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### Permalink

<https://escholarship.org/uc/item/3d91b443>

### Journal

Journal of Experimental Biology, 221(5)

### ISSN

0022-0949

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### Publication Date

2018

### DOI

10.1242/jeb.168344

Peer reviewed

## RESEARCH ARTICLE

# The reluctant visitor: a terpenoid in toxic nectar can reduce olfactory learning and memory in Asian honey bees

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## ABSTRACT

The nectar of the thunder god vine, *Tripterygium hypoglaucum*, contains a terpenoid, triptolide (TRP), that may be toxic to the sympatric Asian honey bee, *Apis cerana*, because honey produced from this nectar is toxic to bees. However, these bees will forage on, recruit for, and pollinate this plant during a seasonal dearth of preferred food sources. Olfactory learning plays a key role in forager constancy and pollination, and we therefore tested the effects of acute and chronic TRP feeding on forager olfactory learning, using proboscis extension reflex conditioning. At concentrations of 0.5–10 µg TRP ml<sup>-1</sup>, there were no learning effects of acute exposure. However, memory retention (1 h after the last learning trial) significantly decreased by 56% following acute consumption of 0.5 µg TRP ml<sup>-1</sup>. Chronic exposure did not alter learning or memory, except at high concentrations (5 and 10 µg TRP ml<sup>-1</sup>). TRP concentrations in nectar may therefore not significantly harm plant pollination. Surprisingly, TRP slightly increased bee survival, and thus other components in *T. hypoglaucum* honey may be toxic. Long-term exposure to TRP could have colony effects but these may be ameliorated by the bees' aversion to *T. hypoglaucum* nectar when other food sources are available and, perhaps, by detoxification mechanisms. The co-evolution of this plant and its reluctant visitor may therefore likely illustrate a classic compromise between the interests of both actors.

**KEY WORDS:** Plant–pollinator interaction, Triptolid, Toxic honey, *Apis cerana*, Proboscis extension reflex, Memory

## INTRODUCTION

From 8% to 36% of floral nectars contain phenolics and alkaloids (Baker, 1977). These secondary metabolites can serve multiple functions, including ameliorating stress, protecting against microbes, deterring herbivory and influencing pollination (Goyal, 2013). Such compounds can discourage less efficient pollinators or nectar robbers, encourage preferred pollinators, or both (Adler, 2000; Irwin et al., 2004). For example, caffeine is naturally found in the nectar of some plants, and can enhance pollination by increasing honey bee foraging and recruitment (Couvillon et al., 2015). Nectar compounds also influence floral constancy – the tendency of pollinators to return to the same floral species (Wright and Schiestl,

2009). Thomson et al. (2015) showed that bumble bees increased visitation to artificial flowers offering caffeinated sugar solution. Determining how such compounds influence pollinator cognition and behaviour will therefore improve our understanding of plant–pollinator interactions.

Many pollinators can learn to associate rewarding nectar with floral odors (Wright and Schiestl, 2009). Olfactory learning allows pollinators to discover the same rewarding plant species at new locations and, in the social corbiculate bees, facilitates colony recruitment to rewarding plant species (Dornhaus and Chittka, 2004; Frisch, 1967; Nieh, 2004). Plants could therefore benefit by manipulating pollinator olfactory learning. In fact, nectar amino acids such as isoleucine and proline enhance honey bee olfactory learning (Simcock et al., 2014). Caffeine also improves honey bee olfactory memory and encourages forager revisitation and floral constancy (Wright et al., 2013). However, the learning effects can be complex and depend upon compound concentrations and timing. Mustard et al. (Mustard et al., 2012) fed caffeinated sucrose solutions to honey bees and found reduced learning 20–30 min after initial exposure but no long-term effects on memory recall when the same bees were tested 24 h later. The effects of such nectar metabolites on pollinator learning and memory therefore deserve further study.

The thunder god vine, *Tripterygium hypoglaucum*, provides a fascinating case because it contains a diterpenoid epoxide, triptolide (TRP), a defensive chemical that is likely noxious to herbivores (Sun et al., 2009) but may also be toxic to bees, including a common Asian honey bee species, *Apis cerana* (Tan et al., 2007). When bees fed on honey produced from *T. hypoglaucum*, they suffered a significant increase in mortality in comparison with control bees fed on sugar and honey not produced from *T. hypoglaucum* nectar (Tan et al., 2007). *Tripterygium* species are insect pollinated (Roubik, 1995), and, although little is known about their pollination biology, their flowers are frequently and regularly visited by honey bees (largely *A. cerana*) (Tan et al., 2007), Diptera, solitary wasps and ants (K.T., unpublished) when other floral resources are less available. *Apis cerana* normally avoids feeding on *T. hypoglaucum* honey (Tan et al., 2007), and decrease waggle dancing and recruitment for *T. hypoglaucum* honey (Tan et al., 2012). However, *T. hypoglaucum* blooms from May to June when there are few alternative food sources for *A. cerana* in many areas (Tan et al., 2012). Within this period of relative food dearth, foragers will collect and even dance and recruit nestmates to honey made from *T. hypoglaucum* nectar (Tan et al., 2012). Thus, there is likely a pollination mutualism between *A. cerana* and *T. hypoglaucum* because bees will obtain food from this plant and pollinate it when there are few other resources (Tan et al., 2012).

Because TRP is the primary defensive compound in *T. hypoglaucum* nectar and the likely toxic compound in *T. hypoglaucum* honey, we tested the hypotheses that TRP alone can impair honey bee survival and cognitive abilities. *Apis cerana*

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foragers have good olfactory learning (Wang and Tan, 2014) but no studies have examined how TRP may alter their learning and memory. Caffeine fed in artificial nectar can impair olfactory learning (Mustard et al., 2012). We therefore tested the hypothesis that feeding on artificial nectar with TRP would impair forager olfactory learning and memory retention. We used a wide range of TRP concentrations and tested the effects of TRP consumption on bees exposed to a single dose (acute exposure) or over multiple days (chronic exposure). Because *T. hypoglaucum* and *A. cerana* are sympatric, have coevolved and are likely pollination mutualists (Tan et al., 2007), we expected that foragers would be impaired but could also cope with some of the hypothesized negative effects of TRP on olfactory learning and memory.

## MATERIALS AND METHODS

### Colonies and bees

We used three healthy colonies of *Apis cerana* Fabricius 1793, each maintained in a separate single-story wooden box at an experimental apiary at the Southwest Biodiversity Research Center (Kunming, China). Each colony contained ~12,000 bees. We conducted experiments from March to June 2017, when *T. hypoglaucum* blooms. In our learning experiment, the unconditioned stimulus (US) was sucrose solution that did not contain TRP. We therefore simulated a situation in which foragers were fed nectar with TRP (via food exchange with a *T. hypoglaucum* nectar forager or from honey processed from such nectar) once (acute exposure) or over an extended period (chronic exposure).

### Relevance of TRP concentrations used

An analysis of five *A. cerana* colonies found that honey produced from *T. hypoglaucum* nectar has an average of  $0.6 \mu\text{g TRP g}^{-1}$  ( $0.61 \pm 0.11 \mu\text{g g}^{-1} = 0.87 \mu\text{g ml}^{-1}$ , based upon an *A. cerana* honey density of  $1.32 \text{ g ml}^{-1}$ ) (Tan et al., 2007). Unfortunately, the natural concentrations of TRP in *T. hypoglaucum* nectar are not known. In plants, TRP levels have only been quantified in plant roots, where they range from 2 to  $14 \text{ ng g}^{-1}$  (dry root mass) (Brinker and Raskin, 2005). However, in multiple cases, such toxic secondary metabolites are known to degrade significantly in concentration during the conversion from nectar to honey (London-Shafir et al., 2003; Tan et al., 2007). Caffeine and amygdalin are significantly degraded by 90% and 50%, respectively, during the conversion from nectar to honey (Kretschmar and Baumann, 1999; London-Shafir et al., 2003). Natural nectar TRP concentrations may therefore be much higher than  $0.87 \mu\text{g ml}^{-1}$ . If so, concentrations ranging up to  $10 \mu\text{g TRP ml}^{-1}$  (assuming 90% degradation) may be ecologically relevant.

### Experiment 1: acute exposure

We carefully captured returning foragers, each in a separate, clean glass vial, at colony entrances. After capture, each bee was chilled on ice for 3–4 min and then restrained in a small plastic microcentrifuge tube that was cut open at the end to allow just its head and proboscis to emerge (Wang and Tan, 2014). We allowed these bees to rest and to equalize their hunger levels inside a dark incubator [ $25^\circ\text{C}$ , 65% relative humidity (RH)] for 5 h. Before the olfactory learning proboscis extension reflex (PER) tests, these bees were divided into three groups. In a preliminary study with 15 nectar foragers captured upon their return to the nest, we measured a mean crop volume of  $22.9 \pm 0.7 \mu\text{l nectar bee}^{-1}$ . We therefore fed our bees with  $15 \mu\text{l}$  of sugar solution, a slightly lower volume, to ensure full consumption by all bees. The control group was fed with  $15 \mu\text{l}$  of  $2 \text{ mol l}^{-1}$  (55% sucrose w/v) pure sucrose syrup. In the other

groups, each bee was fed with  $15 \mu\text{l}$  of  $2 \text{ mol l}^{-1}$  sucrose syrup that contained 0.5, 1, 5 or  $10 \mu\text{g TRP ml}^{-1}$ . Bees therefore received 7.5, 15, 75 or  $150 \text{ ng TRP bee}^{-1}$ . We then waited 2 h to allow bees to recover their hunger levels, because feeding motivation influences rewarded olfactory learning (Giurfa and Sandoz, 2012). We replicated these treatments three times with each colony, testing the learning of ~30 bees  $\text{treatment}^{-1} \text{ colony}^{-1}$ . In total, we tested the acute effects with 526 bees (sample size details in Fig. 1 legend).

### Experiment 2: chronic exposure

We collected ~100 foragers per trial from each focal colony with clean glass tubes (see Experiment 1) and transferred them to a polystyrene foam microhive ( $25 \times 12 \times 12 \text{ cm}$ ) with one small piece of empty comb ( $10 \text{ cm} \times 5 \text{ cm}$ ) hanging inside. Into this microhive, we placed a 10 ml horizontal feeding tube that provided one of the following treatments in  $2 \text{ mol l}^{-1}$  sucrose solution: 0, 0.5, 1, 5 or  $10 \mu\text{g TRP ml}^{-1}$ . We provided only one treatment per microhive. We placed this microhive into an incubator at  $25^\circ\text{C}$  and 65% RH. After 7 days of chronic exposure, we measured forager olfactory learning.

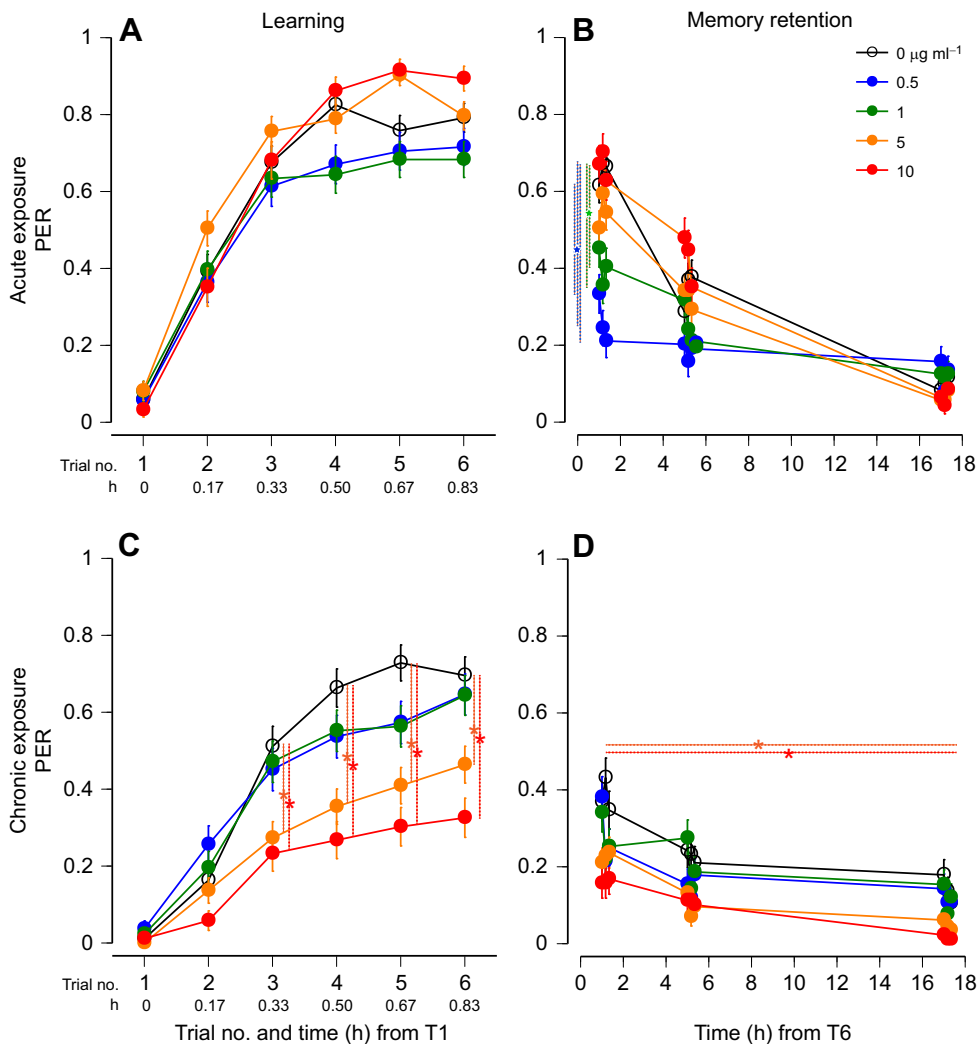
To calculate the average consumption per bee per cage, we followed standard procedures (Williams et al., 2013). Each 24 h, we counted and removed the dead bees (using this data for our survival analysis) and measured the volume of sucrose solution consumed, calculating average consumption per bee per day per cage. To determine the loss of sucrose solution due to evaporation alone, we conducted three separate trials in which we placed identical microhives and feeding tubes in the same incubator under the same conditions and measured evaporative weight loss each day over 7 days. We then corrected our bee daily mean consumption measurements to account for this evaporation. To assess learning and survival, we tested ~30 bees  $\text{treatment}^{-1} \text{ colony}^{-1}$ , for a total of 457 bees. We ended each trial by freezing all surviving bees.

### Olfactory PER conditioning

We used a standard method for assessing honey bee olfactory learning – PER assays (Bitterman et al., 1983). After olfactory conditioning, a bee that has learned to associate an odor with food will extend its proboscis to drink sucrose solution upon detecting the odor alone (Bitterman et al., 1983). For our conditioned stimulus (CS), we used hexanal (98%, Aladdin Reagent Database Inc., Shanghai, China) dissolved in mineral oil (1:10). Although hexanal is not known to be an odorant in *T. hypoglaucum* nectar, honey bees have excellent general olfactory learning that does not typically depend upon the precise odor compound used (Giurfa and Sandoz, 2012). In addition, our experiment simulated the results of a forager exposed to TRP via trophallaxis with another forager or by feeding on TRP honey, and then tested for its ability to generally learn floral odors.

We pipetted  $2 \mu\text{l}$  of our hexanal solution onto a strip of filter paper, which we then placed inside a clean glass Pasteur pipette through which clean and humidified (90% RH) air flowed ( $15 \text{ ml s}^{-1}$ ) into a polyfluorotetraethylene (PFTE) tube. Each individual bee was placed 1 cm away from the outlet of a PFTE tube that provided the CS. To draw this odor away, we placed an exhaust fan 12 cm behind the bee.

In each trial, we first presented the CS alone for 3 s and scored proboscis extension during this period. We then presented the US to the antennae ( $2 \text{ mol l}^{-1}$  pure sucrose solution on a clean toothpick) for 3 s. Both CS and US therefore overlapped for 3 s. Each bee underwent six training trials with an inter-trial interval of 10 min (Menzel, 2001) during the learning phase. Detailed memory-



**Fig. 1. Triptolide (TRP) altered olfactory learning (PER) and memory retention in *Apis cerana* foragers.** Bees were exposed (A,B) acutely (2 h before testing: 120 bees at 0 µg ml<sup>-1</sup>, 88 bees at 0.5 µg ml<sup>-1</sup>, 101 bees at 1 µg ml<sup>-1</sup>, 123 bees at 5 µg ml<sup>-1</sup> and 94 bees at 10 µg ml<sup>-1</sup>) or (C,D) chronically (over 7 days: 92 bees at 0 µg ml<sup>-1</sup>, 82 bees at 0.5 µg ml<sup>-1</sup>, 87 bees at 1 µg ml<sup>-1</sup>, 110 bees at 5 µg ml<sup>-1</sup>, and 86 bees at 10 µg ml<sup>-1</sup>). We used three colonies. We show means ± 1 standard error for the proboscis extension reflex (PER) and give both trial number and elapsed time. The color key gives TRP treatment concentrations and applies to all plots. We used Tukey's honestly significant difference (HSD) tests to make corrected pairwise comparisons. To simplify our presentation, we only compare the control (0 µg TRP ml<sup>-1</sup>) with the TRP treatments within each trial and use color-coded dashed lines and stars to indicate significant differences. Vertical dashed lines show Tukey's HSD test comparisons at each indicated time point (Tukey's HSD test,  $P < 0.05$ ). For memory in chronically exposed bees, we show horizontal dashed lines to indicate differences over all trials (Tukey's HSD test,  $P < 0.05$ ) because there was no significant trial × treatment interaction ( $P = 0.09$ ) in this analysis.

retention curves were obtained by presenting the CS only for 3 s and scoring proboscis extension at the following times after the last learning trial: 1, 1.17, 1.33, 5, 5.17, 5.33, 17, 17.17 and 17.33 h. We tested 10–16 bees in each trial and tested all treatments in each trial. Bees that showed PER to the odor prior to conditioning or failed to show PER to the sucrose solution were discarded and not used (Bitterman et al., 1983). We recorded the number of non-responding bees per treatment.

#### Sucrose responsiveness following chronic exposure

In our chronic experiments, we found a significant effect of TRP on learning. To determine if this could have arisen because TRP reduced forager appetitive motivation, we tested sucrose responsiveness on a separate set of bees. We captured and treated bees exactly as in Experiment 2, chronically giving them five different treatments (0, 0.5, 1, 5 and 10 µg TRP ml<sup>-1</sup>) over 7 days. We restrained them as in Experiment 1 and followed standard sucrose responsiveness testing procedures (Carr-Markell and Robinson, 2014). We then tested their response to a series of pure sucrose concentrations (0%, 0.1%, 0.3%, 1%, 3%, 10% and 30% w/v) provided by tapping the bee on the antenna with a clean wooden toothpick with a small drop of the test solution. No TRP was in the sucrose solutions. Each bee was tested with an ascending series of these sucrose concentrations, with a presentation of pure water (no TRP) between each pair of sucrose concentrations to

minimize sensitization. No bees responded to the interspersed water controls. We recorded all PER responses. We used 600 bees from three colonies and did not reuse these bees in any subsequent experiments.

#### Statistics

To analyze the PER results, we separately analyzed learning and longer-term memory and used a Repeated-Measures ANOVA (REML algorithm) with colony as a random effect and all other effects (treatment and trial) fixed. Although such data can be analyzed with non-parametric tests, our sample sizes were typically more than twice as large as the minimal sizes (40–50 bees per treatment) recommended for such an ANOVA analysis (Matsumoto et al., 2012). Based upon visual inspection of mean learning data (Fig. 1), we suspected that different TRP concentrations changed these curves, and ANOVA allowed us to compare learning curves between the treatments. For our analyses of memory, we applied the same method because we repeatedly measured memory over nine successive time points. To determine the effect of TRP upon memory without the potential issue of habituation, we also ran ANOVAs to analyze just the first memory trial (1 h) in the acute and chronic experiments. For *post hoc* testing, we used Tukey's Honestly Significant Difference (HSD) tests, one per model, to test for pairwise differences while correcting for multiple comparisons (Zar, 1984).

To analyze sucrose responsiveness, we ran an ANOVA (REML algorithm) on the sucrose responsiveness score (sum of all PER responses for each bee to the sequence of six sucrose concentrations) (Carr-Markell and Robinson, 2014) with TRP concentration as a fixed effect and colony as a random effect.

For our sucrose consumption data, we used an ANOVA (REML algorithm) with colony as a random effect and treatment as a fixed effect. To determine if TRP altered the proportion of bees that did not respond to sucrose, we used GLM (binomial distribution, reciprocal link, maximum likelihood estimation) and included colony as a factor. We also report these results as Log-Rank (L-R)  $\chi^2_{\text{degrees of freedom}}$ . To test the effects of TRP on survival, we used a Proportional Hazards survival analysis with colony and treatment as fixed effects. We report the L-R  $\chi^2_{\text{degrees of freedom}}$  and applied the Dunn–Sidak correction (Zar, 1984) to correct for multiple comparisons between the control treatment and TRP treatments ( $k=4$ ). Tests that remain significant after this correction are denoted 'DS'. Because each trial ended after 7 days, we show data on two different measurements of survival: the mean day of death of bees that died within the 7 day period; and the mean day of death for all bees (including those bees that we froze at the end of the trial, censored data).

Throughout our paper, we give means $\pm$ 1 standard error.

## RESULTS

### Experiment 1: acute TRP effects

#### Acute consumption of TRP reduced memory retention

In the acute experiment, bees exhibited significant overall learning (trial effect:  $F_{5,2605}=328.36$ ,  $P<0.0001$ , Fig. 1A). There were significant TRP effects on memory (treatment effect:  $F_{4,407}=5.40$ ,  $P=0.0003$ ). In addition, the interaction treatment  $\times$  trial was significant ( $F_{20,2605}=2.51$ ,  $P=0.0002$ ). However, there were no significant pairwise differences between the control treatment and any TRP treatment in any trial (Tukey's HSD,  $P<0.05$ ). The significant interaction arose because the slopes of some learning curves differed between treatments. Colony accounted for <1% of model variance.

As expected, given our nine memory tests, there was a significant decay in memory retention over 17 h (trial effect:  $F_{8,4215}=126.62$ ,  $P<0.0001$ , Fig. 1B). TRP significantly impaired memory retention (treatment effect:  $F_{4,419}=10.30$ ,  $P<0.0001$ ). The interaction treatment  $\times$  trial was significant ( $F_{32,4215}=6.53$ ,  $P<0.0001$ ). Pairwise comparisons revealed that memory retention significantly declined 1 h, 1.17 h and 1.33 h after the last learning trial in bees that fed on 0.5 or 1  $\mu\text{g TRP ml}^{-1}$  as compared with control bees (Tukey's HSD test,  $P<0.05$ , Fig. 1B). There were no significant differences in memory retention at later time points. Colony accounted for <1% of model variance.

A separate model run with just the first memory test (1 h) revealed a similar result: memory was significantly impaired in 0.5  $\mu\text{g TRP ml}^{-1}$  bees as compared with control bees (Tukey's HSD test,  $P<0.05$ ). Thus, 0.5  $\mu\text{g TRP ml}^{-1}$  reduced memory after a single acute exposure (7.5 ng bee $^{-1}$ ) when tested 1–1.33 h after the last learning trial.

### Experiment 2: chronic TRP effects

#### Chronic TRP consumption increased PER non-responsiveness

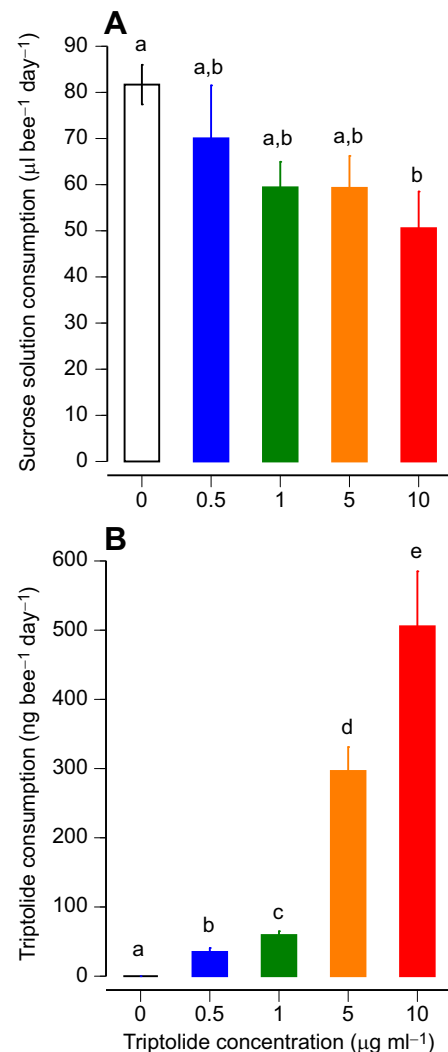
Chronic TRP exposure (7 days) significantly increased the percentage of PER non-responders (23.9 $\pm$ 2.5%) as compared with those exposed acutely for 2 h (1.7 $\pm$ 0.7%, exposure duration: L-R  $\chi^2_1=3.91$ ,  $P=0.048$ ). However, there was no significant effect of TRP concentration ( $\chi^2_1=0.58$ ,  $P=0.44$ ) and no effect of colony ( $\chi^2_2=0.02$ ,

$P=0.99$ ). The interaction TRP concentration  $\times$  exposure duration was also not significant ( $\chi^2_1=0.16$ ,  $P=0.69$ ). Elevated TRP concentrations therefore did not alter non-responsiveness in the acute or chronic experiments.

#### Chronic TRP consumption reduced learning and memory retention

In the chronic experiment, bees showed significant overall learning (trial effect:  $F_{5,2260}=165.41$ ,  $P<0.0001$ , Fig. 1C). TRP significantly impaired this learning (treatment effect:  $F_{4,452}=15.43$ ,  $P<0.0001$ ). The interaction treatment  $\times$  trial was not significant ( $F_{32,3744}=1.35$ ,  $P=0.09$ ). Pairwise comparisons revealed that the control treatment had significantly higher PER than the 5 or 10  $\mu\text{g TRP ml}^{-1}$  treatments in trials 3–6 (Tukey's HSD tests,  $P<0.05$ , Fig. 1C). Colony accounted for <1% of model variance.

Memory retention decreased over time, just as in the acute consumption experiment (trial effect:  $F_{8,3744}=28.86$ ,  $P<0.0001$ , Fig. 1D). TRP reduced memory retention (treatment effect:  $F_{4,465}=8.86$ ,  $P<0.0001$ ). The interaction treatment  $\times$  trial was not



**Fig. 2. Consumption of triptolide (TRP) in 2 mol l $^{-1}$  sucrose solution.**

(A) Mean consumption of treatment solutions by volume per living bee per day (averaged per cage). (B) Mean consumption of TRP (ng bee $^{-1}$  day $^{-1}$ ). Different letters show significant differences (Tukey's HSD tests,  $P<0.05$ ). In total, we tested 675 (0  $\mu\text{g ml}^{-1}$ ), 365 (0.5  $\mu\text{g ml}^{-1}$ ), 370 (1  $\mu\text{g ml}^{-1}$ ), 658 (5  $\mu\text{g ml}^{-1}$ ) and 675 (10  $\mu\text{g ml}^{-1}$ ) bees from three colonies. TRP concentrations follow the color coding used in Fig. 1.

significant ( $F_{32,3744}=1.35$ ,  $P=0.09$ ). Pairwise comparisons of the different treatment doses revealed that 5 and 10  $\mu\text{g TRP bee}^{-1}$  resulted in significantly reduced memory as compared with the control treatment (Tukey's HSD tests,  $P<0.05$ , Fig. 1D). Colony accounted for <1% of model variance. A separate model run with just the 1 h memory data showed a similar result: memory was significantly impaired in 10  $\mu\text{g ml}^{-1}$  bees as compared with control bees (Tukey's HSD test,  $P<0.05$ ).

#### Bees consumed less nectar when it had a high TRP level

There was a significant effect of treatment on sucrose solution consumption per bee per day ( $F_{4,15}=3.09$ ,  $P=0.049$ ). Pairwise comparisons revealed that control bees consumed significantly more pure sucrose solution than sucrose solution with 10  $\mu\text{g TRP ml}^{-1}$  (Tukey's HSD test,  $P<0.05$ , Fig. 2A). Colony accounted for <1% of model variance.

However, bees still consumed higher quantities of TRP when fed with sucrose solutions containing higher concentrations of TRP (treatment effect:  $F_{4,15}=684.29$ ,  $P<0.0001$ , Fig. 2B). All pairwise comparisons were significantly different (Tukey's HSD test,  $P<0.05$ ). Colony accounted for <1% of model variance.

#### Chronic 10 $\mu\text{g TRP ml}^{-1}$ exposure reduced sucrose responsiveness

There was a significant effect of TRP concentration ( $F_{4,474}=3.01$ ,  $P=0.02$ ) on sucrose responsiveness. However, only the highest concentration of 10  $\mu\text{g TRP ml}^{-1}$  significantly decreased sucrose responsiveness as compared with the control (Tukey's HSD test,  $P<0.05$ , Fig. 3). Colony accounted for 3% of model variance.

#### TRP slightly increased bee survival

TRP significantly altered bee survival (L-R  $\chi^2_4=64.86$ ,  $P<0.0001$ , Fig. 4A), but in an unexpected way. For 5  $\mu\text{g TRP ml}^{-1}$  versus the control treatment, there was no significant difference in survival (L-R  $\chi^2_1=0.59$ ,  $P=0.44$ ). However, all other TRP concentrations resulted in slightly improved survival as compared with the control (L-R  $\chi^2_1\geq 8.47$ ,  $P\leq 0.0036^{\text{DS}}$ , Fig. 4B). Over the 7 day trial, control bees lived for a mean of 5.17 days, and bees exposed to concentrations

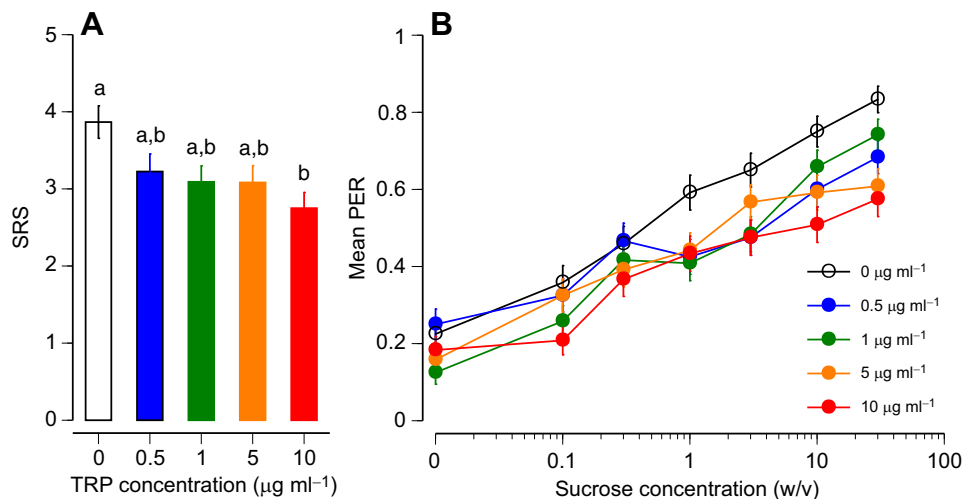
of 0.5–10  $\mu\text{g ml}^{-1}$  had 1–15% longer mean lifespans than control bees. If one considers only the bees that died within each trial (excluding living bees that were frozen at the end of the trial), TRP resulted in similar mean mortality trends (Fig. 4B).

#### DISCUSSION

TRP, a terpenoid that is naturally found in nectar visited by *A. cerana*, significantly impaired olfactory learning at a concentration (0.5  $\mu\text{g ml}^{-1}$ ) found in the honey of bees that collected nectar from *T. hypoglaucum* inflorescences and at higher concentrations that may be found in *T. hypoglaucum* nectar. We examined the effects of both acute and chronic exposure and found, perhaps not surprisingly, a higher overall effect in bees chronically fed TRP. However, the strongest memory effect occurred following acute exposure: memory retention decreased by 56% for the 0.5  $\mu\text{g ml}^{-1}$  dose as compared with the control 1 h after the last learning reinforcement trial (Fig. 1B). In the chronic exposure experiment, learning and memory retention were only impaired at the higher doses of 5 and 10  $\mu\text{g ml}^{-1}$  (Fig. 1D). Interestingly, TRP consumption slightly but significantly increased survival over 7 days (Fig. 4). These results suggest that TRP alone may not account for increased mortality in bees fed *T. hypoglaucum* honey.

#### Acute exposure results

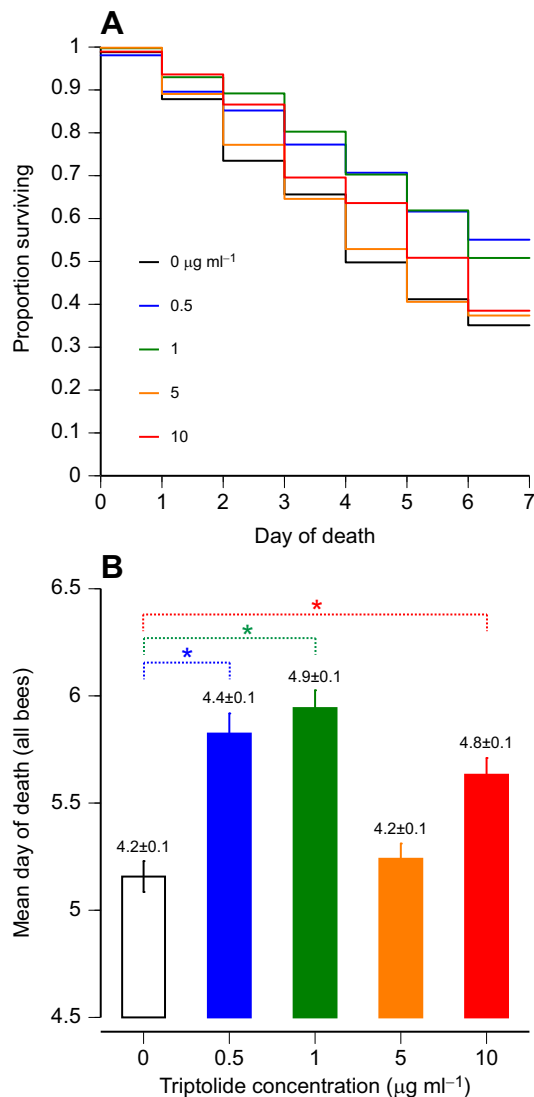
In the acute experiment, TRP did not affect learning, perhaps because a longer period of exposure is necessary, as suggested by our chronic exposure results. However, acute exposure to TRP decreased memory retention when olfactory memory was tested 1–1.33 h after the final learning trial (Fig. 1B). There was a similar pattern of memory decrease with lower TRP concentrations at 1–1.33 h and at 5–5.33 h (Fig. 1B). The differences at 5–5.33 h may not have been significant because memory generally decreased, including in the control group. Memory tests are unrewarded, and the nine memory tests that we conducted may have contributed to this decline. Testing memory fewer times could have resulted in higher memory levels and thus a longer-lasting effect of TRP on memory. Interestingly, our data show that there were no memory



**Fig. 3. Effect of chronic triptolide (TRP) treatments on forager sucrose responsiveness.** Responsiveness was measured (A) as the sucrose responsiveness score (SRS), which is the sum of all proboscis extension reflex (PER) responses to sucrose solutions per bee (different letters indicate significant differences, Tukey's HSD test,  $P<0.05$ ) or (B) as the mean PER responses at each sucrose concentration, using a log scale to better show the data. We show means  $\pm 1$  standard error in each plot. For bees treated chronically with 0, 0.5, 1, 5 and 10  $\mu\text{g TRP bee}^{-1}$ , the mean lowest sucrose concentrations that elicited PER were  $3.0 \pm 0.6\%$ ,  $2.7 \pm 0.7\%$ ,  $3.6 \pm 0.7\%$ ,  $2.1 \pm 0.6\%$  and  $3.0 \pm 0.7\%$  sucrose (w/v), respectively. We tested bees with 0%, 0.1%, 0.3%, 1%, 3%, 10% and 30% sucrose (w/v). The color key gives TRP treatment concentrations and applies to all plots. We replicated our results with three colonies and measured the sucrose responsiveness of 40 bees per TRP treatment per colony (120 bees per TRP concentration) for a total of 600 bees.

effects at higher doses (5 and 10  $\mu\text{g TRP ml}^{-1}$ ). We speculate that acute exposure to higher concentrations of TRP may have stimulated detoxification pathways that were not activated by lower concentrations.

Mustard et al. (Mustard et al., 2012) followed a similar olfactory conditioning design to test the acute effects of caffeine and found that average learning was reduced after the third learning trial. Our results suggest a potential decrease in learning after the third trial following acute consumption of 0.5  $\mu\text{g TRP ml}^{-1}$  (Fig. 1A), although this difference was not significant. Mustard et al. (Mustard et al., 2012) found no effect of caffeine on bee memory, tested 24 h later. We similarly found no significant impairment of memory 18 h after the last learning trial, although our memory trial design could have reduced our ability to discern an effect at this time point (Fig. 1B,D).



**Fig. 4. Triptolide (TRP) slightly increased survival over a 7 day period.** (A) The survival data are shown. (B) The mean day of death (with standard error bars) is shown for all bees. Above each bar, we provide the mean day of death  $\pm 1$  standard error for bees that died within the 7 day trial. Dashed lines with stars connect significantly different treatments as compared with the control ( $P \leq 0.0036$  <sup>Dunn-Sidak corrected</sup>). In total, we tested 675 (0  $\mu\text{g ml}^{-1}$ ), 365 (0.5  $\mu\text{g ml}^{-1}$ ), 370 (1  $\mu\text{g ml}^{-1}$ ), 658 (5  $\mu\text{g ml}^{-1}$ ) and 675 (10  $\mu\text{g ml}^{-1}$ ) bees from three colonies.

### Chronic exposure results

Chronic exposure showed a more expected effect: higher TRP concentrations resulted in progressively poorer learning. Reduced memory likely resulted from this poorer learning. In the chronic experiment, 5 and 10  $\mu\text{g TRP ml}^{-1}$  consistently impaired memory retention (Fig. 1D). However, these doses are much higher than what bees would likely encounter. Chronic TRP consumption could therefore harm learning but only at very high concentrations.

If chronically fed bees were unwilling to feed from sugar solutions with TRP, learning reduction may have resulted from decreased appetitive motivation, not learning impairment. However, only the highest TRP concentration (10  $\mu\text{g TRP ml}^{-1}$ ) significantly reduced sucrose responsiveness as compared with the control treatment (Fig. 3A). The similarity of the mean responses at each sucrose concentration for all lower TRP concentrations and the control (Fig. 3B) suggests that the learning differences observed following exposure to 5  $\mu\text{g TRP ml}^{-1}$  (Fig. 1C) were unlikely to arise from reduced sucrose responsiveness.

Why would there be acute but no chronic effects at 0.5  $\mu\text{g TRP ml}^{-1}$ ? It is possible that honey bees exposed repeatedly over a long period of time to even low levels of TRP can activate detoxification mechanisms. Our chronic memory retention results could have arisen if higher concentrations (5 or 10  $\mu\text{g TRP ml}^{-1}$ ) are too high for this hypothesized mechanism to handle. Although we do not know of a specific mechanism for TRP detoxification in bees, honey bee foragers have enzymes such as glutathione S-transferases and mixed-function oxidases that may break down the toxic compounds found in natural nectar (Smirle and Winston, 1988). Many insects have evolved ways to detoxify plant toxins (Dowd et al., 1983), and *A. cerana* has likely been exposed, over evolutionary time, to *T. hypoglaucum* nectar.

Our survival results differ from Tan et al. (Tan et al., 2007), who found that feeding caged bees honey candy consisting of honey derived from bees foraging on *T. hypoglaucum* nectar and powdered sugar mixed 1:1 by mass (resulting in 0.3  $\mu\text{g TRP g}^{-1}$ ) reduced survival. In contrast, we did not find any decreases in survival but slight, although significant, increases in survival over 7 days (Fig. 4). The dissimilar methods of feeding may account for these differences, particularly if higher water consumption via liquid food is needed to help detoxify TRP. In *Apis mellifera*, different feeding methods can significantly alter the mortality of caged foragers (Abou-Shaara, 2017). Feeding *A. mellifera* sucrose versus honey can influence the survival of bees consuming aflatoxin (Johnson et al., 2012). Based upon these *A. mellifera* results, one might expect honey feeding, as compared with sucrose feeding, to increase survival in *A. cerana*, although our results suggest the opposite. Species differences may play a role.

The slight increase in survival may have arisen from hormesis – a phenomenon in which stressors can stimulate opposite and sometimes beneficial responses at low versus high doses (Guedes and Cutler, 2013). Hormesis is widespread in insects and depends upon multiple factors, particularly the precise exposure levels and durations (Cutler and Rix, 2015). For example, low doses of the pesticide spirotetramat increased drone production in bumble bees but decreased it at higher doses (Ramanaidu and Cutler, 2012). Organic copper salts fed at low concentrations to honey bees can help control *Varroa jacobsoni* infestations but these salts are toxic at higher levels (Bounias et al., 1995). The memory-enhancing effects of caffeine at low doses (Wright et al., 2013) can harm learning at higher doses (Mustard et al., 2012). Another possibility is that some secondary metabolites found in nectar, such as anabasine and gelsemine, can improve survival in bees infected with parasites

(Manson et al., 2010; Richardson et al., 2015). However, our bees came from healthy colonies.

Finally, both our lower and higher doses slightly increased survival, suggesting that we did not test sufficiently long exposure durations, high levels, or both. We speculate that survival benefits from short-term TRP exposure are temporary, given that we only measured its effects over 7 days, and will be reduced when long-term survival is analysed. *Tripterygium hypoglaucum* blooms over a period of 2 months and thus bees could be exposed to TRP for very extended periods. Moreover, individual avoidance of *T. hypoglaucum* honey and colony-level reluctance to recruit for *T. hypoglaucum* honey when other resources are available (Tan et al., 2012) strongly suggest fitness costs to consuming TRP.

Alternatively, it is possible that other compounds found in honey derived from *T. hypoglaucum* nectar (e.g. additional nectar compounds, TRP or other compound degradation products, or both) caused the increased mortality and other effects previously reported (Tan et al., 2007, 2012). For example, *Ranunculus* pollen is toxic to adult honey bees but Sedivy et al. (2012) showed that a major toxic component, ranunculin, did not explain the increased death of bees fed such pollen. Future analyses and experiments should therefore explore toxic potential of other components of *T. hypoglaucum* nectar and honey.

### Ecological significance

Toxic compounds in plant nectar can have multiple ecological roles. Such compounds may deter nectar robbers (Kaczorowski et al., 2014), encourage visitation by more efficient pollinators (Sun and Rychtář, 2015) or reduce herbivory (Adler, 2000). In *T. hypoglaucum*, TRP is present in multiple plant tissues and is at the highest concentrations in stems ( $91.4 \mu\text{g g}^{-1}$ ), leaves ( $17.5 \mu\text{g g}^{-1}$ ) and roots ( $142.6 \mu\text{g g}^{-1}$ ) (Sun et al., 2009). TRP may therefore have evolved primarily to give the plant protection against herbivores, not to influence pollinator behavior.

In our study, we did not provide TRP in the unconditioned stimulus. Thus, we simulated a situation in which foragers were exposed inside the nest to TRP from trophallaxis or from consuming honey with TRP but did not repeatedly visit *T. hypoglaucum* inflorescences. We think this scenario is interesting because it examines the potential harm and effects of TRP on foragers who are primarily pollinating other plants, not *T. hypoglaucum*. Our results suggest that natural TRP concentrations in nectar may not seriously harm plant pollination because lower levels of TRP did not impair honey bee olfactory learning when bees were exposed acutely or chronically (Fig. 1). Lower levels of  $0.5$  and  $1.0 \mu\text{g ml}^{-1}$  impaired memory 1–1.33 h after the last learning trial but this should not pose a major problem for the *T. hypoglaucum* plant because bees, when they have few other resources, will accept nectar with low TRP concentrations and can continue to visit and recruit multiple nestmates for this nectar (Tan et al., 2012). This memory impairment may reduce floral constancy for foragers that are exposed to TRP inside the nest but then visit other plant species. However, if inflorescences of *T. hypoglaucum* or other plants continue to provide nectar, learning should be reinforced, and learning was not impaired (Fig. 1). Future studies examining the efficacy of *A. cerana* in pollinating this species and determining the primary pollinator for *T. hypoglaucum* would be beneficial.

For *A. cerana* colonies, the effects of longer-term TRP exposure remain to be determined. Larvae may be sensitive to TRP, and studies have shown that bees exposed as larvae, even to very small quantities of toxins, can have impaired olfactory learning as adults (Tan et al., 2015; Yang et al., 2012). Toxic nectar may not affect

foragers but could harm brood or young nurse bees (Sharma et al., 1986). Singaravelan et al. (2006) reported that nicotine did not affect the hatching success of larvae and honey bee survival at trace concentrations but had negative effects at higher concentrations.

If *A. cerana* has evolved to use *T. hypoglaucum* when foragers have fewer alternative food choices (Tan et al., 2012), it may have evolved detoxification mechanisms that are not present in allopatric bee species, such as *A. mellifera*. Indeed, it is unclear if *A. mellifera* can recognize and avoid nectar or honey with TRP. We hypothesize that *A. mellifera* will be more cognitively impaired by TRP than *A. cerana* – a prediction that has implications for understanding the co-evolution of plant nectar metabolites and pollinators and for the management of *A. mellifera*, which is now widely reared throughout China (Huang, 2005).

### Acknowledgements

We would like to thank Katrina Klett and the anonymous reviewers for their comments, which have significantly improved the manuscript.

### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: K.T., Z.W., P.W., J.N.; Methodology: Z.W.; Formal analysis: J.N.; Investigation: J.Z., Z.W., Y.Q.; Resources: K.T.; Writing - original draft: K.T., J.Z., Z.W., J.N.; Writing - review & editing: K.T., J.N.

### Funding

K.T. was supported by the Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, the Chinese Academy of Sciences (CAS) 135 program (no. 2017XTBG-T01) and the China National Research Fund (31770420). Z.W. was supported by the China National Research Fund (31772682) and the CAS 'Light of West China' Program (Y6XB081K01).

### Data availability

Data are available from the Dryad Digital Repository (Zhang et al., 2018): doi:10.5061/dryad.c8b66

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