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THE EFFECTS OF NECTAR–NICOTINE ON COLONY FITNESS OF CAGED HONEYBEES

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Abstract—Nectar of many bee flowers contains secondary compounds, which are considered toxic for honeybees on repeated exposure. Although many anecdotal reports indicate the toxicity of secondary compounds to bees, only a few studies have tested the extent of toxicity at different honeybee ages, especially at the larval stages. Honeybees encounter nicotine at trace concentrations (between 0.1 and 5 ppm) in floral nectar of a few *Nicotiana* spp. and in *Tilia cordata*. Adult honeybee workers tolerate these nicotine concentrations. In controlled nonchoice feeding experiments with caged bees, we investigated the effect of nicotine on hatching success and larval and forager survival. Naturally occurring concentrations of nectar–nicotine did not affect hatching success of larvae or their survival, but the latter was negatively affected by higher concentrations of nicotine (50 ppm). Concentrations of nicotine in fresh honey samples from the hives were 90% lower than the concentrations in the offered experimental sucrose solutions. Our results indicate that honeybees can cope with naturally occurring concentrations of nicotine, without notable mortality, even when consumed in large quantities for more than 3 weeks.

Key Words—Nectar, secondary compounds, nicotine, *Nicotiana* spp. honeybees, toxicity.

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INTRODUCTION

Nectar of several plants contains secondary compounds (hereafter SC), which are suspected to be toxic to bees and other pollinators (Baker and Baker, 1975). The prevalence of SC in bee nectars has given rise to the hypothesis that bees are less susceptible to SC than are flower-inconstant butterflies (Baker and Baker, 1975). Conceivably, bees developed counteradaptations to SC (Rhoades and Bergdahl, 1981). Still, it is unclear whether bees consume SC deliberately or only for lack of alternative food sources. The actual concentrations of SC in nectar are known for only a few plants, yet, they are frequently reported to be toxic to honeybees (Adler, 2000). Detzel and Wink (1993) tested a wide array of nectar SC for their toxic (LD_{50}) effects on adult honeybees and found that most SC (particularly alkaloids) are highly toxic to bees across a wide range of concentrations.

Most reports on toxicity of SC to honeybees have not addressed the extent of toxicity in relation to the age of the bees. Toxic nectar may not affect forager bees but may still be toxic to brood or young nursing bees (Sharma et al., 1986). Conversely, anecdotal accounts indicate that SC may be fatal for foraging bees (Adler, 2000). Accordingly, it is important to test the age-related toxicity of SC on bees as they undergo clear-cut transitions in tasks and physiological plasticity with age (Smirle and Winston, 1988). The shift from the protected hive environment to the open field exposes honeybees to numerous challenges, such as pesticides and SC in nectar. The bees' ability to withstand exposure to such toxicants may be a critical factor determining colony foraging performance (Smirle and Winston, 1988). Plant SC and pesticides have been shown to induce detoxifying enzymes in honeybees (Terriere and Yu, 1974).

Nicotine is encountered by foraging honeybees in floral nectar of some *Nicotiana* species at concentrations between 0.1 and 5 ppm (Detzel and Wink, 1993; Tadmor-Melamed et al., 2004). Although birds and Lepidoptera are the typical pollinators of most *Nicotiana* species, honeybees also consume nectar of *N. tabacum* (Bhuiyan et al., 2002; Oddo et al., 2004) and possibly of *N. sylvesteris* (Jakobsen et al., 1995) and *N. suaveolens* (Loughrin et al., 1991, 1993). Honeybees also actively collect nectar containing nicotine from *Tilia cordata* (Naef et al., 2004; the precise concentration is not known).

Detzel and Wink (1993) assessed the toxicity of nicotine in sucrose solutions for caged bees after an exposure of 48 hr. They found that nicotine was toxic to adult bees at LD_{50} concentration of 2000 ppm, a dosage that foragers probably never experience in nectar. Lower concentrations (<300 ppm) were tolerated for over a week without increased mortality (Detzel, 1990). Free-flying honeybees tolerated and even prefer nicotinated sucrose solutions at their naturally occurring concentration in nectar (Singaravelan et al., in press). Forager bees unload the collected (toxic) nectar to young workers in the hive,

who process and store it as honey, but they also feed it directly to young developing larvae. An effect can, therefore, be expected even in hive-bound adult bees and the developing brood. Developing larvae were more sensitive to such toxic constituents in the food than adult workers (Sharma et al., 1986; Miranda et al., 2003; Amir and Peveling, 2004), perhaps because of the low level of induced physiological specialization they have to manage. A remarkable increase was found in the activities of detoxification enzymes (glutathione *S*-transferases and the mixed-function oxidases) in forager bees. The detoxifying power proved positively correlated with workers' age (Smirle and Winston, 1988). Hence, because toxic nectar is also consumed by the developing brood (larvae), they should be more susceptible to it than adult worker bees.

Here, we investigated the effect of natural concentrations of nectar–nicotine on the hatching success of bee larvae and on survival of larvae and forager bees (*Apis mellifera ligustica*). Knowing the effect of nicotine at the colony level is essential for understanding the interactions between honeybees and nicotine-containing plants.

METHODS AND MATERIALS

Maintenance of Caged Hives. Minibeehives (400–2000 bees) of four-frame strength were obtained from an apicultural farm (Noga Reuven, Manoot, Israel). In the fall, experimental hives were randomly assigned to four nylon-meshed outdoor cages (3 × 3 m, allowing natural environments) erected at the Oranim Campus of the University of Haifa, Israel. Each beehive was introduced into a cage, and the bees were offered sucrose solution (20%) and pollen on Petri dish plates. In addition, pollen substitute (Bee-Pro^{R}) was provided *ad libitum* on plastic bowls placed inside the hives. To prevent ants from gaining access to test solutions, we applied tangle foot at the base of the table on which the solutions were provided. We routinely removed mites (*Varrora* spp.) and caterpillars of lesser wax moth (*Achroia grisella*) from the experimental colonies.

Experimental Procedure. The work schedule is shown in Table 1. To investigate the direct effect of nicotine, after 1 d of adjustment, the bees in each cage were fed exclusively with one of the following solutions: control (20% sucrose) or treated 20% sucrose laced with three concentrations of nicotine—0.5, 5, and 50 ppm. To trace the fate of individual eggs, after 10 d of bees' exposure to the test solutions, we mapped all frame cells on drawn sheets. We marked the cells of 15–30 new eggs randomly on two or three frames each day for 3 d using color dyes. We marked the cells of 75 (± 10 SD) new eggs in each colony. The developmental stage of each individual was classified as follows: "egg 1" to "egg 3" for 3 d in the egg stage; "L1d" through "L5d" for a 5-d larval period; and "CL" for capped larvae. We monitored the marked cells until

TABLE 1. WORK SCHEDULE WITH CAGED HIVES

Days	Tasks[a]
Day 1	Acclimatizing bees to the cage environment
Days 2–10	Exposure session
Days 11–14	Mapping, marking cells of new eggs
Days 15–17	Monitoring the cells for egg hatching
Days 18–26	Monitoring the cells for larval survival (capping)

[a]Note that during the entire session, the hives were exposed to a single source of nectar.

the egg became a capped larva (usually about 9–10 d after the "egg 1" stage). Disappearances of eggs and larval death were noted regularly. To test the effect of nicotine concentrations on survival of forager bees, we numbered a minimum of 20–25 foragers in each colony in all replicates, and these were monitored for about 15 d. When our experiments were completed, we returned the hives to the apicultural farm. This setup was repeated four times (between August and November 2004), each time with new beehives that were randomly placed in the cages. In two cases, we terminated the experiment early, as the bees remained outside the hive. Therefore, the 5- and 50-ppm nicotine treatments had only three replicates.

Nicotine Concentration in Fresh Honey Samples. To determine the concentrations of nicotine in stored honey, we collected fresh honey samples from the combs with a blunt-tipped syringe. The samples were dried by a speed-vac (VR-Maxi, Heto, Allerod, Denmark) and kept at −20°C. Methanol (150 or 250 μl) was added to each of the dried samples, and after vortexing, the samples were centrifuged at 13,000 rpm for 5 min. Some 50 μl of the supernatant was derivatized, and the following solutions were sequentially added: 25 μl 4 M acetate buffer (pH 4.7); 10 μl 1.5 M potassium cyanide in water; 10 μl 0.4 M chloramine-T in water; 50 μl 50 mmol l^{-1} thiobarbituric acid in water/acetone (50:50, v/v). The contents were mixed and incubated for 5 min; the reaction was stopped by the addition of 10 μl 0.1 M sodium metabisulfite in water.

High-performance liquid chromatography (HPLC) analysis was performed exactly 3 min after the reaction had stopped. The HPLC configuration (HPLC, Beckmann system gold, Beckmann, Fullerton, CA, USA) for determination of nicotine consisted of an HPLC pump (Beckmann 125P) connected to a photodiode array detector (Beckmann 168; wavelength, 505 nm). The mobile phase–linear gradient was water/acetonitrile from 0 to 100% acetonitrile in 15 min (flow rate, 1 ml min^{-1}). The column used was Merck LiChroCART RP-18 (5 μm, 250 × 4 mm; Merck, Darmstadt, Germany). The injection volume was 20 μl. The column was equilibrated for 3 min prior to each injection. Concentrations of nicotine were determined by calibration curves by using standards at concentrations between 0.3 and 50 ng μl^{-1}.

Data Analysis. The differences in the percentages of larval hatching and larval survival between the control and the different treatment groups and the differences in percent mortality among larval instars exposed to 50-ppm nicotine were analyzed with one-way analysis of variance on arcsine square-root-transformed data, followed by Tukey's multiple comparison mean separation test.

RESULTS

Foraging Activities and Survival of Forager Bees. Bees collected the test solutions until the end of the experiment except in the colonies exposed to 50-ppm nicotine; there, the bees reduced their usual flight activities as well as their food uptake after 15 d. We monitored the survival of foragers with minimal sample size (as it is already known that foragers tolerate nicotine up to 300 ppm without increased mortality; Detzel, 1990) for about 15 d and found that more than 80% of the bees survived (Table 2).

Hatching Success. Larvae hatched from about 70% of the eggs in all colonies. Exposures to nicotinated sucrose solutions did not affect the hatching success ($F_{3,10} = 2.858$, $P > 0.05$; Figure 1A). A slight nonsignificant reduction in hatching was found in the 50-ppm treatment (Figure 1A).

Larval Survival. Naturally occurring concentrations of nicotine (0.5 and 5 ppm) in nectar did not affect larval survival. About 70% of larvae survived. However, 50-ppm nicotine reduced larval survival 30% more than other concentrations ($F_{3,10} = 28.23$, $P < 0.001$; Figure 1B). Mortality rates differed significantly among larval instars ($F_{4,10} = 20.95$, $P < 0.001$) exposed to 50-ppm nicotine. High mortality occurred mainly in 3-d-old larvae (Tukey's multiple comparison, $P < 0.05$; Figure 2).

Concentrations of Nicotine in Fresh Honey Samples. On average, there was more than a 90% reduction in concentrations of nicotine in honey samples compared with the concentrations of the test solutions offered. The average concentration of nicotine traced in honey samples of colonies treated with 50-ppm nicotine was 3.23 ± 0.41 ppm (in wet mass; mean ± SE; $N = 15$), whereas

TABLE 2. PERCENT SURVIVAL OF NUMBERED FORAGERS MONITORED IN DIFFERENT COLONIES (POOLED DATA)

Colonies	Foragers monitored	Foragers survived	Percentage
Control	79	68	86.1
0.5 ppm	84	72	85.7
5 ppm	63	51	80.9
50 ppm	58	49	84.5

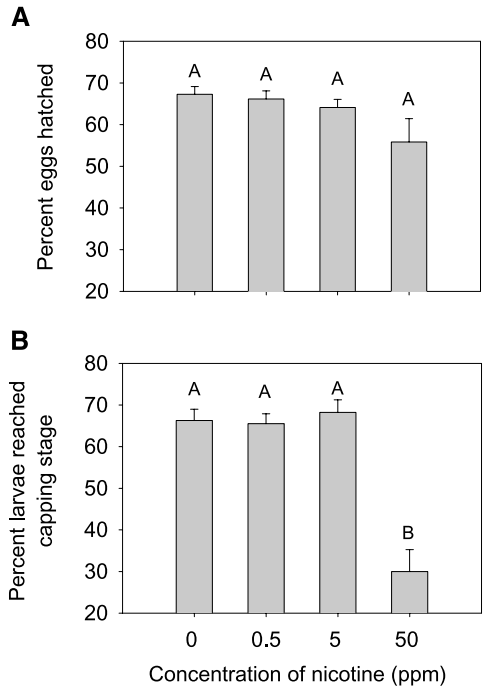

FIG. 1. Effect of nicotine consumption on honeybees. (A) Hatching success; (B) larval survival ($P < 0.05$, Tukey's multiple comparison). $N = 4$ hives with 0- and 0.5-ppm nicotine and $N = 3$ hives with 5- and 50-ppm nicotine. Values given as mean ± SE. Bars followed by different letters are statistically significant.

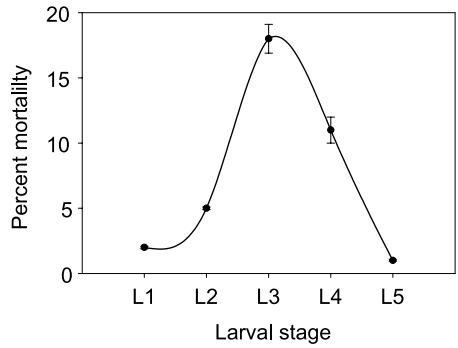

FIG. 2. Percent mortality (mean ± SE) of different larval instars in the colonies exposed to 50 ppm of nicotine.

concentrations in honey samples of colonies treated with 0.5- and 5-ppm nicotine were hardly detectable.

DISCUSSION

The results indicate that bees' consumption of nicotine in its naturally occurring concentrations in floral nectar does not adversely affect hatching success and larval survival, even in the absence of alternative nectar sources for more than 3 wk. However, the effect of nicotine was dose-dependent, and a higher concentration (50 ppm) significantly reduced larval survival (Figure 1). Adult forager honeybees proved unaffected at any of the tested nicotine concentrations. Previously, we found that free-flying bees tolerated and were even stimulated by naturally occurring concentrations of nicotine in artificial nectar (Singaravelan et al., 2005). Adult forager honeybees may escape the acute toxic effects of nicotine because they unload the collected nectar almost completely to receiver bees immediately upon returning to the hive (von Frisch, 1965). By contrast, nectarivorous sunbirds that consume naturally occurring concentrations of nicotine were adversely affected (Tadmor-Melamed et al., 2004). Unlike honeybees, birds immediately digest the consumed nectar (Tadmor-Melamed et al., 2004) and, hence, are more liable to suffer negative effects.

Baker and Baker (1975) suggested that the level of tolerance to SC in nectar by pollinators is related to their pollination efficiency. In our experiments, we used the European race of bees (*A. mellifera ligustica*), which may encounter nicotine naturally in nectar of *Nicotiana tabacum* (Detzel and Wink, 1993) and *T. cordata* (Naef et al., 2004). Although these plants are native to tropical America and Europe, respectively, they have been introduced and are cultivated in many areas, including Israel. General pollinators usually serve invasive plants in similar strategies that are used in their natural habitats (Richardson et al., 2000). Accordingly, interpreting the reciprocal adaptations of different flower visitors to tolerate nectar–nicotine in respective host plants is more complex and entails several lines of considerations. For just a few, this can be illustrated by the following questions: Is a particular pollinating species more tolerant or better adapted to nectar–nicotine concentrations in areas where it has coevolved with the host plant than in areas where it has not? If the host plant has an array of pollinating species in a given area, how does the ability of the different species to tolerate the nectar–nicotine level vary? Most nectar SC have proved toxic for honeybees across a wide range of concentrations (Detzel and Wink, 1993). Nicotine is toxic for adult workers at LD_{50} concentration of 2000 ppm (Detzel and Wink, 1993). Detzel and Wink (1993) chose a high concentration range probably because bees also encounter the SC in pollen, in which their concentrations are many times higher than in nectar (Detzel

and Wink, 1993; Kretschmar and Baumann, 1999; London-Shafir et al., 2003). Because high concentrations of nicotine and other SC cause severe toxicity in forager bees, it has been suggested that they are not especially adapted to cope with them (Detzel and Wink, 1993). However, if the bees encounter SC only in nectar, it is reasonable to consider the toxic nature of SC only within the concentration range actually found in nectar. We focused on nicotine concentrations actually found in floral nectar of *Nicotiana* spp. because it is unknown whether the bees collect and consume the pollen of these plants. If they collect both nectar and pollen of a particular plant that contains SC, one has to consider the concentration ranges of SC of both. Pollen is vital for plant reproduction, so the higher concentrations of SC in pollen may reflect a differential allocation providing high protection from being consumed.

What factors may enable larvae to tolerate naturally occurring concentrations of nicotine? In nature, bees collect nectar from various plants, which may be mixed in the hive to reduce the concentrations of SC. In our experiment, they had no alternative nectar source, but only the experimental test solutions. We found a drastic reduction (>90%) in the concentration of nicotine in honey as compared with the consumed experimental solutions. This is consistent with previous observations on reduction in concentrations of SC, such as amygdalin (50% reduction) and caffeine (90% reduction) in honey (including fresh honey samples) compared with the nectar (Kretschmar and Baumann, 1999; London-Shafir et al., 2003). The actual mechanisms whereby bees reduce SC concentrations in honey are still unknown. Larvae are fed with honey containing reduced SC concentrations. Moreover, induction of detoxifying enzymes known in larval bees (Nielsen et al., 2000) should help to cope with SC toxicity.

In summary, our study demonstrates that naturally occurring concentrations of nicotine in nectar do not affect colony fitness of honeybees, even when such nectar has been consumed for nearly 3 wk and no alternative nectar was offered. However, higher concentrations of nicotine cause high larval mortality. Further studies are essential for clearer insights into the effect of SC in nectar and pollen on both adult honeybees and developing brood regarding their differential susceptibility and/or physiological resistance. In the future, it will be important to study what concentration spectra of different SC the bees encounter in both nectar and pollen and what concentration spectra of these SC they can handle at all developmental stages.

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