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Honeybee (*Apis cerana*) Foraging Responses to the Toxic Honey of *Tripterygium hypoglaucum* (Celastraceae): Changing Threshold of Nectar Acceptability

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Abstract To investigate honeybee foraging responses to toxic nectar, honey was collected from *Apis cerana* colonies in the Yaoan county of Yunnan Province, China, during June, when flowers of *Tripterygium hypoglaucum* were the main nectar source available. Pollen analysis confirmed the origin of the honey, and high-performance liquid chromatography showed the prominent component triptolide to be present at a concentration of 0.61 μg/g± 0.11 SD. In cage tests that used young adult worker bees, significantly more of those provided with a diet of *T. hypoglaucum* honey mixed with sugar powder (1:1) died within 6 d (68.3%) compared to control groups provided with normal honey mixed with sugar powder (15.8%). Honeybees were trained to visit feeders that contained honey of *T. hypoglaucum* (toxic honey) as the test group and honey of *Vicia sativa* or *Elsholtzia ciliata* as control groups (all honeys diluted 1:3 with water). Bees preferred the feeders with normal honey to those with toxic honey, as shown by significantly higher visiting frequencies and longer imbibition times. However, when the feeder of normal honey was removed, leaving only honey of *T. hypoglaucum*, the foraging bees returned to the toxic honey after a few seconds of hesitation, and both visiting frequency and imbibition time increased to values previously recorded for normal honey. Toxic honey thus became acceptable to the bees in the absence of other nectar sources.

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Introduction

Apart from sugars and amino acids, secondary compounds such as phenolics, alkaloids, and terpenoids are of widespread occurrence in nectar (Baker and Baker 1982; Nicolson and Thornburg 2007). Thus far, studies of toxic nectars have focused on the possible fitness benefits to the plants that produce them (Adler 2000) rather than on the sensitivity of natural floral visitors to the nectars. Rhoades and Bergdahl (1981) argued that the ability of specific pollinators to tolerate toxic or repellent compounds would serve as a coevolutionary mechanism to exclude nectar thieves and encourage legitimate pollinators. Adler's (2000) review of toxic nectar emphasized that there have been, first, many studies on the chemistry of secondary compounds in nectar and, second, many demonstrations of nectar toxicity to honeybees (in which the responsible compounds are usually not identified). Less common, however, are studies in a third category: those that make the link between chemistry and toxicity by presenting nectar toxins in sucrose solutions to pollinators in the field (Stephenson 1982; Masters 1991; Hagler and Buchmann 1993; Singaravelan et al. 2005; Liu et al. 2007).

The efficient recruitment behavior of honeybees ensures that good nectar sources are exploited rapidly: colonies focus on the best nectar sources in a large area by working relatively few patches at any one time and making frequent adjustments to the number of foragers at those patches (Visscher and Seeley 1982). The response of bees to a particular nectar depends on the ecological context, especially other available nectar sources (Gegear et al. 2007). This is an important consideration for experiments that involve feeder choices in a natural environment. In a classic study, Lindauer (1948) showed that the threshold sucrose concentration for eliciting recruitment behavior in honeybees declined from ~55% w/w during the main nectar flow in spring to around 3.5% in mid-summer when bee forage became scarce and competition was intense.

During periods of dearth, colonies of honeybees have a considerably more restricted range of plant choices, and in the course of the seasons, they are inevitably forced by floral circumstances to readjust their threshold of nectar acceptability. It is in this context of natural dearth in southern China that honeybees, *Apis cerana*, forage on the toxic nectar of a perennial vine, *Tripterygium hypoglaucum* (Level.) Hutch (Celastraceae). Powdered roots of this plant, also known as Lei Gong Teng ('Thunder God Vine'), have been used in traditional Chinese medicine for 2,000 years, and the main active ingredient is a potent diterpenoid, triptolide (Zhen et al. 1995; Qiu and Kao 2003; Brinker and Raskin 2005; Brinker et al. 2007). In this paper, we report the results of experiments on the foraging sensitivity of honeybees to the honey of this plant and its toxicological effects, and assess whether the bees alter their nectar acceptability threshold under conditions of dearth.

Methods and Materials

The toxic honey of *T. hypoglaucum* was collected from five *A. cerana* colonies in Yaoan county, Yunnan Province, China in June 2006, when *T. hypoglaucum* was one of only very few plants providing nectar for foraging insects. Pollen grains from both *T. hypoglaucum* flowers and the honey derived from this plant species were examined microscopically to confirm their common origin.

Determination of Triptolide The concentration of triptolide in honey derived from *T. hypoglaucum* was measured by using high-performance liquid chromatography (HPLC), preceded by a solid-phase extraction step. A 25-g sample of toxic honey was placed in a centrifuge tube with 40 ml water, and when it was completely dissolved, 40 ml ethyl acetate and 30 ml petroleum ether were added to the honey solution. The sample was treated ultrasonically to accelerate dissolution and then centrifuged for 10 min at 3,000 rpm. Finally, the supernatant was transferred to another centrifuge tube and was re-extracted with 30 ml ethyl acetate and 20 ml petroleum ether. The supernatant was concentrated in a rotary evaporator, and the residue was redissolved in ethyl acetate and filtered with 20 ml ethyl acetate through a neutral aluminum column, which was immersed in ethyl acetate. The eluant was collected and concentrated to dryness, then dissolved in 1 ml methanol and filtered through a 0.45-μm millipore membrane.

For standard solutions, 2.5 mg of triptolide (Medical Research Institute, Fujian Province, China, Fig. 1) were dissolved in 5 ml methanol to obtain a 0.5 mg/ml solution and kept at 4°C. Chromatographic conditions were as follows: Symmetry® C18 column (4.6×150 mm, 5 μm), acetonitrile–water (30:70) as the mobile phase, flow rate of 1 ml/min, detection at a wavelength of 218 nm, and sample size of 20 μl. The same method was used to test for triptolide in the honey of *Vicia sativa* L. (Fabaceae) and *Elsholtzia ciliata* (Thunb.) Hyl. (Lamiaceae): three samples were tested for each species.

Cage Tests To establish toxicity of the *T. hypoglaucum* honey samples, six groups of 40 young adult worker bees were collected randomly from one beehive and placed into each of six cages. A test group of three cages were given toxic honey as food and a control group of three cages received normal honey as food. Honey and powdered sugar were mixed (1:1) to make the cake to feed the bees. Cages were kept in an incubator at 25°C and 70% relative humidity. The number of dead bees in each cage was counted three times a day for 6 d.

Feeder Experiments Honeybee foraging preferences for normal and triptolide honey were tested in a series of experiments. A feeder containing 30% w/w sucrose solution was placed on a small square of blue board, 15×15 cm, at the entrance of an *A. cerana* observation hive. When a few foraging workers had been lured to it and had begun to imbibe the solution, the feeder and bees were moved to a site 5 m away. After having consumed

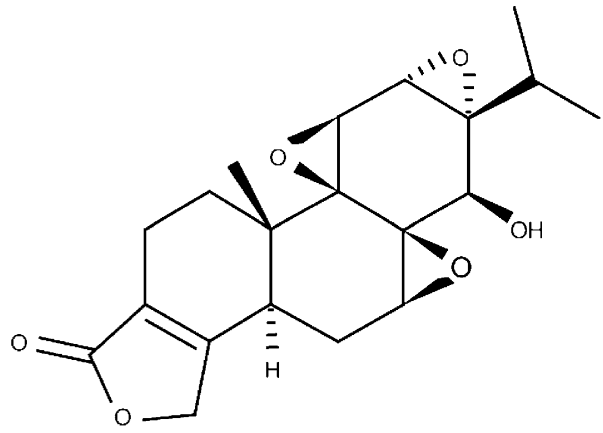

Fig. 1 Structure of triptolide

sufficient sucrose solution, these bees returned to their hive, and about 15 min later, additional, newly recruited foragers arrived at the feeder. At this point, we began to use paired feeders (A and B), which were placed at the same site. Visiting frequencies (the number of bees visiting in 10 min) and imbibition times of individual bees (sec) were recorded at both feeders. The ambient temperature was around 25°C during these tests.

Initially, both feeders (A and B) contained the same 30% sucrose solution. Then, the position of the feeders was reversed, and recordings continued for three more exchanges of position to assess whether there might be a position bias in the bees' responses. We then compared honeybee responses to the toxic honey of *T. hypoglaucum* and the nontoxic normal honeys of *V. sativa* and *E. ciliata* (all diluted 1:3 with water). We also compared the responses to 30% sucrose solution, with and without added triptolide at a concentration of 0.6 μg/g. This triptolide concentration is higher than in the diluted honey of *T. hypoglaucum*, but additional toxic compounds are likely to be present in the honey. All test solutions were of a similar sugar concentration, thus excluding effects of viscosity on imbibition time. Finally, we simulated the dearth conditions under which the bees actually forage on *T. hypoglaucum* by removing the normal honey and measuring responses to *T. hypoglaucum* honey alone.

Statistical Analysis Data are presented as mean±SD. Pearson χ^2 tests were used to test for significant differences in the percentage mortality of worker bees between the control and test groups. Log-linear *G* test analysis was used to test for homogeneity of mortality rates within the groups (Sokal and Rohlf 1995).

Analysis of variance (ANOVA) procedures with dependent samples (feeders A and B) were used to test for significant group effects in the visiting frequencies and imbibition times of foragers. Levene's test was used to test for homogeneity of the variances, and heterogeneity of the variances was stabilized after a square–root transformation of the visiting frequencies. Scheffé post-hoc multiple comparison tests were used for significant group effects. In one comparison, small sample size necessitated the use of a Mann–Whitney *U* test. All tests were performed by using Statistica© (StatSoft 2006).

Results

Determination of Triptolide Pollen grains from both *T. hypoglaucum* flowers and the honey derived from these flowers were examined, and it was confirmed microscopically that the honey had the same pollen grains as the flowers.

In the HPLC tests, 20, 10, 5, 2, and 1 μl of the authentic triptolide solution (0.5 mg/ml) were run to obtain the standard chromatogram for the pure compound. Using the peak area as the ordinate and concentration as the abscissa, a regression equation (y=2,511,038x+ 165,934, r=0.999) was obtained demonstrating linearity over the range of 1–20 μg/g. Repeated HPLC analyses of the authentic toxin established that the relative standard deviation (RSD) of the peak area was 2.63%. Samples (20 μl) of the toxic *T. hypoglaucum* honey were injected three times, with a 30-min run. The results showed that the retention time and the peak area were both stable; the RSD of the peak area was 0.61%. Triptolide was present in honey at a concentration of 0.61±0.11 μg/g (n=5) but was absent from the honeys of *V. sativa* and *E. ciliata*.

Cage Tests The toxicity of *T. hypoglaucum* honey was reflected in the numbers of dead bees in each cage. The results showed that significantly more worker bees in the test group (toxic honey) died within 6 d (68.3±25.0%) compared to the control group with nontoxic *E. ciliata* honey, where only 15.8±12.3% died (χ^2=67.9, *df*=1, *P*<0.001). The mortality rates were homogeneous among the cages in the control group (*G* test: G^2=4.5, *df*=2, *P*=0.107), while in the test group (*G* test: G^2=15.3, *df*=2, *P*<0.001), two cages were homogeneous, but in one cage a large number of bees (22) died on the sixth day.

Feeder Experiments When both feeders A and B contained 30% sucrose, Scheffé post-hoc results showed no significant differences in the visiting frequencies and imbibition times of foragers visiting the feeders (number of visits in 10 min: A=145.1±41.9, B=143.7±42.1, *N*=9 replicates; imbibition times: A=26.2±10.2 sec, B=27.8±13.6 sec, *N*=20 bees; Scheffé: *P*>0.05). This establishes that there was no position bias in the worker bees.

Subsequent tests measured selectivity in the foraging behavior of worker honeybees for the test groups (*T. hypoglaucum*, sucrose solution with triptolide) and control groups (*V. sativa* and *E. ciliata* honeys, sucrose solution only). ANOVA showed significant differences in the visiting frequencies and imbibition times of foragers between the control and test groups (number of visits: $F_{1,\ 24}$=72.3, *P*<0.001, Fig. 2; imbibition times: $F_{1,\ 115}$=43.0, *P*<0.001, Fig. 3) and significant group and food type interaction effects (number of visits: $F_{4,\ 24}$=13.1, *P*<0.001, Fig. 2; imbibition times: $F_{4,\ 115}$=6.6, *P*<0.001, Fig. 3).

Post-hoc test results showed that visiting frequencies to the feeders with normal honey were significantly higher than to those with toxic honey (*V. sativa*=275.3±75.6 bees per 10-min period, *T. hypoglaucum*=54.2±29.4, and *E. ciliata*=304.0±179.3, *T. hypoglaucum*=118.8±75.8, all *N*=6; Scheffé: *P*<0.001, Fig. 2). Likewise, the imbibition times of foragers at the feeders with normal honey were longer than at those with toxic honey (*V. sativa*=24.6±

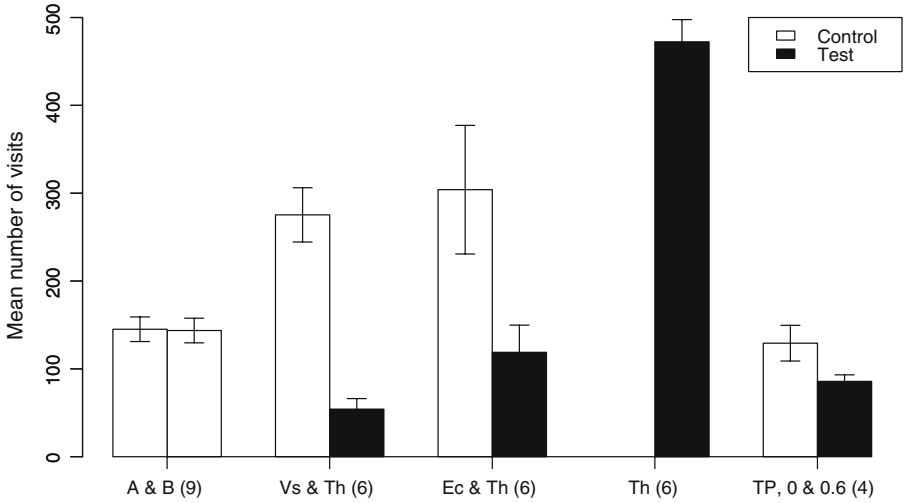

Fig. 2 Bee visits to feeders (10-min counts, means±SE, number of replicates in *parentheses*). In all cases, the control food is shown by *clear bars* and the toxic food by *black bars*. *A & B* Identical feeders containing 30% w/w sucrose solution, *Vs & Th* honeys of *Vicia sativa* and *Tripterygium hypoglaucum*, *Ec & Th* honeys of *Elsholtzia ciliata* and *Tripterygium hypoglaucum*, *Th* honey of *Tripterygium hypoglaucum.* All honeys were diluted 1:3 with water. *TP, 0 & 0.6* 30% sucrose solution and the same solution containing 0.6 μg/g triptolide

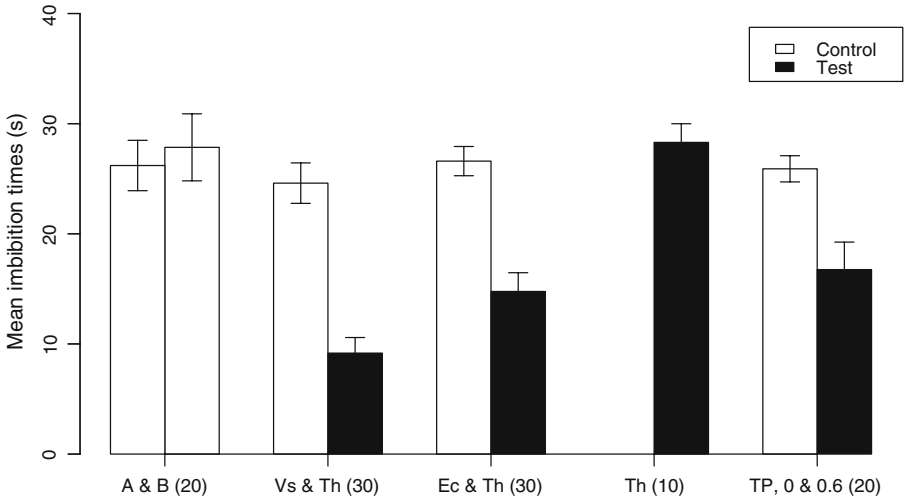

Fig. 3 Imbibition times (sec, means±SE, number of bees in *parentheses*). In all cases, the control food is shown by *clear bars* and the toxic food by *black bars*. *A & B* Identical feeders containing 30% w/w sucrose solution, *Vs & Th* honeys of *Vicia sativa* and *Tripterygium hypoglaucum*, *Ec & Th* honeys of *Elsholtzia ciliata* and *Tripterygium hypoglaucum*, *Th* honey of *Tripterygium hypoglaucum.* All honeys were diluted 1:3 with water. *TP, 0 & 0.6* 30% sucrose solution and the same solution containing 0.6 μg/g triptolide

10.1 sec, *T. hypoglaucum*=9.2±7.7 sec, and *E. ciliata*: 26.6±7.3 sec, *T. hypoglaucum*=14.8±9.3 sec, all N=30; Scheffé: P<0.001, Fig. 3).

There were significant differences in the visiting frequencies of foragers to feeders that contained sucrose solution with and without triptolide (normal=129.2±40.6, triptolide=85.7±14.9, N=4; Mann–Whitney: U=1.0, P=0.043). There was likewise a significant difference in imbibition times (normal=25.9±5.3 sec, triptolide=16.7±11.1 sec, N=20; Scheffé: P=0.050).

When the feeder of normal honey of *E. ciliata* was removed from the feeding site, leaving only the feeder of toxic *T. hypoglaucum* honey, within a few seconds, all of the foraging bees landed and began to imbibe the toxic honey. The visiting frequency and imbibition period increased significantly, reaching and even exceeding levels previously recorded at the feeder of normal honey (ANOVA: visits [N=6]=472.3±61.9, $F_{2,\ 15}$=13.5, P<0.001; imbibition times [N=10]=28.3±5.3 sec, $F_{2,\ 47}$=16.3, P<0.001). Post-hoc test results showed a significant increase in mean visiting frequencies and imbibition times of foragers at the feeders with toxic honey after *E. ciliata* honey was removed (visits, Scheffé: P<0.001; imbibition times, Scheffé: P<0.001) but no significant difference in mean visiting frequencies and imbibition times between toxic honey after *E. ciliata* was removed and normal honey when toxic honey was also present (visits, Scheffé: P=0.077; imbibition times, Scheffé: P=0.923).

Discussion

Terpenoids, especially monoterpenoids such as linalool, are common constituents of floral scent (Raguso and Pichersky 1999), and it is usually assumed that their presence in nectar is due to passive acquisition from scented corollas (Raguso 2004). Kessler and Baldwin (2006) recently reported a large number of secondary compounds in the headspace, nectar,

and floral parts of *Nicotiana attenuata* (Solanaceae), including many terpenoids. When defensive compounds are produced in other plant tissues and appear in phloem, it is likely that they will also occur in nectar (Adler 2000). Triptolide is presumably present in nectar as a consequence of its occurrence in the leaves and roots of *T. hypoglaucum*: the roots are used for commercial extraction of this compound (Brinker and Raskin 2005). It was suggested by Detzel and Wink (1993) that terpenoids may be less toxic or repellent to bees than other secondary compounds because of the role they play in the attraction of pollinators by floral volatiles. The olfactory responses of bees to terpenoids in floral volatiles are well known (Pham-Delegue et al. 1997; Laska et al. 1999). However, to our knowledge, the study by Detzel and Wink (1993) is the only previous test of the responses of bees to terpenoids in artificial nectar.

Although the triptolide concentration in nectar of *T. hypoglaucum* is unknown, we cannot assume that it is much lower than in honey because of the concentrating process. Phenolics in the nectar of *Aloe littoralis* appear to be deactivated during the manufacture of honey (Liu et al. 2005), and nicotine concentrations in fresh honey have been recorded as 90% lower than the concentrations in experimental diets from which that honey was manufactured (Singaravelan et al. 2006). Almond honey also contains lower amygdalin concentrations than the nectar (London-Shafir et al., 2003). Naef et al. (2004) compared the biochemistry of nectar, honeybee crop contents, and ripe honey and attributed changes in the chemistry of terpenoids and other compounds to the highly oxidative atmosphere in the hive.

Visiting frequencies measured at feeders demonstrated a strong preference of the worker bees for normal sucrose solution or diluted honey as opposed to the toxic equivalent. Similarly, in all comparisons, the imbibition rates were significantly shorter on the toxic honey and sucrose solution when the bees had a choice between toxic and nontoxic diets. Because any honey is a complex mixture of many compounds, it was necessary to demonstrate that pure triptolide had the same effects as did the toxic honey. This suggests that the bees perceive differences between the diets and reduce the risks associated with a toxic honey by reducing the amount of uptake. This is supported by the fact that when there was no alternative to a toxic honey, the visiting frequencies and imbibition times increased to the same levels as for normal honey. Indeed, this is precisely what happens under dearth conditions when there is little flower choice, and people in southern China avoid collecting honey during the flowering of *Tripterygium*. Although our cage and feeder experiments showed that toxic nectar of *T. hypoglaucum* has a significant toxicological effect on honeybees and acts as a deterrent, the seasonal context is important.

Similar results to ours were reported by London-Shafir et al. (2003) for almond flowers, whose nectar contains amygdalin, a cyanogenic glycoside. Preference tests with sugar solutions containing various concentrations of amygdalin showed avoidance of amygdalin in early summer. However, bees did not differentiate among the different amygdalin concentrations in mid-summer, when floral resources are scarce and the bees are less discriminating. This also meant that the authors had to use a lower sugar concentration for their experiments in mid-summer (15 compared to 25%) to avoid overcrowding at the feeders.

Secondary compounds that are repellent to bees at some concentrations can sometimes stimulate them to feed. Recently, Singaravelan et al. (2005) tested the responses of honeybees to four compounds found naturally in floral nectar: nicotine, anabasine, caffeine, and amygdalin. Except for anabasine, naturally occurring concentrations had no deterrent effect, and low concentrations of nicotine and caffeine elicited a significant feeding preference. These compounds were presented to the bees in 20% sucrose solutions. In

addition, feeding experiments with caged bees showed no deleterious effect of natural concentrations of nicotine (Singaravelan et al. 2006).

Most studies (including the present one) use a single sugar concentration for feeding experiments with bees. However, an important recent finding is that the response of *A. cerana* to phenolics in artificial nectar depends on the sugar concentration of the nectar (Liu et al. 2007). Phenolics had an inhibitory effect at low and high sugar concentrations but an attractant effect in the mid-range of 15–35% w/w. Therefore, the attractive or deterrent effect of a secondary compound in nectar depends not only on the concentration of that particular compound but also on the sugar concentration.

In a recent study of the complex nectar chemistry of *N. attenuata* and its effect on pollinators, Kessler and Baldwin (2006) found that visit frequency and visit time were inversely correlated for hawkmoths and hummingbirds presented with choices involving various secondary metabolites. These authors suggest that if nectar repellents decrease visit time and thus the amount of nectar removed, while at the same time increasing visit frequency, pollination can be achieved with smaller nectar rewards. This does not, however, apply to *T. hypoglaucum*, where the presence of triptolide decreases both visit time and visit frequency of honeybees. The ecological consequences for reproduction in this plant with toxic nectar are not clear. However, our feeder experiments show that the bees are only partially deterred by nectar of *T. hypoglaucum* when given the option of good-quality nectar, and in the absence of alternative food sources, the threshold for acceptability of toxic honey is rapidly reduced. The responses of honeybees to differences in nectar concentration and to the variety of chemicals present in nectar are complex and depend on the ecological context.

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