First posted online on 22 January 2018 as 10.1242/jeb.168344
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.168344

The reluctant visitor: an alkaloid in toxic nectar can reduce olfactory learning and memory in Asian honey bees

Junjun Zhang[1,2], Zhengwei Wang[1*], Ping Wen[1], Yufeng Qu[1], Ken Tan[1*], James C. Nieh[3]

1. Chemical Ecology Group, Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming 650000, China

2. University of Chinese Academy of Sciences, Beijing, 100049, China

3. Division of Biological Sciences Section of Ecology, Behavior, and Evolution University of California, San Diego La Jolla, California, USA

*Author for correspondence.

Email: kentan@xtbg.ac.cn, wangzhengwei@xtbg.ac.cn

SUMMARY STATEMENT

Triptolide, the hypothesized toxic compound in nectar of the thunder god vine, can reduce bee memory of a rewarded odor.

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, 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. Chronic exposure did not alter learning or memory, except at high concentrations (5 and 10 μg TRP/ml). 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, alkaloids, toxic honey, *Apis cerana*, olfactory learning, memory

INTRODUCTION

From 8-36% 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 (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 to 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 (Tan pers. comm.) 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* since 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* foragers have good olfactory learning (Wang and Tan, 2014), but no studies have examined how TRP may alter their learning and memory. Caffeine, another alkaloid, 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 *A. cerana*, each maintained in a separate single-story wood box at an experimental apiary at the Southwest Biodiversity Research Center (Kunming, China). Each colony contained approximately 12,000 bees. We conducted experiments from March to June of 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 triptolide concentrations used

An analysis of five *A. cerana* colonies found that honey produced from *T. hypoglaucum* nectar has an average of 0.6 μg TRP/g (0.61±0.11 μg/g = 0.87 μg/ml, based upon an *A. cerana* honey density of 1.32 g/ml) (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 2-14 ng/g (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 μg/ml. If so, concentrations ranging up to 10 μg TRP/ml (assuming 90% degradation) may be ecologically relevant.

Exp. 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°C, 65% relative humidity) 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±0.7 µl nectar per bee. We therefore fed our bees with 15 µl of sugar solution, a slightly lower volume, to ensure full consumption by all bees. The control group was fed with 15 µl of 2 M (55% sucrose w/v) pure sucrose syrup. In the other groups, each bee was fed with 15 µl of 2 M sucrose syrup that contained 0.5, 1, 5, 10 µg TRP/ml. Bees therefore received 7.5, 15, 75, or 150 ng TRP/bee. We then waited 2 h to allow bees to recover their hunger levels, since feeding motivation influences rewarded olfactory learning (Giurfa and Sandoz, 2012). We replicated these treatments three times with each colony, testing the learning of approximately 30 bees/treatment/colony. In total, we tested the acute effects with 526 bees (sample size details in Fig. 1 legend).

Exp. 2: Chronic exposure

We collected approximately 100 foragers per trial from each focal colony with clean glass tubes (see experiment 1) and transferred them to a polystyrene foam micro-hive (25 × 12 × 12 cm) with one small piece of empty comb (10 cm × 5 cm) hanging inside. Into this microhive, we placed a 10-ml horizontal feeding tube that provided one of the following treatments in 2 M sucrose solution: 0, 0.5, 1, 5, or 10 µg TRP/ml. We provided only one treatment per microhive. We placed this micro-hive into an incubator at 25°C and 65% relative humidity. After 7 d 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 micro-hives and feeding tubes in the same incubator under the same conditions and measured evaporative weight loss each day over 7 d. We then corrected our bee daily mean consumption measurements to account for this evaporation. To assess learning and survival, we tested approximately 30 bees/treatment/colony, 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 µl 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 mL/s) into a PFTE tube. Each individual bee was placed 1 cm away from the outlet of a polyfluorotetraethylene (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 M 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 intertrial interval of 10 min (Menzel, 2001) during the learning phase. Detailed memory 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 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) over 7 d. 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 wood 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 analysed learning and longer-term memory and used a Repeated-Measures Analysis of Variance (ANOVA, REML algorithm) with colony as a random effect and all other effects (treatment and trial) fixed. Although such data can be analysed 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 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 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 Log-Rank (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). Because each trial ended after 7 d, we show data on two different measures of survival: the mean day of death of bees that died within the 7-d 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 mean±1 standard error.

RESULTS

Exp. 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*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 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*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 µg TRP/ml as compared to control bees (Tukey 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 µg TRP/ml bees as compared to control bees (Tukey HSD test, P<0.05). Thus, 0.5 µg TRP/ml reduced memory after a single acute exposure (7.5 ng/bee) when tested 1-1.33 h after the last learning trial.

Exp. 2: Chronic TRP effects

Chronic TRP consumption increased PER non-responsiveness

Chronic TRP exposure (7 d) significantly increased the percentage of PER non-responders (23.9±2.5%) as compared to those exposed acutely for 2 h (1.7±0.7%, exposure duration: L-R χ^2_1=3.91, P=0.048). However, there was no significant effect of TRP concentration (χ^2_1=0.58, P=0.44) and no effect of colony (χ^2_2=0.02, P=0.99). The interaction TRP concentration*exposure duration was also not significant (χ^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*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 µg TRP/ml treatments in trial 3-6 (Tukey 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*trial was not significant ($F_{32,3744}$=1.35, P=0.09). Pairwise comparisons of the different treatment doses revealed that 5 and 10 µg TRP/bee resulted in significantly reduced memory as compared to the control treatment (Tukey 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 µg/ml bees as compared to control bees (Tukey 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 µg TRP/ml (Tukey 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 HSD test, P<0.05). Colony accounted for <1% of model variance.

Chronic 10 μg TRP/ml exposure reduced sucrose responsiveness

There was a significant effect of TRP concentration ($F_{4,474}$=3.01, P=0.02). However, only the highest concentration of 10 μg TRP/ml significantly decreased sucrose responsiveness as compared to the control (Tukey 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 χ^2_4=64.86, P<0.0001, Fig. 4A), but in an unexpected way. For 5 μg TRP/ml vs. the control treatment, there was no significant difference in survival (L-R χ^2_1=0.59, P=0.44). However, all other TRP concentrations resulted in slightly improved survival as compared to the control (L-R $\chi^2_1 \geq 8.47$, $P \leq 0.0036^{DS}$, Fig. 4B). Over the 7-day trial, control bees lived for a mean of 5.17 d, and bees exposed to concentrations of 0.5-10 μg/ml 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

Triptolide (TRP), an alkaloid that is naturally found in nectar visited by *A. cerana*, significantly impaired olfactory learning at a concentration (0.5 μg/ml) 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 μg/ml dose as compared to 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 μg/ml (Fig. 1D). Interestingly, TRP consumption slightly but significantly increased survival over 7 d (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 effects at higher doses (5 and 10 μg TRP/ml). 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 3rd learning trial. Our results suggest a potential decrease in learning after the 3rd trial following acute consumption of 0.5 μg TRP/ml (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).

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 μg TRP/ml 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 μg TRP/ml) significantly reduced sucrose responsiveness as compared to 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 μg TRP/ml (Fig. 1C) were unlikely to arise from reduced sucrose responsiveness.

Why would there be acute, but no chronic effects at 0.5 μg TRP/ml? 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 μg TRP/ml) 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 μg TRP/g) reduced survival. In contrast, we did not find any decreases in survival, but slight, though significant, increases in survival over 7 d (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 *A. mellifera*, different feeding methods can significantly alter the mortality of caged foragers (Abou-Shaara, 2017). Feeding *A. mellifera* sucrose vs. 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 to sucrose feeding, to increase survival in *A. cerana*, though 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 d, and will be reduced when long-term survival is analysed. *T. hypoglaucum* blooms over two 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—additional nectar compounds, TRP or other compound degradation products, or both—caused the increased mortality and other effects previously reported (Tan et al., 2007; Tan et al., 2012). For example, *Ranunculus* pollen is toxic to adult honey bees, but Sedivy et al. (Sedivy et al., 2012) showed that a major toxic component, ranunculin, did not explain the increased death of bees fed such pollen (Sedivy et al., 2012). 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 μg/g), leaves (17.5 μg/g), and roots (142.6 μg/g) (Sun et al., 2009). TRP may therefore have evolved primarily to give the plant protection against herbivores, not to influence pollinator behaviour.

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 μg/ml 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 honeybee 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 this manuscript.

COMPETING INTERESTS

All authors declare that they have no competing interests.

FUNDING

This work was supported by the Key Laboratory of Tropical Forest Ecology, Xishuangbanna Tropical Botanical Garden, the CAS 135programm(No.2017XTBG-T01), China National Research Fund (31770420) to Ken Tan, China National Research Fund (31772682) and CAS "Light of West China" Program (Y6XB081K01) to Zhengwei Wang.

AUTHOR CONTRIBUTIONS

ZW, PW, KT, and JN conceived of and designed the experiments. JZ, ZW, and YQ performed the experiments. ZW and JN analysed the data. ZW, PW, KT, and JN contributed reagents, materials, or analysis tools. ZW, JZ, KT, and JN wrote the paper.

DATA AVAILABILITY

All data are available as a supplemental file included with this paper.

REFERENCES

Abou-Shaara, H. F. (2017). Effects of various sugar feeding choices on survival and tolerance of honey bee workers to low temperatures. *J Entomol Acarol Res* **49**, 1–11.

Adler, L. S. (2000). The ecological significance of toxic nectar. *Oikos* **91**, 409–420.

Baker, H. G. (1977). Non-sugar chemical constituents of nectar. *Apidologie* **8**, 349–356.

Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107–119.

Bounias, M., Navone-Nectoux, M. and Popeskovic, D. S. (1995). Toxicology of cupric salts in honeybees. I. Hormesis effects of organic derivatives on lethality parameters. *Ecotox. Environ. Safe.* **31**, 127–132.

Brinker, A. M. and Raskin, I. (2005). Determination of triptolide in root extracts of *Tripterygium wilfordii* by solid-phase extraction and reverse-phase high-performance liquid chromatography. *Journal of Chromatography A* **1070**, 65–70.

Carr-Markell, M. K. and Robinson, G. E. (2014). Comparing reversal-learning abilities, sucrose responsiveness, and foraging experience between scout and non-scout honey bee (*Apis mellifera*) foragers. *J. Ins. Behav.* **27**, 736–752.

Couvillon, M. J., Toufailia, Al, H., Butterfield, T. M., Schrell, F., Ratnieks, F. L. W. and Schürch, R. (2015). Caffeinated forage tricks honeybees into increasing foraging and recruitment behaviors. *Curr. Biol.* **25**, 2815–2818.

Cutler, G. C. and Rix, R. R. (2015). Can poisons stimulate bees? Appreciating the potential of hormesis in bee-pesticide research. *Pest. Manag. Sci.* **71**, 1368–1370.

Dornhaus, A. and Chittka, L. (2004). Information flow and regulation of foraging activity in bumble bees (*Bombus* spp.). *Apidologie* **35**, 183–192.

Dowd, P. F., Smith, C. M. and Sparks, T. C. (1983). Detoxification of plant toxins by insects. *Insect Biochemistry* **13**, 453–468.

Frisch, von, K. (1967). *The dance language and orientation of bees*. 2nd ed. Cambridge, Massachusetts: Belknap Press.

Giurfa, M. and Sandoz, J. C. (2012). Invertebrate learning and memory: fifty years of olfactory conditioning of the proboscis extension response in honeybees. *Learn. Mem.* **19**, 54–66.

Goyal, S. (2013). Ecological role of alkaloids. In *Natural Products: Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes*, pp. 149–171. Berlin, Heidelberg: Springer Berlin Heidelberg.

Guedes, R. N. C. and Cutler, G. C. (2013). Insecticide-induced hormesis and arthropod pest

management. *Pest. Manag. Sci.* **70**, 690–697.

Huang, Y. G. (2005). Harm of introducing the western honeybee *Apis mellifera* L. to the Chinese honeybee *Apis cerana* F. and its ecological impact. *Acta Entomol. Sinica* **48**, 401–406.

Irwin, R. E., Adler, L. S. and Brody, A. K. (2004). The dual role of floral traits: Pollinator attraction and plant defense. *Ecology* **85**, 1503–1511.

Johnson, R. M., Mao, W., Pollock, H. S., Niu, G., Schuler, M. A. and Berenbaum, M. R. (2012). Ecologically appropriate xenobiotics induce cytochrome P450s in *Apis mellifera*. *PLOS ONE* **7**, e31051–9.

Kaczorowski, R. L., Koplovich, A., Sporer, F., Wink, M. and Markman, S. (2014). Immediate effects of nectar robbing by Palestine sunbirds (*Nectarinia osea*) on nectar alkaloid concentrations in tree tobacco (*Nicotiana glauca*). *J. Chem. Ecol.* **40**, 325–330.

Kretschmar, J. A. and Baumann, T. W. (1999). Caffeine in *Citrus* flowers. *Phytochem.* **52**, 19–23.

London-Shafir, I., Shafir, S. and Eisikowitch, D. (2003). Amygdalin in almond nectar and pollen – facts and possible roles. *Plant Syst. Evol.* **238**, 87–95.

Manson, J. S., Otterstatter, M. C. and Thomson, J. D. (2010). Consumption of a nectar alkaloid reduces pathogen load in bumble bees. *Oecologia* **162**, 81–89.

Matsumoto, Y., Sandoz, J.-C. and Giurfa, M. (2012). Revisiting olfactory classical conditioning of the proboscis extension response in honey bees: A step toward standardized procedures. *J. Neurosci. Method.* **211**, 159–167.

Menzel, R. (2001). Massed and spaced learning in honeybees: The role of CS, US, the intertrial interval, and the test interval. *Learn. Mem.* **8**, 198–208.

Mustard, J. A., Dews, L., Brugato, A., Dey, K. and Wright, G. A. (2012). Consumption of an acute dose of caffeine reduces acquisition but not memory in the honey bee. *Behav. Brain Res.* **232**, 217–224.

Nieh, J. C. (2004). Recruitment communication in stingless bees (Hymenoptera, Apidae, Meliponini). *Apidologie* **35**, 159–182.

Ramanaidu, K. and Cutler, G. C. (2012). Different toxic and hormetic responses of *Bombus impatiens* to *Beauveria bassiana*, *Bacillus subtilis* and spirotetramat. *Pest. Manag. Sci.* **69**, 949–954.

Richardson, L. L., Adler, L. S., Leonard, A. S., Andicoechea, J., Regan, K. H., Anthony, W. E., Manson, J. S. and Irwin, R. E. (2015). Secondary metabolites in floral nectar reduce parasite infections in bumblebees. *Proc. Roy. Soc. B* **282**, 20142471–20142471.

Roubik, D. W. (1995). *Pollination of cultivated plants in the tropics. No. 118*. Food & Agriculture Organization.

Sedivy, C., Piskorski, R., Müller, A. and Dorn, S. (2012). Too low to kill: concentration of the secondary metabolite ranunculin in buttercup pollen does not affect bee larval survival. *J. Chem. Ecol.* **38**, 996–1002.

Sharma, O. P., Raj, D. and Garg, R. (1986). Toxicity of nectar of tea (*Camellia thea* L.) to honeybees. *J. Apic. Res.* **25**, 106–108.

Simcock, N. K., Gray, H. E. and Wright, G. A. (2014). Single amino acids in sucrose rewards modulate feeding and associative learning in the honeybee. *J. Ins. Phys.* **69**, 41–48.

Singaravelan, N., Inbar, M., Ne'eman, G., Distl, M., Wink, M. and Izhaki, I. (2006). The effects of nectar–nicotine on colony fitness of caged honeybees. *J. Chem. Ecol.* **32**, 49–59.

Smirle, M. J. and Winston, M. L. (1988). Detoxifying enzyme activity in worker honey bees: an adaptation for foraging in contaminated ecosystems. *Can. J. Zool.* **66**, 1938–1942.

Sun, S. and Rychtář, J. (2015). The screening game in plant–pollinator interactions. *Evol. Ecol.* **29**, 479–487.

Sun, Y. M., Xu, J. Z., Wang, Z. A. and Yu, X. P. (2009). Determination of triptolide in diifferent ages of root and different parts of *Tripterygium wilfordii* Hook f. by HPLC. *Chin J Mod Appl Pharm* **26**, 904–906.

Tan, K., Chen, W., Dong, S., Liu, X., Wang, Y. and Nieh, J. C. (2015). A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. *Sci. Rep.* **5**, 1–8.

Tan, K., Guo, Y. H., Nicolson, S. W., Radloff, S. E., Song, Q. S. and Hepburn, H. R. (2007). Honeybee (*Apis cerana*) foraging responses to the toxic honey of *Tripterygium hypoglaucum* (Celastraceae): changing threshold of nectar acceptability. *J. Chem. Ecol.* **33**, 2209–2217.

Tan, K., Wang, Z., Yang, M., Fuchs, S., Luo, L., Zhang, Z., Li, H., Di Zhuang, Yang, S., Tautz, J. R., et al. (2012). Asian hive bees, *Apis cerana*, modulate dance communication in response to nectar toxicity and demand. *Anim. Behav.* **84**, 1589–1594.

Thomson, J. D., Draguleasa, M. A. and Tan, M. G. (2015). Flowers with caffeinated nectar receive more pollination. *Arth. Plant Interac.* **9**, 1–7.

Wang, Z. and Tan, K. (2014). Comparative analysis of olfactory learning of *Apis cerana* and *Apis mellifera*. *Apidologie* **45**, 45–52.

Williams, G. R., Alaux, C., Costa, C., Csáki, T., Doublet, V., Eisenhardt, D., Fries, I., Kuhn, R., McMahon, D. P., Medrzycki, P., et al. (2013). Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions. *J. Apic. Res.* **52**.

Wright, G. A. and Schiestl, F. P. (2009). The evolution of floral scent: the influence of

olfactory learning by insect pollinators on the honest signalling of floral rewards. *Func. Ecol.* **23**, 841–851.

Wright, G. A., Baker, D. D., Palmer, M. J., Stabler, D., Mustard, J. A., Power, E. F., Borland, A. M.Stevenson, P. C. (2013). Caffeine in floral nectar enhances a pollinator's memory of reward. *Science* **339**, 1202–1204.

Yang, E.-C., Chang, H.-C., Wu, W.-Y. and Chen, Y.-W. (2012). Impaired olfactory associative behavior of honeybee workers due to contamination of imidacloprid in the larval stage. *PLOS ONE* **7**, e49472.

Zar, J. H. (1984). *Biostatistical analysis*. Englewood Cliffs, New Jersey: Prentice-Hall.

Figures

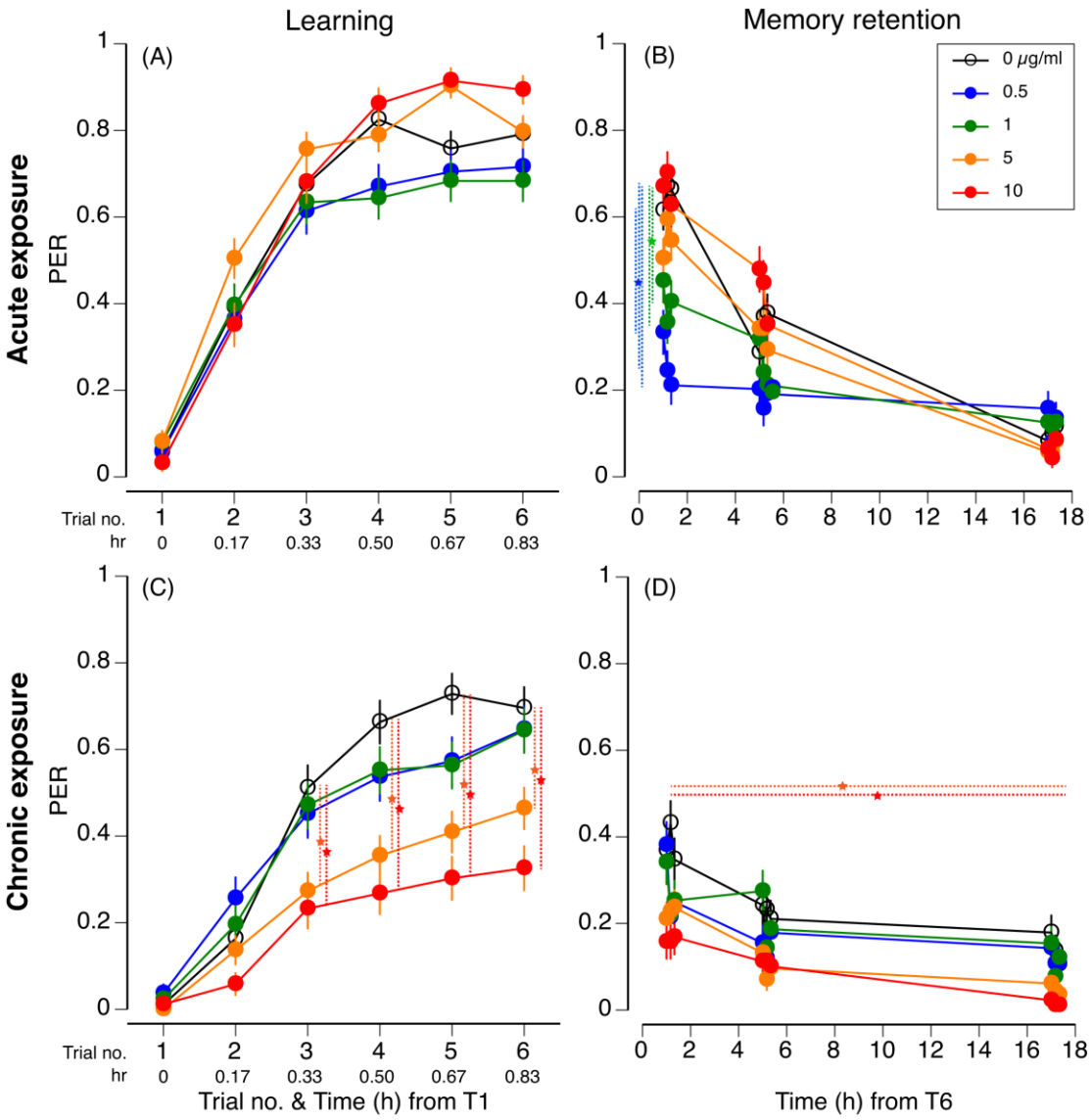

Figure 1. Triptolide (TRP) altered olfactory learning (PER) and memory retention in *A. cerana* foragers exposed (A, B) *acutely* (2 h before testing: 120 bees at 0μg/ml, 88 bees at 0.5μg/ml, 101 bees at 1μg/ml, 123 bees at 5μg/ml, and 94 bees at 10μg/ml) or (C, D) *chronically* (over 7 days: 92 bees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

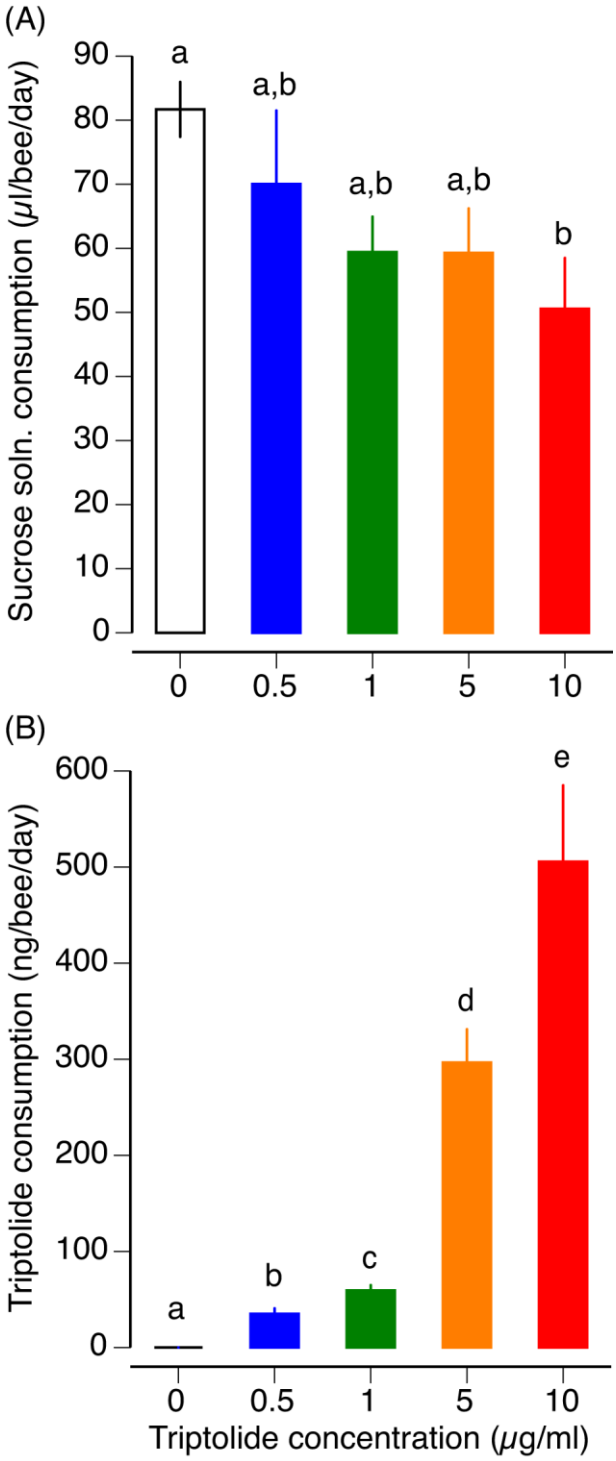

Figure 2. Consumption of triptolide (TRP) in 2 mol/l sucrose solution. (A) Mean consumption of treatment solutions by volume per living bee per day (averaged per cage). (B) Mean consumption of TRP (ng/bee/day). Different letters show significant differences (Tukey HSD tests, $P<0.05$). In total, we tested 675 (0 µg/ml), 365 (0.5 µg/ml), 370 (1 µg/ml), 658 (5 µg/ml), and 675 (10 µg/ml) bees from three colonies. TRP concentrations follow the color coding used in Fig. 1.

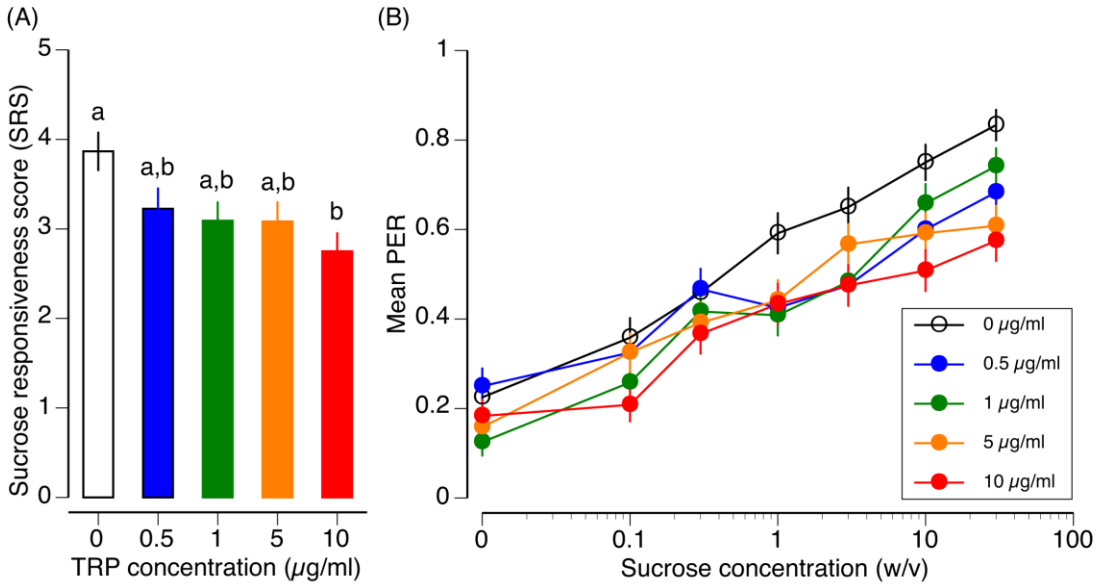

Figure 3. Effect of chronic TRP treatments on forager sucrose responsiveness (A) measured as the sucrose responsiveness score (SRS), which is the sum of all PER responses to sucrose solutions per bee (different letters indicate significant differences, Tukey HSD test, $P<0.05$) or (B) as the mean PER responses at each sucrose concentration, using a log scale to better show the data. We show mean±1 standard error in each plot. For bees treated chronically with 0, 0.5, 1, 5, and 10 μg TRP/bee, the mean lowest sucrose concentrations that elicited PER were 3.0±0.6, 2.7±0.7, 3.6±0.7, 2.1±0.6, and 3.0±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 legend 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.

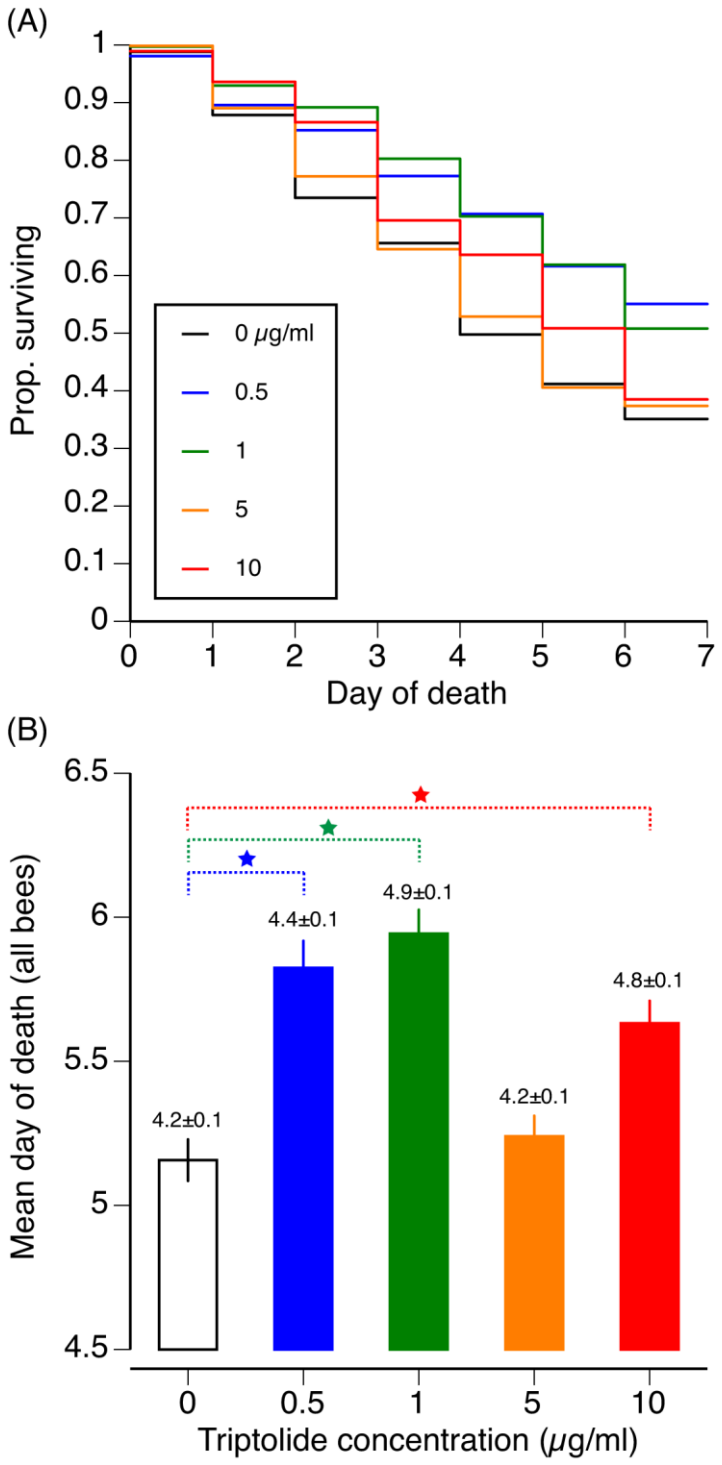

Figure 4. Triptolide (TRP) slightly increased survival over a 7-d period. (A) The survival data are shown. (B) The mean day of death (with standard error bars) is shown for all bees. Above each bar, we provide the mean day of death±1 standard error for bees that died within the seven-day trial. Dashed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