.

Nectar robbing was found to be relatively common in the Palestine sunbird population (on average about 40 % of floral visits; Tadmor-Melamed 2004) in our study area, where we also grew the *N. glauca* plants. Further, in Southern double-collared sunbirds (*Cinnyris chalybea*) about 60 % of floral visits involved nectar robbing (Geerts and Pauw 2009). However, other sunbird species may nectar rob very infrequently, as found in dusky sunbirds (*Cinnyris fuscus*, less than 10 % of floral visits) or, perhaps, as in malachite sunbirds (*Nectarinia famosa*), not at all (Geerts and Pauw 2009). The nectar robbing sunbirds may face the cost of increased PSM concentrations in the floral nectars they consume. There is very little evidence for additional costs to nectar robbers, as it is generally assumed that only the plant has the potential to experience negative effects in this interaction (Irwin et al. 2010). Nectar robbing may result in positive, negative or neutral effects on plant fitness (reviewed in Maloof and Inouye 2000), but meta-analyses have found that, overall, nectar robbing tends to have more negative effects on plant fitness (Burkle et al. 2007; Irwin et al. 2001). Nectar robbing can negatively affect plant fitness through many potential mechanisms; such as damaging reproductive structures, altering resource allocation, reducing pollen production and/or quality, or indirectly influencing pollinator visitation (reviewed in Irwin et al. 2010). Depending upon the type of nectar robber and the plant species being robbed, nectar may remain in the flower following nectar robbing (see Maloof and Inouye 2000). Pollinators collecting this remaining nectar may be affected by the immediate release of PSMs into floral nectar after nectar robbing, potentially decreasing plant fitness. The frequency of nectar robbing on plants in nature can range from 0 to 100 % depending upon the plant species, location, year or season (Irwin and Maloof 2002). It is not always clear which factors lead to this extensive variation. The increase in nectar PSMs following nectar robbing could potentially contribute to the variation in nectar robbing frequency, depending upon factors such as PSM type and concentration, as well as the sensitivity of the animal.

This study demonstrates that floral damage, even the minor damage produced by a nectar robber, can have immediate effects on the alkaloid concentrations in the nectar. Further studies are needed to determine whether this increase in alkaloid concentrations has significant effects on the nectar robber, other floral visitors or plant fitness.

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