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# FEEDING RESPONSES OF FREE-FLYING HONEYBEES TO SECONDARY COMPOUNDS MIMICKING FLORAL NECTARS

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**Abstract**—The role of secondary compounds (SC) in deterring herbivores and pathogens from vegetative parts of plants is well established, whereas their role in plant reproductive organs such as floral nectar is unclear. The present study aimed to reveal the response of free-flying honeybees to naturally occurring concentrations of four SC in floral nectar. We selected nicotine, anabasine, caffeine, and amygdalin, all of which are found in nectar of various plants. In repeated paired-choice experiments, we offered 20% sucrose solution as control along with test solutions of 20% sucrose with various concentrations of the above SC. Except for anabasine, naturally occurring concentrations of SC did not have a deterring effect. Furthermore, low concentrations of nicotine and caffeine elicited a significant feeding preference. SC can, therefore, be regarded as postingestive stimulants to pollinators, indicating that the psychoactive alkaloids in nectar may be a part of their mutualistic reward. Further studies are needed to test our hypothesis that psychoactive alkaloids in nectar impose dependence or addiction effects on pollinators.

**Key Words—**Nectar, secondary compounds, naturally occurring concentrations, honeybees, attraction, deterrence.

## INTRODUCTION

Many studies have focused on elucidating the role of secondary compounds (SC) in deterring herbivores and pathogens from plants (e.g., Rosenthal and Berenbaum, 1991). Others have determined the costs and benefits of producing

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these compounds in the context of plant–herbivore interactions (Van Dam et al., 1996; Agrawal et al., 1999). The current concept is that the wide varieties of SC produced by higher plants play a multifunctional role in the complex biotic and abiotic interactions of plants (Izhaki, 2002; Harrison and Baldwin, 2004; Holopainen, 2004). The myriad challenges that plants face seem to promote natural selection for SC that possess multiple functions (Wink, 1999; Adler et al., 2001; Gronquist et al., 2001; Izhaki 2002). Although the role of SC in deterring herbivores and pathogens is well established (Karban and Baldwin, 1997), their role as mediators of plant–pollinator mutualistic relationships has been widely overlooked (Adler, 2000).

SC are not uncommon in floral nectar. Depending on the specific compound, SC have been found in 9–55% of surveyed species (Baker and Baker, 1975; Baker, 1977, 1978). The nectar of some plants may be deterrent or even toxic to floral visitors (Adler, 2000), and widespread "toxic nectar" is puzzling considering its attractive role in pollination (Faegri and van der Pijl, 1979). The deterrence and toxicity of SC in nectar may be activated through unpalatability or through an effect on the central nervous system of the flower visitors (Baker and Baker, 1975). Consequently, SC in nectar may mediate plant–pollinator interactions affecting the fitness of both plants and pollinators (Baker, 1977).

Several adaptive hypotheses have been proposed to explain the ecological and evolutionary roles of SC in nectar (Rhoades and Bergdahl, 1981; Adler, 2000). The most common claims that SC deter nectar robbers and generalists, or inefficient pollinators. Baker and Baker (1975) suggested that the level of tolerance to SC in nectar by pollinators is related to their efficiency in transferring conspecific pollen. This "pollinator fidelity" hypothesis holds that the nontolerant pollinators are also less efficient in transferring conspecific pollen in comparison to pollinators that are more tolerant to SC (Adler, 2000). However, toxic nectar may have no adaptive function but instead be a consequence of production and mobilization of SC in other plant tissues (Adler, 2000).

Most SC studied so far (e.g., alkaloids, glycosides, phenolic substances) actually deter bees (*Apis mellifera*) within a wide range of high concentrations (Detzel and Wink, 1993). The effects of SC on bees are dose- and season-dependent (e.g., Singaravelan et al., 2006). Low concentrations of phenolic substances such as caffeic and genistic acids elicited preference, whereas high concentrations deterred honeybees (Hagler and Buchmann, 1993). Likewise, bees preferred low concentrations of amygdalin during early summer but not later (London-Shafir et al., 2003). Some alkaloid-containing nectars attracted bees in the field even when alternative nectar sources were available (Ish-Am and Eisikowitch, 1998). This circumstantial evidence indicates that bees cope with naturally occurring concentrations of SC in nectar. Despite evolutionary and ecological implications, the interaction between bees and SC in nectar has not

been widely studied. Specifically, this study was designed to test the responses of honeybees to natural concentrations of SC in nectar, with *a priori* prediction that the latter will not impose strong deterrent effects.

In repeated paired-choice experiments with artificial nectar, we studied feeding preferences of free-flying honeybees (*A. mellifera*) under natural conditions. We offered the bees artificial nectar of 20% sucrose solution as a control, simultaneously with test solutions of 20% sucrose containing various concentrations of four SC. We tested nicotine and anabasine, naturally occurring in the nectar of *Nicotiana* spp. (Detzel and Wink, 1993; Tadmor-Melamed et al., 2004), caffeine that is most common in the nectar of *Citrus* spp. (Kretschmar and Baumann, 1999), and amygdalin that characterizes almond (*Amygdalus* sp.) nectar (London-Shafir et al., 2003). We examined the effects of naturally occurring concentrations of these SC on foraging behavior of honeybees.

## METHODS AND MATERIALS

*Experimental Arena and Training Procedures.* We conducted the experiments on a flat rooftop of a building in the Oranim campus of University of Haifa, Israel, between January and April 2004. Hourly temperatures were recorded with a maximum–mininum thermometer. We conducted the experiments only when the ambient temperature was above 18°C. Honeybees were trained to feed on sucrose solutions (20% sucrose) from 250-ml translucent plastic beakers (6.5-cm diam). The mouth of the solution-filled beakers was covered with a Petri dish (8.6-cm diam and 1.3 cm deep) and turned upside down. The bees fed from the nectar trough formed around the beaker's mouth. Nectar spontaneously filled the trough whenever its level dropped below the mouth. This feeder allowed 70–80 bees to feed simultaneously. Each feeder was placed on a colored plastic plate (14-cm diam) that was placed on a white plastic tray (36 $\times$ 26 cm) on the floor. We started by training bees to feed from a 20% sucrose solution in one station. Then, we split them into five separate groups, each feeding from a feeder that was placed on different colored plate. Later, we gradually separated five feeders about 20 m apart. In a preliminary experiment, we marked 50 bees at each feeding station and monitored their visits for about a week to ensure that they established independent feeding groups. Indeed, about 85% of the bees fed only in the feeding station where they were marked, whereas only some (<15%) were observed also in another feeding station.

*Secondary Compounds.* We tested the bees' response to four SC: nicotine (Aldrich Ltd), anabasine, caffeine, and amygdalin (Sigma Ltd). We chose these SC because honeybees frequently visit flowers and feed on the nectar of *Nicotiana* spp., *Citrus* spp., and *Amygdalus* sp. that contain them, and their natural concentration in floral nectar is known (Table 1).

TABLE 1. NATURALLY OCCURRING CONCENTRATIONS OF SC AND THE CONCENTRATIONS TESTED IN EXPERIMENTS I AND II OF THE PRESENT STUDY

| Secondary compounds | Plant species | Naturally occurring concentration in nectar (ppm) | References | Concentration tested in the present study (ppm) | |
|---|---|---|---|---|---|
| | | | | Experiment I | Experiment II |
| Nicotine | *Nicotiana tabacum* | 0.166[a] | Detzel and Wink (1993) | 2.5, 5, 10, 20 | 0.5, 1, 2, 5 |
| | *Nicotiana glauca* | 0.56 ± 0.12 (0 to 2.5) | Tadmor-Melamed et al. (2004) | | |
| Anabasine | *Nicotiana tabacum* | 0.166[a] | Detzel and Wink (1993) | NT | 2.5, 5, 10, 25 |
| | *Nicotiana glauca* | 5.4 ± 0.90 (0 to 50) | Tadmor-Melamed et al. (2004) | | |
| Caffeine | *Citrus paradisi* | 94.26 ± 2.90 | Kretschmar and Baumann (1999) | 50, 100, 150, 200 | 12.5, 25, 50, 100 |
| | *Citrus maxima* | 17.61 ± 0.97 | | | |
| | *Citrus limon* | 11.61 ± 0.39 | | | |
| Amygdalin | *Amygdalus communis* | 4 to 10 | London-Shafir et al. (2003) | 5, 10, 25, 50 | 2.5, 5, 7.5, 10 |

Natural concentration: mean ± SE; range, when available, is given in parentheses.
NT: not tested.
[a]Total alkaloid concentration.

*Food Preference Trials.* At each feeding station, we offered the bees simultaneously one feeder with a control solution (20% sucrose) and one feeder with a test solution (20% sucrose with a known concentration of one SC). In each experimental session, only one SC was tested, and in each experimental trial, the same concentration of the same SC was tested against the control in all five feeding stations. Thus, each test was replicated five times. We preferred the paired-choice design, as it is a compromise between multiple-choice tests, which simulate the natural situation but suffer from lack of independence among observations, and single-choice tests, which often underestimate preferences because of the lack of real choice (Manly, 1993). In the first experiment, we examined the response of bees to a wide range of concentrations (experiment I, Table 1) to obtain concentration–response information. This enabled us to determine the threshold minimal deterring concentration for each SC. In the second experiment, we repeated the same experimental design testing the range of naturally occurring concentrations of each SC (experiment II, Table 1).

To determine the consumption rate, we weighed (Precisa Instruments Ltd, Switzerland, electronic balances) each feeder before and after 1 hr of bee feeding. Simultaneously with the control solution, a particular concentration of SC test solution was offered in all stations simultaneously for 1 hr. The tested concentrations were changed after each hour. Whenever we changed the tested concentrations, we also changed the relative position of the control and test feeders randomly on the plastic tray to shun any possible association of any solution type with a certain position by bees. Each experimental session that tested a range of concentrations of a SC lasted 3–5 consecutive days. Between any two experimental sessions, we had an average time lag of 5 d, during which we offered only control solutions to the bees. On each experimental day, we tested all concentrations of a particular SC for its selected range. We changed the order of presentation of the various concentrations during each experimental day.

To detect correlations between number of bees and consumption rate of test solutions, we counted the number of bee visits in each station with a tally counter and stopwatch. Bees were counted for 1 min at the control feeder and 1 min at the tested solution feeder.

*Data Analyses.* We considered each experimental station as an independent replicate, as >85% of marked bees remained feeding only in one station. Thus, we obtained five replicates for each concentration of each SC. We calculated the percentage differences in food intake and in the number of bees per minute between control and experimental solutions for each preference trial and each station. We averaged the differences for the three experimental days. We analyzed these differences by two-tailed one-sample $t$ test. One-way ANOVA was used to detect effects of SC concentrations on food consumption followed by Tukey's multiple comparison test ($P < 0.05$). We related the number of bees that visited the feeders and the consumption rate of test solutions with Pearson's

correlation. All proportions were arcsin-square-root-transformed prior to statistical analyses for normal distribution. Results are presented as mean ± SE.

## RESULTS

*Experiment I—Wide Range of Concentration Series.* In this experimental series, the bees were not deterred from naturally occurring concentrations of nicotine, caffeine, and amygdalin (Figure 1). On the contrary, bees consumed these concentrations more than that of the control solutions. Bees were deterred by concentrations of nicotine and caffeine that were higher than natural concentrations in nectar (>2.5 and >100 ppm, respectively). The deterrence effect tended to increase with concentration by an order of magnitude. Notably, bees were not deterred by any of the tested amygdalin concentrations. The relative differences in consumption of treated solutions per hour from that of control varied significantly across concentrations for all SC except for amygdalin (Figure 1).

*Experiment II—Natural Range of Concentration Series.* Of the four SC tested within their natural range of concentration, bees significantly preferred the lower concentrations of nicotine and caffeine over the control. They were significantly deterred by three of four concentrations of anabasine. Although bees consumed more amygdalin at all tested concentrations than controls, the differences were not significant (Figure 1). This preferential intake of experimental solutions was significant for 0.5 and 1 ppm of nicotine and for 25 ppm of caffeine. Moreover, in this experiment, bees were not deterred by any of the tested concentrations of caffeine and amygdalin. The consumption of tested solutions relative to that of controls varied significantly across concentrations for all SC except amygdalin (Figure 1).

*Consumption Rate vs. Number of Bees.* We found a positive and significant correlation between number of bees feeding and consumption rate across concentrations and SC (nicotine: $R = 0.89$, $N = 40$, $P < 0.001$; caffeine: $R = 0.85$, $N = 40$, $P < 0.001$; amygdalin: $R = 0.40$, $N = 40$, $P < 0.01$; anabasine: $R = 0.82$, $N = 20$, $P < 0.001$). The number of bees feeding on the tested solutions relative to control solutions showed a similar pattern to that of the relative consumption of the solutions and is, therefore, not presented here.

## DISCUSSION

*Preference vs. Deterrence.* Honeybees use multiple cues to identify food. They associate and/or memorize many chemical stimuli with sucrose to recognize or discriminate among differing mixtures of reward (Jakobsen et al.,

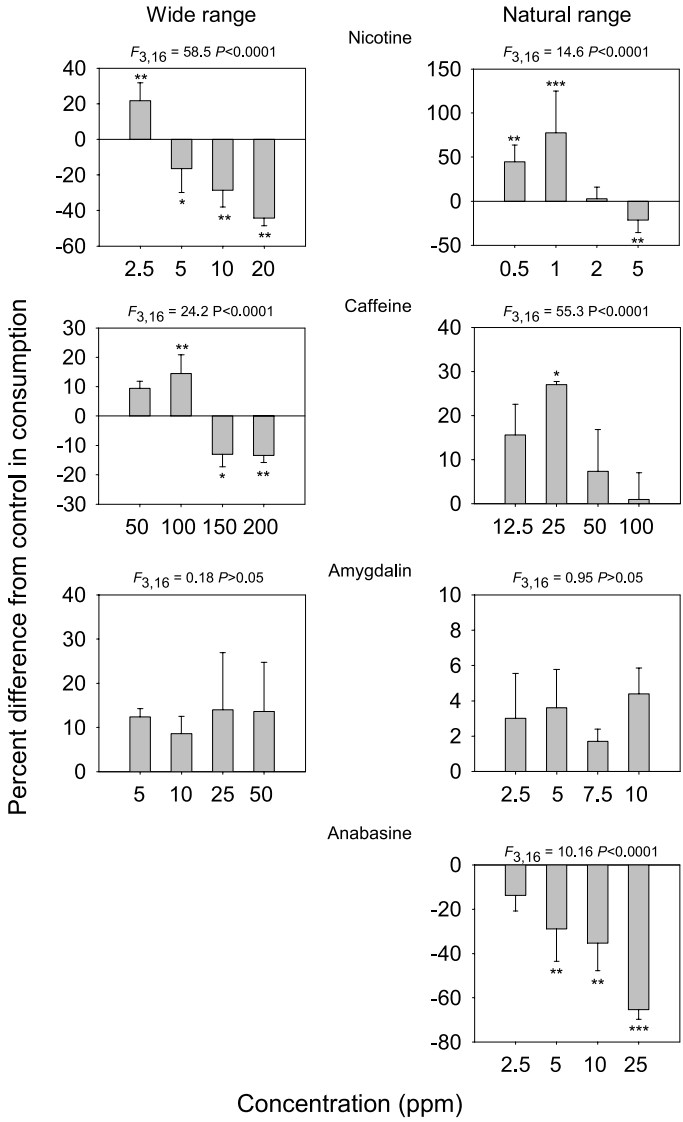


FIG. 1. Responses of *Apis mellifera* to various concentrations of four secondary compounds in artificial nectar of 20% sucrose. The relative differences (%) in nectar intake of the test solutions from controls were subjected to one sample, two-tailed $t$ test, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Bars represent mean ± SE; positive bars indicate preference, and negative bars indicate deterrence. The effects of concentrations are presented as $F$ and $P$ values of one-way Anova.

1995; Laska et al., 1999; Paldi et al., 2003). For foraging bees, natural concentrations of SC are associated with artificial reward imitating natural floral nectar. Our results indicate that except for the strong deterrent effect of anabasine, which acts as a selective nicotinic acetylcholine receptor (nAChR) agonist for insects (Sultana et al., 2002), honeybees were not deterred by nicotine, caffeine, or amygdalin in their natural range of nectar concentrations. Furthermore, honeybees significantly preferred solutions with low concentrations of nicotine and caffeine over control (20% sucrose) solution. A similar but nonsignificant pattern was detected also for all concentrations of amygdalin (Figure 1). When offered a wide range of concentrations, bees consumed nicotine and caffeine solutions only within their natural concentration range and were deterred by higher concentrations. For amygdalin, bees were not deterred even by higher concentrations. It appears that some SC in nectar may furnish foraging cues to mutualistic pollinators as has been hypothesized for fruits and their frugivores (Cipollini and Levey, 1997).

Bees appeared to modulate their response to the differing concentration spectra of SC, as they chose the lower concentrations of nicotine and caffeine in both ranges. Such modulatory and/or differential responses across different concentration spectra are known for nectar compounds in general (Masson et al., 1993; Menzel, 1993) and SC in particular (London-Shafir et al., 2003). Nonetheless, the differential responses cannot be an outcome of choice behavior or side bias by bees to the feeders during choice experiments, as we noted a consistency in deterrence response to concentrations deviating from the natural range of nicotine and caffeine.

*Response to Nicotine.* The biphasic, dose-dependent response in nicotine intake might be the result of a dual motivational effect of nicotine (Laviolette and van der Kooy, 2004), rewarding (preference) at low concentrations on the one hand and aversive (deterrence) at higher concentrations on the other. Nicotine acts on endogenous nAChR prevalent in the central and peripheral nervous systems in almost all animal species (Laviolette and van der Kooy, 2004). The highly inducible nicotine, which acts as a feeding deterrent to herbivorous insects, can be found in vegetative plant parts in relatively high (300–5000 ppm) concentrations (Ohnmeiss and Baldwin, 2000). Our results demonstrated that honeybees were deterred even by much lower concentrations (≥5 ppm) of nicotine in sucrose solution.

Low concentrations of nicotine act as positive reinforcers both through intravenous and oral self-administration (Halladay et al., 1999; Laviolette and van der Kooy, 2004). Nicotine drinking has induced active nicotine preference in rats (Halladay et al., 1999). In insects, the actions are likely to be CNS-specific, where they appear to play a major excitatory role (Wolf and Heberlein, 2003). Repeated exposure to nicotine enforces subsequent neuronal changes in the mesolimbic dopamine system of the brain, which, in turn, evokes com-

pulsive nicotine-seeking behaviors in mammals (Laviolette and van der Kooy, 2004). Although the mechanism in insects has yet to be elucidated, there are some indications that invertebrates such as the nematode *Caenorhabditis elegans* (Schafer, 2004) and insects such as *Drosophila* (Bainton et al., 2000) adopt addictive behaviors when exposed to low concentrations. Given this, it is paramount to test rigorously whether nicotine in nectar imposes dependence or addiction effects on pollinators. The mammalian literature on addiction characterizes it as a progressive increase in preferential intake of psychoactive substances despite its toxic effects and/or even after deprivation of drugs over a stipulated period (Heyne and Wolffgramm, 1998). However, addiction (if any) to substances such as nicotine in nectar by pollinators needs to be studied in detail. It should be noted that natural concentrations of nicotine (Table 1) do not affect the fitness of caged honeybees (Singaravelan et al., 2006).

*Response to Anabasine.* The bees in our experiments were deterred even by naturally occurring concentrations of anabasine. Thus, we cannot rule out the possibility that certain SC at their natural concentrations deter honeybees. Indeed, anabasine is a selective nAChR agonist for insects with insecticidal activity at relatively high concentrations (Sultana et al., 2002) and an effective antifeedant (Gonzalez-Coloma et al., 2004). Nonetheless, anabasine and nicotine are both constituents of *Nicotiana* nectar; anabasine is the predominant compound in *N. glauca*, whereas nicotine is the major one in *N. tabacum* (Bush and Crowe, 1992). Notably, honeybees visit only the flowers of the latter. It would be of interest to study the response of bees to combinations of nicotine and anabasine simulating the natural situation.

*Response to Caffeine.* Caffeine acts as a mild reinforcer and psychostimulant to mammals such as rats (Vitiello and Woods, 1977). In contrast, it may function as an antifeedant to insects (Bernays et al., 2000), although it is not efficiently effective against insect pests of coffee (Guerreiro and Mazzafera, 2000). Caffeine in relatively high concentrations is deterrent ($ED_{50}$ at 300 ppm) and even toxic to honeybees ($LD_{50}$ at 2000 ppm; Detzel and Wink, 1993). In our study, bees preferred caffeinated 20% sucrose solutions, within its natural concentration range in nectar, over control 20% sucrose (Table 1). In natural situations, honeybees collect caffeine containing *Citrus* nectar (Ish-Am and Eisikowitch, 1998) and even prefer it to alternative nectar resources. Moreover, in Israel, during winter, when nectar resources are limited, honeybees often forage in trash bins on sweetened Coca-Cola (personal observations) that contains 103 ppm of caffeine (http://www.coca-cola.com; accessed 24 March 2005). These factors may help bees in dealing with caffeine in floral nectars.

*Response to Amygdalin.* Bees showed a nonsignificant higher intake of amygdalin-laced 20% sucrose solutions than 20% sucrose controls in both natural and wider concentration ranges. A previous study showed a variable seasonal response of honeybees to amygdalin. The intake of amygdalin-laced sucrose

varied with the availability of other seasonal nectar sources (London-Shafir et al., 2003). The cyanogenic glycoside amygdalin also did not have strong deterrent effect on folivorous orthopterans (Bernays, 1983), but reduced food intake in two noctuid caterpillars (Glendinning and Slansky, 1994). The preference and performance of a frugivorous cedar waxwing bird (*Bombycylla cedrorum*) were not affected by even high concentrations of amygdalin (Struempf et al., 1999).

*SC in Nectar vs. Pollen.* Detzel and Wink (1993) found that bees were deterred by many SC, but mostly at higher concentrations that we tested. Our concentration range was based on naturally occurring concentrations in floral nectar, whereas Detzel and Wink (1993) examined higher ones that occur mainly in pollen. However, foraging bees probably do not encounter in nectar high $ED_{50}$ concentrations of SC (mentioned in their study). From an evolutionary perspective, to increase fitness, plants might have evolved higher concentrations of SC in pollen to deter pollen eaters and lower concentration in nectar to increase attractiveness to pollinators.

*Possible Mechanisms.* We found that honeybees can discriminate well among various concentrations of SC (Figure 1), as reported earlier (Hagler and Buchmann, 1993; London-Shafir et al., 2003). Such discrimination might be based on the universally bitter taste of alkaloids (Kingsbury, 1964). How do honeybees overcome this unpalatability? The presence of carbohydrates (sugars and sugar alcohols) can "mask" the unpleasant taste of some SC to herbivorous insects (Glendinning, 2000), as carbohydrates inhibit the response of deterrent taste cells (Shields and Mitchell, 1995). It appears that sucrose (20%) might have masked the unpalatable nature of low concentrations of nicotine and caffeine. Further studies should reveal the full spectrum of this tradeoff by evaluating the bee's responses to various concentrations of SC in various concentrations of sugar.

*Ecological and Evolutionary Implications.* As predicted, naturally occurring concentrations of nectar SC do not have a strong deterrent effect on bees (with the exception of anabasine); rather, some low concentrations of nicotine and caffeine even significantly stimulate them. Although honeybees are generalist pollinators, a few *Nicotiana* sp., *Citrus* spp., and *Amygdalus* spp. depend on bees for pollination (Detzel and Wink, 1993; Kretschmar and Baumann, 1999; London-Shafir et al., 2003). Thus, our results provide some support for the "pollinator fidelity" hypothesis, as honeybees are not deterred by SC and were even stimulated by the natural concentrations of nicotine and caffeine mimicking nectar. Notably, nicotine and caffeine are not restricted to nectars of *Nicotiana* spp. and *Citrus* spp. These alkaloids are distributed in nectars of other plant species (Naef et al., 2004). Thus, further studies should focus on the hypothesis that plants produce these compounds in nectar to "addict" faithful pollinators. Many insects are addicted to SC (Boppré, 1999; Renwick and

Lopez, 1999; Renwick, 2001), and plants may use SC to mediate various insect–plant relationships by a method of differential allocation of SC concentrations to different plant parts (Harborne, 1993; Boppré, 1999). Thus, SC in nectar may govern the selection of the best mutualistic partners. The prediction of a pollinator fidelity hypothesis remains to be studied.

In summary, pollinators are stimulated by a variety of constituents in nectar at substance-specific spectra of concentrations. They are stimulated mainly by substances such as sugars and amino acids to fulfill their energetic and nutritional demands (Baker and Baker, 1975) and are controlled by taste thresholds (Gardener and Gillman, 2002). They are also stimulated by essential oils (Detzel and Wink, 1993) and other volatiles/scents mediated by olfactory sense (Heinrich, 1979). These may be considered as "preingestive stimulants." In a similar manner, some SC, particularly the psychoactive alkaloids in nectar, may act as "postingestive stimulants" mediated possibly by their concentration-specific rewarding (pleasuring) effects on flower visitors. Conceivably, a considerable number of alkaloids in nectar (e.g., nicotine, caffeine, cannabinoids) have both addictive and aversive properties and have not yet been studied in an ecological context. It is a question of considerable interest whether preferential intake of low concentrations of nicotine and caffeine could impose dependence or addiction effects on bees.

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