



# Synergistic and Dichotomous Effects of Nectar Phenolics on Honey Bee Colonies

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## ABSTRACT

Nectar phenolics have a widespread effect on honey bees and their colonies. Because of their complex, non-linear interactions, it is difficult to assess honey bee health risks from exposure to real-world floral nectar with complex phenolic mixture. In the study, we investigate the bee losses of *Apis mellifera* in the flowering period of the Mexican sunflower *Tithonia diversifolia* in southwestern China, and use data mining approach to model the relationships between nectar phenolics and bee losses. The results show that bee losses are closely related to the phenolics of isochlorogenic acid, *p*-coumaric acid, chlorogenic acid and galangin, identified from the sunflower nectar. The nectar phenolics do not cause bee-poisoning to death, but can trigger bee colonies to explore food sources at risk. Also, each of these phenolics acts in a dichotomous mode, with above a certain value destructing colonies and below such value affecting little. This study provides new insight into the mechanism underlying the catastrophic events of bee losses or honey harvests, which have been reported worldwide.

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## Authors' Contribution

FL conceived and designed the study. FL, XJ and JG designed methodology. FL and XJ worked on formal analysis. FL and JG investigated the whole work. MZS and XZ performed feeding trials. FL and JG wrote and edited the manuscript.

## Key words

*Apis mellifera*, Catastrophic events, Data mining, Dichotomous effects, Nectar phenolics, *Tithonia diversifolia*.

## INTRODUCTION

Global declines of various pollinators have been well documented in literature (Brown and Paxton, 2009; Cameron *et al.*, 2011). The honey bee colonies of *Apis mellifera* is the most economically valuable pollinator of crops in many parts of the world (Brukle *et al.*, 2013). They are thought to have a great adaptive potential, but have experienced unprecedented losses over the past decade. Potential causal factors, including but not limited to, pesticides, disease, parasites, malnutrition and environmental stresses, have been extensively examined. It is still poorly understood the puzzling phenomenon, termed as colony collapse disorder (CCD), in which adult worker bees abruptly disappeared from their hives (Evans *et al.*, 2009).

Diet effects on honey bees have recently been the subject of considerable debate (Fine *et al.*, 2018; Lopez-Urbe *et al.*, 2020). As the main food of worker bees, floral nectar contains rich plant secondary metabolites,

such as phenolic compounds that are associated with plant defense against herbivores (Nicolson and Thornburg, 2007). Numerous studies have examined their effects on honey bees using common feeding experiments, in which honey bees or their colonies are fed with sugars solution containing individual phenolics or their combinations (Gao *et al.*, 2010; Liu and Liu, 2010; Zhang *et al.*, 2016). High levels of phenolics isolated from nectar usually couple with each other or with other nectar constituents, such as sugars, inhibit individual bees' feeding, and can cause colony-level disorder, such as worker-queen conflicts in colonies (Liu *et al.*, 2005, 2007, 2015). The complex, non-linear phenolics in nectar pose a challenge for us to assess and predict their effects on honey bees.

Data mining, also called "knowledge discovery from datasets", is basically a process to use machine learning algorithms to extract information from a dataset and recognize the data pattern for decision support. Many algorithms have been developed to process data from medicine, molecular biology, toxicology and others, in order to predicting the effects or properties of samples with complex features and unknown interrelations (Heinonen *et al.*, 2012). For example, artificial neural network is applied to model yield and environmental emissions from lentil

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cultivation (Elhami *et al.*, 2017). Support vector machines has advantages in recognizing patterns from complex data with high dimensionality, small sample size and nonlinear relationships, and is becoming a powerful tool for classifying the relationship of the real-world complex environment and human health (Zheng *et al.*, 2013). A combination of machine learning with feature selection of random forest (RF) is explored to chemical analysis of floral nectar and honey (Palmer-young *et al.*, 2019).

In the study, we use data mining approach to model the relationships between the bee losses of *Apis mellifera* and nectar phenolics during the period of the Mexican sunflower, *Tithonia diversifolia*. The plant species, occurring naturally from Mexico to Brazil, is widely distributed in the tropics of Asia (Chukwuka *et al.*, 2007). It flowers for more than one month in southwestern China. Because its full bloom is in December when few other flowering plants are available, it becomes a dominant honey source for overwintering colonies of *A. mellifera* in the region. But colonies often suffer rapid bee losses in the flowering season in some years. The main aim of this study was to determine whether and how nectar phenolics destruct bee colonies.

## MATERIALS AND METHODS

### *Field survey on bee losses*

Our field survey was conducted in the Xishuangbanna tropical region (21°55'N, 101°15'E, 550 m in elevation) from 2004-2008. We focused on the apiaries that were at least 1.5-3 km away from farmlands. Also, the apiaries were usually kept 150-220 colonies, and 14 apiaries were included our analysis. For each apiary, the difference of bee frames (~2,500 bees per frame) before and after the sunflowers opened was divided by a total of bee frames before the sunflowers opened, and then was averaged over all sampled colonies as the loss rate of an apiary.

In our field survey, some foraging bees were frequently found to stay on the sunflowers for several hours. To determine whether these foraging bees returned to their hives at night, we conducted an evening investigation at the 8<sup>th</sup> apiary along a 100-m path for 3 times at night. Because such apiaries were far away from villages, such investigation only lasted consecutive 2 nights. Simultaneously, colonies were randomly selected to monitor whether dead bees were presented at the beehive entrances.

### *Identification of nectar phenolics*

During the flowering period of *T. diversifolia*, we sampled nectar from foraging bees, *i.e.* capturing foraging

bees while returning to their hives and then forcing them to expel their crop load into a centrifuge tube. For a whole apiary, at least 50 g nectar sample was pooled to analyze with high performance liquid chromatography (HPLC) (Liang *et al.*, 2009). Briefly, each pooled sample (5 mg) for a given year was extracted with 0.5 mL of methanol at room temperature for 1 h, and sonicated for 15 min and filtered. 10 µL of each sample filter was injected onto a Zorbax SB-C18 column in an Agilent 1100 liquid chromatography system. Nectar phenolics were separated by methanol and aqueous acetic acid as the mobile phase at 1.0 mL/min, and were detected by an electrochemical detector set at 1.0 V in the oxidative mode.

Also, 500 g honey was sampled from the 8<sup>th</sup> apiary to determine whether it contained pollen grains from other honey-source plants in the season by identifying pollen grains, and to examine whether there were pesticide residues by chemistry analysis.

### *Modelling the relationships between bee losses and nectar phenolics*

It is well known that there are complex interactions between nectar phenolics and honey bee colonies (Liu *et al.*, 2015). RF is a widely used classification and regression method in detecting patterns from data with highly dimensional and nonlinear relationships (Breiman and Cutler, 2018). To rank nectar phenolics in their contribution to bee losses, we categorized the surveyed apiaries as the normal ones that lost < 40% adult bee losses in the flowering period and as the destructed ones with > 40% adult bee losses, and then built a RF classification model based on nectar phenolics identified in the samples from the two categories. The relative importance of each phenolic compound with respect to colony losses was evaluated by the caret package v6.0-86 in R software (Kuhn, 2018).

RF-based recursive feature elimination (RF-RFE) is an ideal approach to selection of the features (Guyon, 2003). We implemented the RF-RFE procedure by fitting a RF classification model on the data set of nectar phenolics based on two apiary groups, computing the importance score of each phenolic compound and removing the weakest one. Then, we started second iteration, *i.e.* rebuilding a RF model and deleting the weakest one again. Such iteration continued until the specified number of phenolic compounds is reached.

The RF-RFE was conducted using the caret package, which provides the reference function with two turning parameters of the subset size and refControl (Guyon, 2003). In our case, the specific number of phenolics, *i.e.* the subset size, was set as 1:5, 8, 11, respectively, to determine

the optimum number of phenolics for the RF models. In refControl, the method of 10-fold cross-validation with 5 times was used to assess model performance. The RF classification models with respect to bee losses were also built and evaluated with the caret package v6.0-86 in R software (Kuhn, 2018).

To reveal partial effects of individual phenolics on honey bees, we modelled the relationships between nectar phenolics and the loss rates of the surveyed apiaries using randomForest package v4.6-14 (Breiman and Cutler, 2018) in R software, version 3.6.1 (R Core Team, 2018), by setting the parameters of 500 trees and 3 variables at each split.

#### *Effects of nectar phenolics on brood and young bees*

To determine the effects of nectar phenolics on brood rearing and newly emerged bees, we conducted a feeding trial in a flight cage at the experimental farm of the Institute of Sericulture and Apiculture (23°24'N, 103°17'E, 1260 m in elevation) from July through September of 2010. Our feeding trial included three replications, in each of which, two queen-right, adult-equalized (4 Langstroth-frames of adult bees) and brood-deprived colonies were fed in a flight cage (25 × 10 × 5 m) after all combs with stored food were removed from the hives. One was fed with 25% (w/w) sucrose syrup (S-fed), and the other was fed with the syrup, containing the top important phenolics (P-fed). Their phenolic contents used for the trial were those found in the sunflower nectar. On each day, 200g of the test solutions and 20mg of uniform pollen substitute (corn pollen moulded into dough using a 50% sucrose solution) were given to each colony. Water was available *ad libitum* for colonies in the flight cage. In the following three weeks, we examined the brood combs once a week. The feeding trial included three replications, each having two colonies. The brood rearing rates were compared between the S- and P-fed colonies using Chi-square test.

Also, we tested whether nectar phenolics disorientate bees based on the orientation flights of young bees, engaging before they become foragers (Winston, 1987), using the following procedure. After feeding for 20 days, combs with mature pupae (the period from eggs to pupae requires 21 days) were taken from hives and placed in incubators (RQH-250, Shanghai, China) set to 35°C and 50% RH. 500 newly emerged bees (Edding 751 paint marker) were marked from the S- and P-fed colonies, respectively, and all were transferred to one colony in an apiary that had 13 colonies. A colony rarely discriminates and excludes young bees from other colonies if these are less than 72 h post-emergence (Breed, 1983). In early mornings when no bees left the hives for food collection, the marked bees were examined on the day after introduction, and on

subsequent days, at intervals of three days.

Based on the results of the three replications, we used generalized linear models and binomial errors to test whether the losses of newly emerged bees from their host hives were related to food phenolics, and compared bee losses between two feeding scenarios on subsequent days using Fisher's exact test. Statistics were conducted using R software, version 3.6.1 (R Core Team, 2018).

## RESULTS

### *Colony losses*

The bee losses of the surveyed apiaries are summarized in Table I. In some years most apiaries experienced serious bee losses. In 2004 and 2008, for example, most apiaries lost more than 70% of worker bees in the flowering season. In 2005, 2006 and 2007, however, apiaries lost less than 40% of bees. The apiaries that were suffered serious losses displayed similar symptoms as those of CCD. The queen and young bees seemed to be health, because a large number of brood was nursed, and much food was stored in the hives (Fig. 1A, B).

Our evening survey showed that few dead bees were found to present at the hive entrances. However, numerous bees stayed on the sunflowers both in the daytime and night (Fig. 1C, D). These bees usually aggregated together on the sunflowers. 11-16 worker bees were usually found to concentrate on a flowering branch for more than 5-7 h.

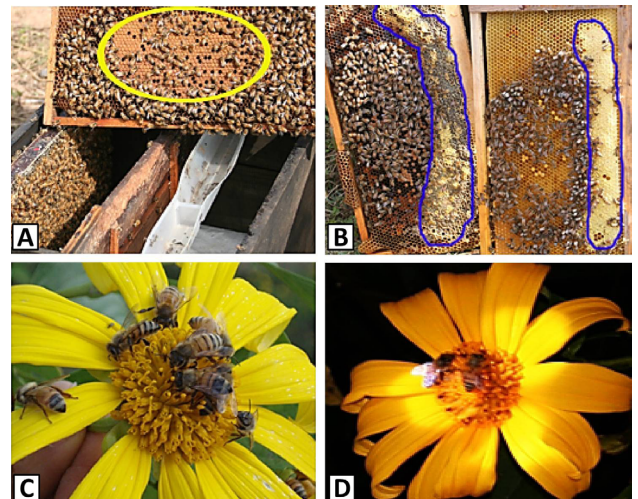


Fig. 1. Rapid decline of *Apis mellifera* colonies during the flowering period of *Tithonia diversifolia*. A, A small cluster of nurse bees were living with queen and brood (yellow ellipse); B, Much honey (blue circle) was stored in the hive; C, Bees aggregated in a flower for more than six hours; D, Bees stayed in a flower at night.

**Table I.- Nectar phenolics and colony losses of 12 surveyed apiaries.**

No. apiary	P	PA	Q	G	GA	CF	F	HB	CA	IA	HVA	Loss rate
1	0.15	0.23	0.5	0.29	0.35	0.49	0.28	2.11	2.3	0	0.05	0.3319
2	0.1	0.09	0.55	0.27	0.32	0.33	0.03	1.679	1.85	2.11	0.02	0.6919
3	0.08	0.08	0.84	0.24	0.36	0.37	0.01	2.73	2.23	0	0.11	0.2233
4	0	0	0.67	0.32	0.24	0.21	0.103	1.879	2.03	1.39	0.09	0.6043
5	0	0.09	0.45	0.32	0.26	0.26	0.086	4.12	1.89	1.27	0.03	0.5702
6	0	0	0.32	0.23	0.22	0.32	0.04	3.22	1.0	0	0.02	0.2312
7	0	0	0.21	0.22	0.49	0.28	0.01	1.78	1.08	1.36	0.03	0.2571
8	0.13	0.28	0.69	0.27	0.82	1.18	0.053	2.63	2.03	2.43	0.06	0.7372
9	0	0.11	1.05	0.23	0.44	0.37	0.02	2.76	2.06	1.76	0.07	0.6398
10	0.08	0.27	0.63	0.25	1.2	0.49	0.09	3.56	1.04	3.66	0.04	0.8357
11	0	0.152	0.41	0.25	0.32	0.32	0.03	3.22	2.08	0	0.08	0.4394
12	2.16	0.05	0.39	4.52	1.71	2	0.19	5.46	1.52	0.86	0.12	0.1387
13	0.17	0.12	0.45	0.39	0.46	0.36	0.106	3.96	2.09	2.27	0.03	0.5312
14	0	0.03	0.22	0.27	0.21	0.22	0.04	2.15	1.26	1.13	0.02	0.3112

P, protocatechuic acid; PA, *p*-coumaric acid; Q, quercetin; G, galangin; GA, gallic acid; CF, caffeic acid; F, ferulic acid; HB, 4-hydroxybenzoic acid; CA, chlorogenic acid; IA, isochlorogenic acid A; HVA, 4-hydroxy-3-methoxyphenylethanol; phenolic content (n=5, mean), mg·g<sup>-1</sup>.

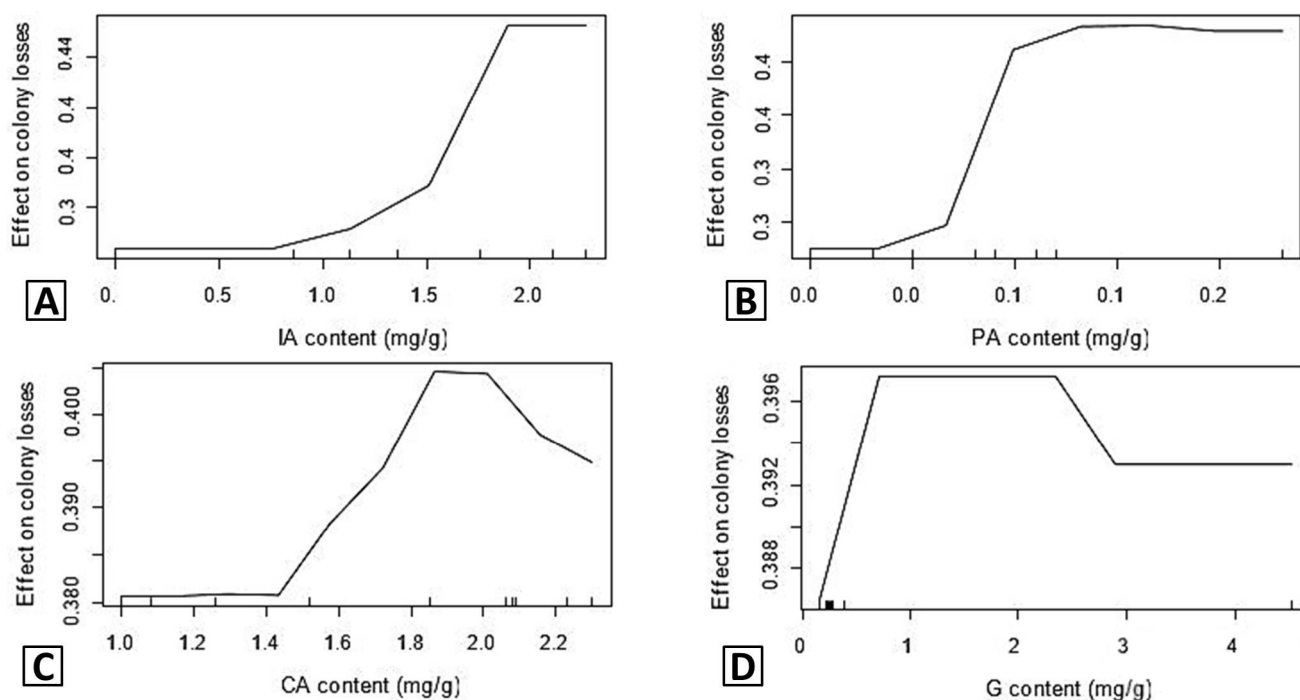


Fig. 2. Partial plots for the four most influential phenolics based on the outcomes of the Random Forests analysis. Abbreviations are defined in Table I.

#### Relationships between bee losses and nectar phenolics

Identifying pollen grains from honey showed that > 99% of pollen was from *T. diversifolia*, indicating that honey bees almost exclusively visited *T. diversifolia* in the winter season. The widely used pesticides in the region, such as imidacloprid and Cyhalothrin, were not detected

in the honey harvested by *A. mellifera*.

11 phenolics were identified in the nectar samples from both the normal and destroyed apiaries (Table I). On the basis of the RF classification model, IA was the greatest contribution to bee losses, followed by PA, G, CA, Q, F, HB, HVA, GA, CF and P. Their mean decrease gini with



respect to bee losses were 1.36, 0.97, 0.83, 0.62, 0.57, 0.48, 0.42, 0.33, 0.29, 0.23 and 0.22, respectively. According to the RF-RFE algorithm, the best RF model was the one built on the combination of IA, PA, G and CA, achieved 76.09% classification accuracy with respect to bee losses. This indicated that the four phenolics synergistically cause bee losses.

The partial effect analysis showed that there was a dichotomous relationship between IA and bee losses, with above the mean content of IA destructing colonies and with that less than the mean affecting very little (Fig. 3). PA, G and CA acted as the similar patterns of IA, although the thresholds were different. Their high contents were positively associated with bee losses (Fig. 3).

#### Effects of nectar phenolics on brood and young bees

Ratios of sealed brood to total larvae were not different between the two treatments during the trial period (4700 cm<sup>2</sup>/17400 cm<sup>2</sup> for the S-fed vs. 4900 cm<sup>2</sup>/17800 cm<sup>2</sup> for the P-fed; Chi-square test,  $\chi^2 = 1.002$ , df: 1,  $P = 0.317$ ). This indicated that phenolic-laced syrup had little effect on brood rearing. Newly emerged adult bees from S- and P-fed colonies were equally accepted by the host colonies (Fisher's exact test:  $P = 0.258$  at 1<sup>st</sup> day; Fig. 3A).

During the orientation flights, bees losses were significantly related to the syrup containing phenolics or not (generalized linear models:  $P < 0.001$ ). The bees introduced from P-fed colonies showed less returns to host hives than those raised in S-fed colonies (Fisher's exact test:  $P < 0.001$  at 4<sup>th</sup> day,  $P < 0.001$  at 7<sup>th</sup> day, and  $P = 0.04$  at 10<sup>th</sup> day; Fig. 3A). As compared to the S-fed bees, the P-fed were more likely to drift to other hives ( $P < 0.001$  at 4<sup>th</sup> day,  $P < 0.001$  at 7<sup>th</sup> day, and  $P < 0.001$  at 10<sup>th</sup> day; Fig. 3B).

## DISCUSSION

The sunflower family (Asteraceae) represents the largest family of flowering plants with ~24,000 species, which are widespread into various terrestrial habitats. In most cases, the sunflowers, either wild or cultured, are the excellent sources of nectar for the western honey bees of *A. mellifera* in the place of origin. Also, their pollen has been shown to help bees enhancing natural resistance to pathogens (Giacomini *et al.*, 2018). But our study shows that the sunflowers of *T. diversifolia* could cause massive bee losses from their hives in some years.

Our RF model demonstrates that bee losses were closely related to the four phenolics of IA, PA, G and CA. Our feeding trail shows that these phenolics did not affect brood rearing of a colony. However, the marked bees from the P-fed colonies lost more rapidly than those raised in the S-fed ones. For example, the bees raised by the P-fed colonies reduced to 59 individuals, but those from the S-fed ones remained 160 after they were transfer into the same hive for 4 days. The result of feed trail further confirms that rapid bee losses were indeed related to nectar phenolics.

It is well known that nectar phenolics can deter bees' feeding, suggesting that they are toxic to bees (Liu *et al.*, 2007, 2015). In our field survey and feeding trial, however, no dead bees were observed at hive entrances. Also, some typical bee-poisoning syndromes, including the difficulty in performing the righting reflex, as well as abdomen dragging and curling up, which are commonly induced by nectar toxins (Victoria *et al.*, 2014), were not observed. Moreover, bees aggregating on the sunflowers tended to aggregated together, and did not appear intoxicated and paralyzed, the unusual bee behavior commonly observed

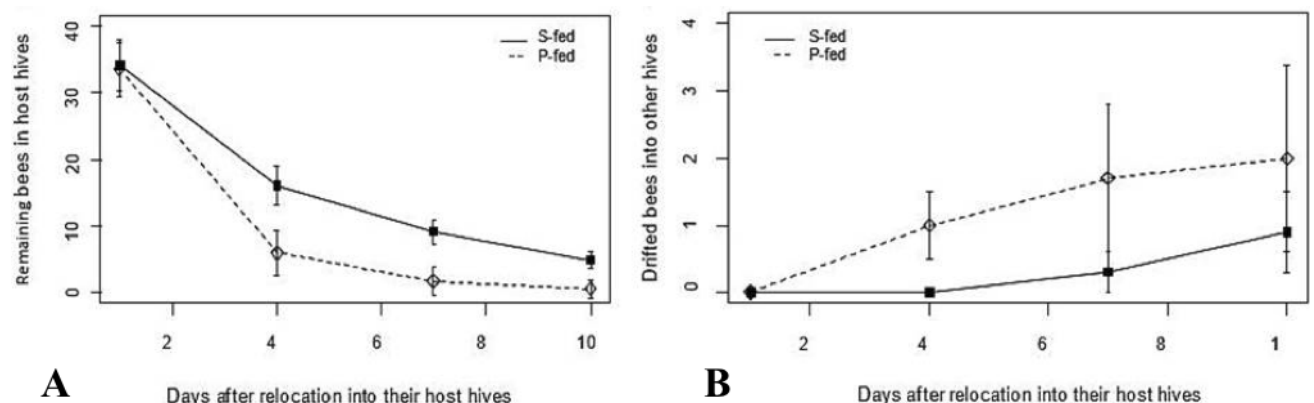


Fig. 3. Effect of nectar phenolics on orientation of young adult bees. **A**, Of 500 young bees ( $\leq 10$ -day old) that fostered in P-fed colonies, a total of 304 bees lost from host colonies; **B**, The drifted bees were found in other hives. Each point represents mean  $\pm$  standard error. S- or P-fed denotes the colonies fed 25% sucrose syrup and those fed the same syrup with the mixture of IA, PA, CA and G, respectively.

on the flowers of *Rhododendron ponticum* (Tiedekenet *al.*, 2016). Both our field survey and feeding trail suggest that bee losses are less likely to be induced by the toxicity of nectar phenolics.

Our feeding trail also shows that the newly emerged bees raised from the P-fed colonies drifted into other neighbouring colonies during their orientation flights. Does this drift result from their disorientation? We noticed that the drifted bees were not randomly distributed across the 11 colonies in the apiary, but tended to select the relative large colonies. In fact, during the period of the feeding trail in the flight cage, the bees from the P-fed colonies were frequently observed to invade the S-fed ones to steal food. The drifted bees in the open apiary were not due to their disorientation, but due to nectar phenolics-driven exploration of food sources.

Previous studies have shown that the effects of nectar phenolics on bees are dose-dependent. Low contents of nectar phenolics usually attract bees' feeding, and high contents deter bees (Liu *et al.*, 2007). Our present study reveals that the four phenolics act as in a dichotomous pattern, with the content above a certain value destructing colonies but below the value no effects occurring. Nectar phenolics-triggered bees' outgoing for food may occur in an abrupt pattern. Further study on the dichotomous effects of nectar phenolics on bee colonies should be warranted.

## CONCLUSIONS

In short, our study shows that rapid bee losses during the sunflower period are related to nectar phenolics. Contrary to most studies of diet effects on bees, which emphasize the bee losses due to food-poisoning, our study reveals for the first time that nectar phenolics can drive bees to go out of their hives to explore food sources at high risk, such as stealing food from other colonies, and that each of nectar phenolics acts as in a dichotomous mode, which may lead to a catastrophic event of bee losses. Plants at the flowering stage are sensitive to weather conditions, and usually produce rich phenolics in nectar under inclement weather. Much attention should be paid to the dichotomous effects of nectar phenolics on bee colonies.

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## Statement of conflict of interest

The authors have declared no conflict of interests.

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