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Adaptive functions of defensive plant phenolics and a non-linear bee response to nectar components

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Summary

1. Secondary compounds such as phenolics, usually present in floral nectar, may act in combination with sugar components to influence the evolution of pollination mutualism.
2. Previous work on the significance of secondary compounds in nectar considers honey bee responses to those compounds alone, but neglects sugar. Our experiments demonstrated that phenolic sugar syrups were attractants to free-flying Asian *Apis cerana* Fab. when sugar concentrations were 15–35%, but were deterrents below or above this range.
3. Synergism between nectar phenolics and sugar may thus provide a novel mechanism for plants to encourage pollinating bees and reduce energy investment in nectar, operating as exaptations by co-opting defence mechanisms against herbivores.

Key-words: *Apis cerana*, bees, nectar constituents, phenolics

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Introduction

Plants produce both antiherbivore secondary compounds and pollinator-attracting nectar sugars in varying concentrations (Baker 1977, 1978). Phenolics occur in a large proportion of floral nectars, are often consumed by pollinators, and appear in honey (Hagler & Buchmann 1993; Vit *et al.* 1997). Indeed, honey bees may seek phenolic nectars (Liu *et al.* 2004). In theory, plants may adjust the secondary compounds, or fragrances, readily expressed in nectar, to selectively deter or attract consumers (Baker 1977, 1978; Rhoades & Bergdahl 1981; Bentley & Elias 1983; Forcone *et al.* 1997; Adler 2000; Bronstein 2001; Gardener & Gillman 2002; Adler & Bronstein 2004; Raguso 2004), and thus varied selective pressures on the plant may arise from diverse species of herbivores, pollinators and inferior flower visitors or damaging nectar robbers. Although non-sugar components, such as phenolics in pollen and nectar, may mediate plant interactions with flower visitors, there has been little experimental work (Adler 2000). The compounds have been assumed to act primarily as deterrents (Kevan & Baker 1983; Gottsberger *et al.* 1984; Inouye & Waller 1984; Haskan 1988; Erhardt & Rusterholz 1998; Liu *et al.* 2006). Because experimental data indicate that nectar with secondary compounds significantly stimulates some bees to feed (Cipollini & Levey 1997; Liu *et al.* 2004), and even alkaloid-containing nectars attract bees in the field when alternative nectar sources are available (Ish-Am & Eisikowitch 1998), it is necessary to evaluate more fully the range of constraints and interactions among flower visitors and a variety of nectar components. Here we present an experiment that tested the interplay between nectar sugar concentrations and phenolic compounds, using feeding assays with a tropical Asian honey bee in its native habitat. To simulate foraging choices in natural conditions, we simultaneously provided forage to free-flying Asian honey bees, *Apis cerana* Fab., that was pure sugar or phenolic-laced syrup. Bee-colony forage intake rates were compared by using standard amounts of phenolic chemicals mixed with various sugar concentrations, or pure sucrose solutions. Our findings suggest that sugar and secondary components in nectar interact, and a non-linear response by foragers to nectar constituents can either augment or diminish pollinator attraction to nectar containing secondary compounds, while reducing plant expenditure in attracting and maintaining the service of pollinators.

Materials and methods

PHENOLICS AND TESTED SYRUPS

Phenolic acids are flavonoid antioxidants characteristic of various honeys, especially buckwheat (Steeg & Montag 1988; Nagai *et al.* 2001; Gheldof *et al.* 2002). The 4-hydroxybenzoic acid of buckwheat honey has a

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high antioxidant capacity (Frankel *et al.* 1998; Nagai *et al.* 2001; Gheldof *et al.* 2002). We used buckwheat phenolics (95%, Shanghai Healthjoy Chemical Co. Ltd) for feeding experiments described here.

To compare the response of bees to various sugar concentrations of pure sugar or phenolic-laced syrups, we made three artificial nectars of pure-sugar, low-phenolic and high-phenolic syrups. The bees seldom selected a syrup of 5%, and often stopped foraging the phenolic syrup of >40% sugar before the dishes were completely depleted during replication, thus we excluded those ranges of sugar concentration from the experiment. The sugar syrup included seven concentrations (10, 15, 20, 25, 30, 35 and 40%, w/w), which represents the lower two-thirds of the range of sugar among naturally foraged nectars (Roubik & Buchmann 1984; Roubik 1989). Low- and high-phenolic syrups were the same seven sugar solutions, but contained 30 mg phenolics/100 g syrup or 80 mg phenolics/100 g syrup, which are within the concentration range in honey (Frankel *et al.* 1998).

BEE SPECIES AND TRAINING PROCEDURE

Feeding experiments were conducted during December, 2005 in the experimental farm of the Institute of Sericulture and Apiculture, Yunnan Academy of Agricultural Sciences (23°N latitude, 1260 m elevation) where hived colonies of *A. cerana* were available. During experiments, air temperature was near 12·7 °C and relative humidity averaged 85%. Bees visited flowers including cultivated and weedy *Rudbeckia laciniata* L. (Asteraceae) and *Eriobotrya japonica* (Thunb.) (Rosaceae).

To promote discovery of the artificial nectar solutions, we removed a frame with many worker bees from a hive and shook it near a dish that contained 20% sucrose syrup. When the bees returned to their hive, we again took a frame with bees to the dish, until worker bees flew to collect the syrup. Feeding experiments were conducted in the following month, during which only the trained colony visited the feeders. We marked foragers and observed them throughout the experiments. No bees skirmished at the feeders, which would occur when multiple colonies forage.

FEEDING EXPERIMENT DESIGN

Pure sugar-solution series were tested in the first feeding experiment. Eight dishes, each containing 20 g syrup, were randomly placed on a board located 5–8 m from the hive. Adjacent dishes were separated by 10–20 cm. Although eight syrups were presented simultaneously to *A. cerana*, their use and depletion by bees followed an orderly sequence between feeding dishes. Social species such as honey bees, which recruit nest mates to resources according to profitability, are ideal for such studies because feeding preferences are clearly indicated by rate of colony foraging, which in turn affects the rate at which standardized amounts of resource are depleted. Feeding experiments were terminated when all syrups were consumed or bees stopped foraging. If the bees stopped foraging before the dish was depleted in one or all replications, we excluded that analysis of depletion rate. The depletion rate by the bees was expressed as g sugar min^{-1}, [(weight of syrup × sugar percentage)/depletion time]. We repeated the same feeding assays to test low-phenolic syrups in the second experiment and high-phenolic syrups in the third. The three test series, first using pure sucrose, then sucrose and low phenolics, then sucrose and high phenolics, were separated by 2–4 days. We repeated this sequence four times using the same colony, because new colonies did not arrive. For the same reason, colony size or genetics (additional factors that affect foraging behaviour), were not introduced. Between any two replicated series, bees were offered nothing. To prevent bees from learning the position of a preferred resource, the arrangement of the eight syrups and the board was changed between replications (Manly 1993).

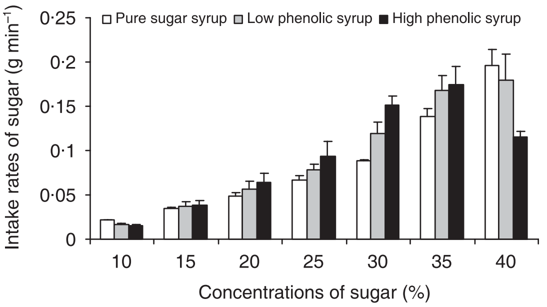

Fig. 1. Feeding responses of honey bees (*Apis cerana*) to various sugar concentrations of pure-sugar, low-phenolic and high-phenolic syrups. Sugar intake is restrained by phenolics in low- or high-sugar syrup, but promoted by phenolics in mid-sugar-concentration syrups. Bars represent non-transformed means and SE.

DATA ANALYSIS

Data were transformed using a square-root transformation before statistical analyses for normal distribution (Pernal & Currie 2001; Singaravelan *et al.* 2005). One-way ANOVA (SPSS 12·0 for WINDOWS) was used to test for differences in syrup-intake rates among series, followed by Tukey's multiple comparison test ($P < 0{\cdot}05$).

Results

CONCENTRATION EFFECTS OF SUGAR AND PHENOLICS

Sugar concentration had a significant influence on the feeding performance of Asian honey bees (Fig. 1). Sugar-intake rates were significantly different among various concentrations of sugar within each series ($F_{6,27} = 69{\cdot}171$, $P < 0{\cdot}001$ for pure syrup series; $F_{6,27} = 11{\cdot}908$, $P < 0{\cdot}001$ for low-phenolic syrup series; $F_{6,27} = 8{\cdot}272$,

$P < 0{\cdot}001$ for high-phenolic syrup series). But comparison of the two different phenolic concentrations in syrups showed they had little effect on the feeding performance of honey bees. Sugar-intake rates were not significantly different between low- and high-phenolic syrups at any level of sugar ($P > 0{\cdot}05$ for all).

NON-LINEAR EFFECT OF SUGAR CONCENTRATION WITH PHENOLICS

The responses of bees to phenolics in syrups depended on sugar concentration. Compared with pure syrups, low-phenolic syrups tended to augment honey bee sugar depletion if sugar concentration was 15–35% (Fig. 1). The sugar intake of low-phenolic syrup was significantly faster than that of pure 30% sugar syrup (Tukey HSD, $P = 0{\cdot}006$). In contrast, phenolic syrups were a deterrent to honey bees if <15% (Tukey HSD, $P = 0{\cdot}008$) or >40% sugar (not statistically significant). High-phenolic syrups showed more pronounced non-linear sugar-dependent effects on honey bees (Fig. 1). When sugar concentrations were within the interval 15–35%, sugar-intake rates were more rapid from high-phenolic solutions than from pure sugar. For example, the sugar-intake rate from high-phenolic syrup was significantly higher than that from pure syrups for 30% sugar (Tukey HSD, $P = 0{\cdot}002$). But when sugar concentrations were outside the 15–35% range, high-phenolic syrups were a deterrent to honey bees; sugar-intake rates from high-phenolic syrups were significantly lower than even those of pure sugar syrups (10%, Tukey HSD, $P = 0{\cdot}004$; 40%, $P = 0{\cdot}041$; Fig. 1), so that syrup viscosity was not involved (Roubik & Buchmann 1984).

PEAK RESPONSES OF BEES TO SUGAR CONCENTRATION

Phenolics in syrups also reduced the sugar concentrations that elicited a peak foraging response in bees. For pure sucrose solutions, the preferred syrup contained 40% sugar (Tukey HSD, $P < 0{\cdot}001$, six paired groups between 40% and 10, 15, 20, 25, 30, 35%). For the low-phenolic syrup, the preferred solution contained 40% sugar (Tukey HSD, $P < 0{\cdot}018$, four paired groups between 40% and 10, 15, 20, 25%). The preferred syrup for high phenolic content had only 35% sugar (Tukey HSD, $P < 0{\cdot}004$, five paired groups between 35% and 10, 15, 20, 25, 40%). Thus the peak foraging response to sugar shifted from 40% (pure sugar syrup) to 35% (high-phenolic syrup) (Fig. 1).

Discussion

ATTRACTION OR DETERRENCE

Few plants have been demonstrated to regulate visitors via nectar secondary compounds, and the ultimate fitness consequences have scarcely been addressed (Adler *et al.* 2001; London-Shafir *et al.* 2003; Singaravelan *et al.* 2006). The sugar component of nectar having secondary compounds (Baker 1977, 1978; Adler 2000) has subsequently been neglected. Our principal finding considering bee behaviour is that honey bees, which prefer a pure sucrose solution of 45–60% sugar content (Roubik & Buchmann 1984; Roubik 1989, 1996), most preferred solutions of only 35–40% sugar when a phenolic constituent was present. The bees usually stopped foraging the phenolic syrups with >40% sugar before the dish was depleted. As mentioned in the Introduction, phenolics may function in both attraction and deterrence. The dual functions evidently depend on the sugar concentration in the phenolic solution, which evokes a non-linear feeding response. High phenolics deterred honey bees when nectar sugar was 40%. Therefore one prediction is that *A. cerana* would tend to abandon a phenolic nectar source if higher-sugar nectars lacking phenolics were available, and a phenolic nectar component may allow plants to discourage such flower visitors when sugar content is relatively high. As discussed below, we believe both generalizations are false.

The present study, in agreement with previous work (Liu *et al.* 2004; Kevan & Ebert 2005), demonstrated that a bee colony can maintain an unexpectedly high level of tolerance and even preference for phenolics in sugar solutions. Studies using European *Apis mellifera* in field conditions have shown that naturally occurring secondary compounds in nectar significantly stimulate bees to feed (Cipollini & Levey 1997; Ish-Am & Eisikowitch 1998). Bees often forage low concentrations of phenolics such as caffeic and genistic acids (Stephenson 1982) and amygdalin (London-Shafir *et al.* 2003). However, alkaloids, glycosides and phenolic substances deter *A. mellifera* at relatively high concentrations (Detzel & Wink 1993), and some nectar is highly toxic to bees (Stephenson 1982; Hagler & Buchmann 1993). Thus attractive or deterrent effects of secondary compounds were thought to be dependent on their doses (Singaravelan *et al.* 2005, 2006). Our results suggest that positive responses by bees to naturally occurring nectar with secondary compounds may depend on sugar concentration, and that the dosage of phenolics is not necessarily low in such nectars.

SHIFT OF SUGAR-RESPONSE THRESHOLDS

Sugar in nectar or honey may mask the unpleasant taste of secondary compounds (Glendinning 2000; Singaravelan *et al.* 2005), and diverse chemicals, like those associated with floral fragrance (Raguso 2004), potentially orchestrate responses to floral rewards and accessory chemicals. These, in turn, may influence plant and forager fitness, including consumers ranging from yeasts to floral herbivores. Interestingly, nectars of arctic and alpine flowers tend to be richer in phenolics than those of temperate counterparts (P. Kevan and H. Baker, personal communication). Phenolics may control nectarivore responses and conserve plant resources.

The high concentration of buckwheat phenolic in this study (80 mg phenolics per 100 g syrup) is the maximum concentration in common honey (Kevan 1995; Frankel *et al.* 1998), and was expected to have a strong deterrent effect. Nonetheless, freely foraging *A. cerana* demonstrated preferences for relatively watery, but 80 mg per 100 g, phenolic syrups in the present study.

Only the social bees make honey, which has only 20–30% water, much less than that in floral nectars (Baker 1978; Roubik 1989). Thus as they evaporate water from nectar, honey-making bees are often faced with a high concentration of phenolics in their stored food (Steeg & Montag 1988; Amiot *et al.* 1989; Liu *et al.* 2006). Carbohydrates inhibit the negative response to deterrents such as bitter phenolics detected by individual taste cells (Shields & Mitchell 1995). Our expectation was therefore that phenolic-laced syrups with high sugar would be preferred. Because the highest sugar concentration in our study (40%) is much below the sugar concentrations that are most profitable to foraging *Apis* (including *A. mellifera*, *A. cerana* and *Apis koschevnikovi*; Roubik 1996), and was a deterrent to *A. cerana*, there was clearly a non-linear interaction between phenolics and sugar. Unsuitable honey stores with high secondary compound content may force bees to seek other sources of sugar, including watery nectar, and to dilute their honey. As an alternative hypothesis, this suggests that a population of plants with phenolic-rich nectar can maintain its pollinators at a considerably lower cost in terms of nectar sugar rewards. If phenolic nectar is common in the habitat, the result with the honey-making bee species may be that more flowers are visited and the cost of providing pollinator reward is reduced. In fact, the acceptance of 10–15% sucrose solutions by *A. cerana* indicated that bees had already shifted their sugar-reward threshold towards very low concentrations, consistent with the hypothesis.

PHENOLIC-MEDIATED POSITIVE FEEDBACK FOR NECTAR COLLECTION

Although the ecological function of secondary compounds in nectar has been a vexing question (Adler 2000), increasing evidence points to pollinator attraction (Cipollini & Levey 1997; Ish-Am & Eisikowitch 1998; London-Shafir *et al.* 2003; Liu *et al.* 2004, 2006; Singaravelan *et al.* 2005). It is well known that honey is frequently rich in both phenolics (Amiot *et al.* 1989; Vit *et al.* 1997) and sugars (»40%). If bees such as *A. cerana* will not use the both phenolic-rich and high-sugar honey, as suggested by their deterrence from 40% sugar phenolic solutions in our study, then they would be more active in collecting fresh nectar. The stored nectar or honey would stimulate bees to visit flowers, rather than being neutral with regard to regulation of nectar collection (*contra* Fewell & Winston 1996).

For angiosperm plants, once the evolutionary investment has been made in producing secondary compounds that occur in phloem sap to deter herbivores, a functional application may be co-opted, as an exaptation, to encourage bee foraging at a decreased energetic cost (Southwick 1984). Rather than serving primarily as a deterrent, plants may obtain a selective benefit from presentation of phenolics in nectar by the diminished sugar concentration in nectar preferred by pollinators, or the diminished acceptance of higher-sugar nectar by certain nectarivores, such as nectar robbers or thieves. In the present study, the demand for nectar sugar by *A. cerana* was reduced by up to 25% (w/w) in phenolic solutions, representing a 41% saving in plant investment in sugar. The relative benefit of the deterrence, and also in nectar sugar production, must be gauged against the response of non-honey-making bees, that is, most species (Michener 2000). It is also necessary to view other potential benefits from phenolics and the other nectar constituents within the context of both solitary and generalist social bee nests, regarding the use and preservation of food within them (Cane & Wcislo 1996; Raguso 2004). Do they deter parasites or microbes? Do they combine with other chemicals to produce compounds that differ in function?

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