Research Article

Received: 25 October 2018 Revised: 26 February 2019 Accepted article published: 18 March 2019 Published online in Wiley Online Library: 13 April 2019

(wileyonlinelibrary.com) DOI 10.1002/ps.5411

Impact of acute oral exposure to thiamethoxam on the homing, flight, learning acquisition and short-term retention of *Apis cerana*

Changsheng Ma,[a] Yongkui Zhang,[a,b] Jia Sun,[a] Muhammad Imran,[c] Huipeng Yang,[a] Jie Wu,[a] Yi Zou,[d*] Hongmei Li-Byarlay[e] and Shudong Luo[a*]

ABSTRACT

BACKGROUND: Thiamethoxam (TMX) represents the second generation of neonicotinoids that has been widely applied in agricultural activities, while how TMX alters the behavior of *Apis cerana*, an important native honey bee species in China, is not clear. We carried out three independent experiments to study the impact of acute oral treatment of 20 μL TMX at concentrations of 2.4 ppb (0.048 ng/bee) and 10 ppb (0.2 ng/bee) on the homing, flight, learning acquisition and short-term retention ability of *A. cerana*. The homing ability was assessed by the catch-and-release method, the flight ability was assessed by flight mills, and the learning acquisition and short-term retention were evaluated by the proboscis extension response method.

RESULTS: When treated with 10 ppb of TMX, bees had a significantly higher average homing time, mean flight velocity, flying distance, and flying duration than the control, whereas 2.4 ppb concentration did not cause any significant effect on homing or flight ability. Bees treated with either 2.4 ppb or 10 ppb TMX had significantly lower learning acquisition and short-term retention ability.

CONCLUSION: Results suggest that acute oral exposure to 10 ppb of TMX altered the short-distance homing time, flight ability, and learning acquisition and short-term retention ability. Our study also highlights the concern that acute oral exposure to a low concentration of 2.4 ppb could have consequences on the behavior of *A. creana*. Those multiple sublethal alterations on *A. cerana's* behavior indicate that TMX are likely having complex but negative consequences on bee health in the field.

Supporting information may be found in the online version of this article.

Keywords: eastern honey bee; flight velocity; homing rate; homing time; proboscis extension response; TMX

1 INTRODUCTION

The honey bee (*Apis* spp.) is one of the most important pollinator groups in the agriculture ecosystem, providing approximately 50% of global crop pollination services.[1,2] The health of honey bee colonies, however, is threatened by the application of pesticides, of which the wide application of neonicotinoids is particularly concerning.[3–7] The Chinese honey bee (also called the eastern honey bee), *Apis cerana*, is an important pollinator species that can be both managed and feral in most of China, providing important pollination services for not only cultivated crops but also wild flowers.[8–10] Compared with the largest commercially managed bee groups, *A. millifera*, the health of *A. cerana* has received much less attention.[11]

Neonicotinoids are a series of neurotoxins that target the central nervous system of insects and have been widely applied as a seed treatment, soil treatment, granular application, dipping of seedlings, by mixing with irrigation water, or through direct spraying in the field.[12] Residues of neonicotinoids are therefore commonly found in water, soil, pollen, and nectar,[13–16] and they have been implicated in the decline of bee populations.[3,5] One representative of the second generation of neonicotinoids is thiamethoxam (TMX), which occupies one of the largest neonicotinoid markets.[12] Likewise, residues of TMX are widely detected in nectar and pollen,[13–19] while the concentration of nectar and pollen collected by bumblebee and honey bee can vary from 0.72 ppb[20] to as high as over 663.8 ppb.[21] Although in China the investigation of residuals of field-realistic levels for TMX is very limited, it has been reported that the level in the beebread of major beekeeping areas was 12.8 ppb.[21]

* *Correspondence to: Y Zou, Department of Health and Environmental Sciences, Xi'an-Jiaotong Liverpool University, Suzhou, China. E-mail: yi.zou@xjtlu.edu.cn or S Luo, Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China. E-mail: luoshudong@caas.cn*

a *Key Laboratory of Pollinating Insect Biology, Ministry of Agriculture, Institute of Apicultural Research, Chinese Academy of Agricultural Sciences, Beijing, China*

b *Guangxi Key Laboratory for Agro-Environment and Agro-Product Safety, Guangxi University, Nanning, China*

c *Department of Entomology, University of Poonch Rawalakot, Azad Jammu and Kashmir, Pakistan*

d *Department of Health and Environmental Sciences, Xi'an-Jiaotong Liverpool University, Suzhou, China*

e *Agricultural Research and Development Program, Central State University, Wilberforce, OH, USA*

Although pesticide authorization procedures require the dose of a pesticide residue in the field to be below the lethal level for the honey bee, a sublethal dose (i.e. one that does not directly cause mortality) may still have significant impact on the foraging behavior and survival of honey bees, by affecting their homing ability,[22,23] flight ability[24], learning acquisition, and short-term retention.[25–32] For example, Henry *et al.*[22] reported that TMX resulted in a reduction of about 20% in the homing ratio for *A. mellifera*, while Stanley *et al.*[33] reported a reduction of 26% in the homing ratio for bumblebee (*Bombus terrestris*), but no significant effect on the homing ability overall. It has also been reported that chronic oral exposure of TMX reduced *A. mellifera* flight distance by 56%, but acute oral TMX treatment increased flight distance by 72%.[24] In addition, studies also reported that TMX impairs learning acquisition and short-term retention and spatial working memory for bumble bees.[25,26] According to our knowledge, there is no study about the effect of TMX on the homing, flight, learning acquisition, and short-term retention abilities of *A. cerana*.

We conducted three independent experiments to investigate the influences of acute oral exposure to TMX on the homing, flight and learning acquisition and short-term retention abilities of *A. cerana*. We hypothesize that acute oral exposure to TMX will reduce (i) the homing ability, (ii) the flight ability, and (iii) the learning acquisition and short-term retention of *A. cerana*.

2 MATERIALS AND METHODS

2.1 Pesticide and honey bee preparation

Two field-realistic TMX concentration levels, 2.4 parts per billion (ppb) and 10 ppb, were selected. The lower concentration was based on pollen collected by honey bees[13] and nectar pots of bumblebee colonies in UK agricultural areas,[20] while the high concentration was measured in the pollen and nectar of many wildflowers and treated crops.[16,34] Both these concentrations have been used previously in studying the effects of TMX on the behavior of bumblebees.[25,26,35] Pesticide solution was made by dissolving 0.3125 g TMX (Hailir Pesticides and Chemicals Group, CAS No.153719-23-4, purity of 96.00% (The most current product has a purity of 98%.)) in 10 mL acetone to make a 0.03 g mL^{-1} pesticide solution, and then diluted with 40% (w/w) sucrose solution to make a concentration of 2.4 ppb and 10 ppb TMX contaminated sucrose solution. A control sucrose solution without TMX was also prepared. The concentration of acetone in both TMX and control treatments was adjusted to the same level, which was 1 mg kg^{-1}, a concentration that has little influence on honey bees.[36]

Healthy experimental beehive colonies were reared at the Institute of Apicultural Research, Chinese Academy of Agricultural Sciences (IARCAAS, 40° 0.362′ N, 116° 11.601′ E), where the study was conducted. To capture forgers, we first closed the entrance and then collected bees by a clean plastic tube at the entrance. To avoid selecting young honey bees, only foragers with pollen loads were captured. Captured bees were minimally chilled on ice (1.5 min; see Tosi *et al.*[24]) and marked with a dot of paint (Testors Enamel, Rockford, IL) on the dorsal surface of the thorax to ensure that all experimental bees were used only once. Marked experimental bees were harnessed on a bottom-removed tube that had a similar width to the bee's head which was able to fix the bee (see Supporting information Appendix S1(a)). After harnessing, bees were starved for 2 h, and then fed 20 µL of either the 40% (w/w) sucrose solution containing 2.4 ppb or 10 ppb of TMX, which was equivalent to a TMX dose of about 0.48 ng or 0.2 ng, respectively, or the control solution. After feeding treatment, marked experimental bees were monitored for 30 min in order to have a complete absorption, and then we commenced the following experiments.

2.2 Experiment 1: Homing ability assessment

The homing ability of *A. cerana* was assessed by the catch-and-release method. In each trial, a 15 × 10 × 12 cm box containing either 25 or 30 marked experimental bees was opened to release the bees simultaneously at 50 m away from the hive. The time between release and entering the hive was recorded for each marked experimental bee.

We conducted a preliminary test to decide for how long we should record after releasing the bees in the experiment. In the preliminary test (for the control treatment), we recorded at 10-min intervals for nine experimental times, with results showing that no marked experimental bees entered the hive after 5 min (homing ratio [mean ± SE]: 97.4% ± 0.5%), with a maximum homing time of 282 s. Thus, the recording was stopped at 5 min during the experiment.

A total of 795 experimental forgers were obtained from three colonies, with each colony tested on three separate trial days. Thus, the experiment was conducted nine times on nine separate trial days. On each trial day, all treatments were conducted once (with either 25 or 30 marked experimental bees released each time). All experiments were carried out on a sunny day (10:00 and 16:00 with the outdoor temperature between 22 and 32 °C) between August and October in 2017 at the IARCAAS.

2.3 Experiment 2: Flight ability assessment

The flight ability of bees was evaluated by flight mills[24,37] (Jiaduo Industry & Trade Co., Ltd, Hebi, China, FXM-2). This procedure was identical to the previous work by Tosi *et al.*.[24] A 1-cm-long hollow Teflon tube was glued to the top of the thorax of each bee and connected to the flight mill (Supporting information Appendix S1(b)). As the glued bees flew on the flight mill, a computer connected to the flight mill sensor recorded maximum and mean velocity, flight duration, and flight distance. All flight mills were located in the same room, with constant light (546.2 ± 1.0 lx) and air temperature (25 ± 1 °C).

Three colonies were used in this experiment, and each colony was tested for six separate trial days. Thus, the experiment was conducted on 18 separate trial days. On each trial day, the captured foragers from the same colony were separated into three groups randomly and fed with 0, 2.4, and 10 ppb TMX contaminated sucrose solution. Seven or eight foragers from these groups were selected to evaluate the effect of TMX on the flight abilities of foragers with the flight mills. In total, the number of experimental bees for each treatment was 133, 135 and 136 for the control, 2.4 ppb, and 10 ppb treatments, respectively.

2.4 Experiment 3: Learning acquisition and short-term retention ability assessment

The learning acquisition and short-term retention of bees were assessed by the proboscis extension response (PER) method.[38] A lemon odor was used as the conditioned stimulus, and a 40% sucrose solution (w/w) reward was used as the unconditioned stimulus (see Li *et al.*[39]). Each experimental forager was tested in six PER trials. The first five trials consisted of reinforced learning, to test short-term learning acquisition, with a 10-min interval in between. Experimental foragers were exposed to the lemon odor for 3 s, and then immediately exposed to both the lemon odor and the sucrose solution for another 3 s. The timing and duration of

the lemon odor and sucrose solution were accurately controlled by an air-pump device.[39] The sixth trial was performed 2 h after the fifth trial to test the short-term retention ability. Bees were exposed to the lemon odor for 3 s without a sucrose solution reward. In all trials, instances of bees showing PER during exposure to the lemon odor were recorded.

In total, 181 foragers were obtained from three colonies (71, 33, and 77 for each colony, respectively). Bees from each colony were evenly and randomly separated into three groups that received a different treatment, resulting in total treated forgers of 59, 60, and 62 bees for 2.4 ppb, 10 ppb, and the control, respectively.

2.5 Data analysis

Linear mixed-effect models (LMM) and generalized linear mixed models (GLMM) were used to test the impact of the TMX treatment on the homing ability of honey bees. Response variables were the flight time of each experimental honey bee to the hive (LMM, Gaussian error distribution with the 'identity' link function) and the accumulated proportion of bees homing successfully within a certain time per sample (GLMM, binomial error distribution with the 'logit' link function). We separately analyzed our 5-min records in a 20 s interval in order to understand the treatment influence for an early or late homing stage, allowing us to have 30 independent analyses at setting accumulated time. Treatment was added as the fixed explanatory variable and the date of the experiment was the random variable. We additionally included colony as a fixed variable in the analysis, and the model showed this variable was not significant ($P > 0.05$). We therefore excluded colony and reported results from the original model.

GLMM were also used to test the impact of TMX on the flight ability of bees. Response variables were (i) the maximum velocity, (ii) the mean velocity, (iii) the total flying time, and (iv) the total flying distance, of which variables (iii) and (iv) were log transformed to fit the normality assumption. Treatment was the fixed explanatory variable and the date of the experiment was the random variable. Colony was again excluded because this factor did not have a significant influence on either response variable when added as a fixed effect factor ($P > 0.05$). A Gaussian error distribution with the 'identity' link function was applied for all models as the (transformed) data fit the normality assumption.

To test the TMX treatment on the bees' learning acquisition and short-term retention ability, we first applied a generalized linear model (GLM) to test the effect of treatment on the colony, and the response variable was the ratio of the number of bees showing PER to the number not showing PER. We pooled data from three colonies in order to have a more robust dataset. Fisher's exact test for testing count data (because the overall tested number was relatively small, see McDonald[40]) was then performed to compare TMX treatments and control. This test was applied separately for each different trial dose.

All analyses were performed in R language,[41] with the 'nlme' package for LMM,[42] and the 'lme4' package for GLMM.[43] Residuals of all models were checked for validity according to the procedure of Zuur *et al.*[44]

3 RESULTS

3.1 Effects of TMX on the short-distance homing ability

For the treatment group receiving 10 ppb of TMX, the average homing time after release was 97.3 ± 4.0 s ($N = 225$), which was about 23% higher than the control (79.1 ± 3.4 s, $N = 237$, $t = 3.81$, $P < 0.001$). However, for the treatment group receiving 2.4 ppb of TMX, the average homing time to the hive (77 ± 3.2 s, $N = 228$,) was not significantly different from that of the control (79.1 ± 3.4 s, $N = 237$, $t = -0.55$, $P = 0.58$). At the recorded 5 min after releasing bees, no significant difference was found between the three groups in the homing proportion of bees (average 89.4% ± 2%, 85.9% ± 4%, and 85.4% ± 1.6% for the control, 2.4 ppb, and 10 ppb groups, respectively; Fig. 1). Although no significant difference was observed between the TMX-treated groups and the control at the late stage after releasing (i.e. 260 s and thereafter), the 10 ppb-treated groups indeed had a lower homing proportion before 260 s (Fig. 1). For example, the homing proportions at 140 s were 66.7% ± 6.8%, 76.2% ± 6.2%, and 77.8% ± 6.1% for 10 ppb, 2.4 ppb, and control, respectively.

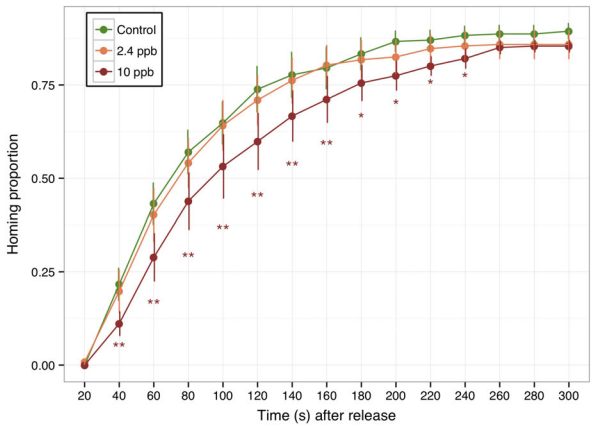

Figure 1. Effects of TMX on the homing proportion of the three different treatment groups at different times after release. Bars refer to standard errors from nine experimental trials. Asterisks show the significance level for the treatment compared with the control based on the GLMM analysis (*≤0.05, **≤0.01, ***<0.001).

3.2 Effects of TMX on flight ability

Results from the flight mill showed that no significant difference was found between the control and TMX-treated bees for the maximum velocity ($P > 0.05$; Fig. 2(a)). However, bees treated with 10 ppb of TMX ($N = 142$) had a significantly higher mean velocity ($t = 3.18$, $P = 0.002$; Fig. 2(b)), flight duration ($t = 2.71$, $P = 0.007$; Fig. 2(c)), and flight distance ($t = 3.39$, $P < 0.001$; Fig. 2(d)) than the control group ($N = 131$). In comparison, no significant difference was found between bees treated with 2.4 ppb of TMX ($N = 131$) and control bees ($N = 131$) in all flight parameters ($P > 0.05$ in all cases, Fig. 2).

3.3 Effects of TMX on learning acquisition and short-term retention

Conditioned PER experiments were carried out in six trials, five of which indicated a significant difference from the controls (Fig. 3). A significantly lower number of honey bees treated with 2.4 ppb of TMX ($N = 59$) showed PER in comparison with the control ($N = 62$) on the fourth ($P = 0.001$) and fifth ($P = 0.005$) learning acquisition trials (Fig. 3, Supporting information Appendix S2). Likewise, a significantly lower number of honey bees treated with 10 ppb of TMX ($N = 60$) showed PER in comparison with the control ($N = 62$) on the third (chi-squared = 4.77, $P = 0.025$), fourth (chi-squared = 11.08, $P < 0.001$), and fifth (chi-squared = 8.81, $P = 0.002$) trials (Fig. 3, Supporting information Appendix S2). TMX-treated bees had a lower number of PER in comparison with the control for the short-term retention trial ($P = 0.002$ and <0.001 for 2.4 ppb and 10 ppb, respectively; Fig. 3).

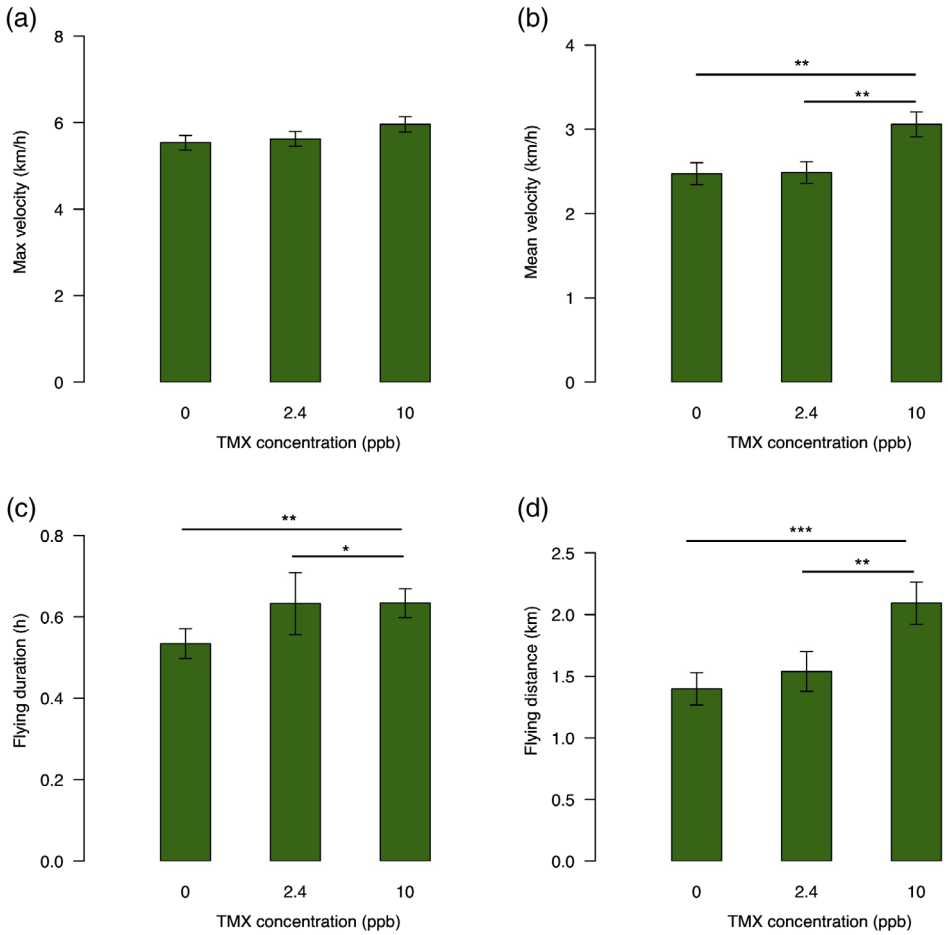

Figure 2. Comparison of the effect of the three different treatment groups on (a) max and (b) mean velocity, (c) flying time, and (d) flying distance of honey bees. Bars refer to standard errors. Asterisks show the significance level for the treatment compared with the control based on the GLMM analysis (*≤0.05, **≤0.01, ***<0.001).

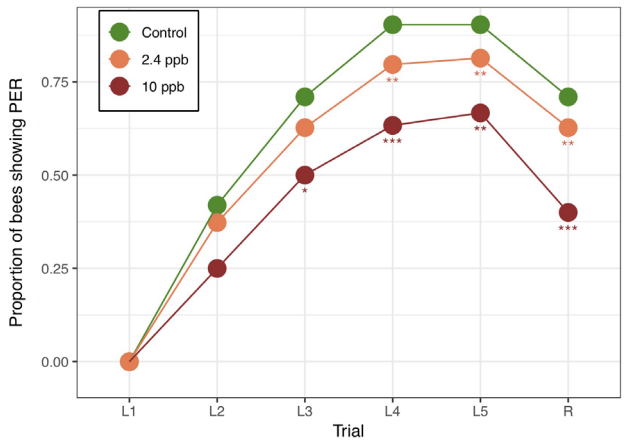

Figure 3. The proportion of bees showing PER in different trials for three different treatment groups. Trials L1 – L5 refer to learning acquisition, whereas trial R refers to short-term retention. Asterisks show the significance level for the treatment compared with the control for each trial, based on Fisher's exact test (*≤0.05, **≤0.01, ***<0.001).

4 DISCUSSION

Our results presented evidence that sublethal doses of TMX significantly affect short-distance homing, flight, and learning acquisition and short-term retention in *A. cerana*. Acute 10 ppb TMX treatment delayed the foraging time to return home by 23% more than that of the control bees. The same homing proportion between the TMX-treated and control groups in our study (about 90% for all three groups), however, indicates that honey bees were finally able to find their hives after acute oral exposure to TMX. Nonetheless, our release distance was relatively short (50 m), while the foraging distance of *A. cerana* can reach several kilometers,[45] which means that 10 ppb TMX exposure will highly likely reduce the homing ratio for *A. cerana* in the field.

Our flight mill results showed that *A. cerana* can fly at a distance of 1.4 km (for the control group), which is comparable with *A. mellifera* (e.g. 1.8 km by Brodschneider *et al.*[46] and 2.1 km by Tosi *et al.*[24]). Flight mill results also showed that acute oral 10 ppb TMX treatment increases the flight speed, distance, and duration. As flight performance depends on muscle activity, the increase in flight ability may have resulted from muscular excitation that was elicited by TMX.[24] This might be because TMX is a partial agonist of the acetylcholine receptors (nAChR) that could lead to neuronal inactivation in the mushroom bodies of the bee brain,[47] and then lead to bee hyperactivity due to hormesis effect.[48–50] Such a hormesis effect could result in an increased temperature of the thoracic muscles, leading to an increase in acute flight ability.[51] An increase in flight ability after TMX treatment has been reported for *A. mellifera* by Tosi *et al.*[24] However, results from the flight mill do not necessarily translate into a better flight performance in the

field because experimental bees were harnessed, while field flight was also determined by locomotion and memory alterations.[52–54] In fact, such an agonist can also disrupt the neural transmission, cognitive ability, and disorientation of honey bees,[47,52,55,56] which explains the impairment effect on bees' homing, learning acquisition, and short-term retention ability, as we observed. In addition, results from acute TMX treatment may differ from chronic treatment, as Tosi *et al.* demonstrated a reduction of flight ability after chronic exposure to TMX for *A. mellifera*.[24] Therefore, those multiple sublethal alterations on *A. cerana's* behavior from our results likely indicate that TMX would have complex but negative consequences on bee health in the field.

In our experiment, honey bees were exposed to one of two concentrations of TMX, 2.4 ppb (20 μL of the solution, 0.048 ng) and 10 ppb (20 μL of the solution, 0.2 ng). The impairment of homing ratio of *A. mellifera* was reported after feeding 20 μL of 67 ppb TMX (1.34 ng),[22] and our results demonstrated that the influential TMX concentration for *A. cerana* can be as low as 10 ppb. Considering the similar sensitivity between *A. cerana* and *A. mellifera* to TMX,[57] the results indicate that the 12.8 ppb field-realistic level of TMX in China[21] would likely cause a negative impact on homing for honey bees. Results nonetheless showed that homing and flight ability of *A. cerana* were impaired by TMX at 10 ppb, but not at 2.4 ppb. These results, however, do not mean that bees are safe when exposed to 2.4 ppb concentration. On the contrary, honey bees treated with 2.4 ppb of TMX had a significantly lower learning acquisition and short-term retention than did the control. Our results contrast with the study by Stanley *et al.*[25] that reported a concentration of 2.4 ppb TMX that did not affect the learning acquisition of bumblebees (*B. terrestris*), which might be because Stanley *et al.* used half the dose (10 μL, 0.024 ng) compared with our study, but this can also result from the different sensitivity to pesticides between bumblebees and *A. cerana*.

We performed three independent experiments to study the impact of acute oral exposure to TMX on the behavior of *A. cerana*. We concluded that acute oral exposure to 10 ppb TMX reduced the short-distance homing ratio and homing time, and affected the flight ability of *A. cerana*. The exposure to both 2.4 ppb and 10 ppb TMX reduced bees' learning acquisition and retention, suggesting a low concentration of neonicotinoids in the field is still cause for concern regarding honey bees and other pollinators. It is not clear, however, whether the impact of TMX on the homing ability of *A. cerana* resulted from the impairment of bees' flight ability or learning and memory ability. Further studies such as radio-frequency identification[23] and harmonic radar tracking[52,56] are recommended to explore this question.

ACKNOWLEDGEMENTS

We thank Peng Tang for his help with fieldwork. This work was supported by the Central Public-Interest Scientific Institution Basal Research Fund (Y2018PT66), the National Natural Science Foundation of PR China (31700363), the China Agriculture Research System (CARS-45), and the Opening Foundation of Guangxi Key Laboratory for Agro-Environment and Agro-Product Safety (17-259-82). H.L.-B. was supported by the US Department of Agriculture – National Institute of Food and Agriculture. We would like to thank Conrad Zagory, Jr., writing specialist at Central State University and Susan Krusemark, managing scientific editor of the Illinois State Geological Survey, for their critical reviews on the English writing of the manuscript.

AUTHOR CONTRIBUTIONS

C.M. and S.L. conceived and designed the experiments. C.M., Yk.Z., and J.S. performed the experiments. Yi.Z. analyzed the data. C.M., Yi.Z., H.L-B., and S.L. wrote the manuscript, and others provided editorial comments.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Kleijn D, Winfree R, Bartomeus I, Carvalheiro LG, Henry M, Isaacs R, *et al.*, Delivery of crop pollination services is an insufficient argument for wild pollinator conservation. *Nat Commun* **6**: 7414 (2015), 7414.
2 Rader R, Bartomeus I, Garibaldi LA, Garratt MPD, Howlett BG, Winfree R, *et al.*, Non-bee insects are important contributors to global crop pollination. *Proc Natl Acad Sci* **113**(1): 146 (2016).
3 Baron GL, Jansen VAA, Brown MJF and Raine NE, Pesticide reduces bumblebee colony initiation and increases probability of population extinction. *Nat Ecol Evol* **1**:1308–1316 (2017).
4 Goulson D, Nicholls E, Botias C and Rotheray EL, Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**:1255957 (2015).
5 Whitehorn PR, O'Connor S, Wackers FL and Goulson D, Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science* **336**:351–352 (2012).
6 Pisa L, Goulson D, Yang E-C, Gibbons D, Sánchez-Bayo F, Mitchell E, *et al.*, An update of the worldwide integrated assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. *Environ Sci Pollut Res* **1**–33 (2017).
7 Tosi S, Démares FJ, Nicolson SW, Medrzycki P, Pirk CWW and Human H, Effects of a neonicotinoid pesticide on thermoregulation of African honey bees (*Apis mellifera scutellata*). *J Insect Physiol* **93-94**:56–63 (2016).
8 Zou Y, Bianchi FJJA, Jauker F, Xiao H, Chen J, Cresswell J, *et al.*, Landscape effects on pollinator communities and pollination services in small-holder agroecosystems. *Agric Ecosyst Environ* **246**: 109–116 (2017).
9 Zheng HQ, Wei WT and Hu FL, Beekeeping industry in China. *Bee World* **88**:41–44 (2011).
10 Yang GH, Harm of introducing the western honey bee, *Apis mellifera* L., to the Chinese honey bee *Apis cerana* F. and its ecological impact. *Acta Entomol Sinica (in Chinese with English abstract)* **48**:401–406 (2005).
11 Tan K, Wang C, Dong S, Li X and Nieh JC, The pesticide flupyradifurone impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. *Sci Rep* **7**:17772 (2017).
12 Simon-Delso N, Amaral-Rogers V, Belzunces LP, Bonmatin JM, Chagnon M, Downs C, *et al.*, Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environ Sci Pollut Res Int* **22**(1): 5–34 (2015).
13 Pilling E, Campbell P, Coulson M, Ruddle N and Tornier I, A four-year field program investigating long-term effects of repeated exposure of honey bee colonies to flowering crops treated with thiamethoxam. *PLoS One* **8**:e77193 (2013).
14 Botias C, David A, Horwood J, Abdul-Sada A, Nicholls E, Hill E *et al.*, Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees. *Environ Sci Technol* **49**(21): 12731–12740 (2015).
15 Stewart SD, Lorenz GM, Catchot AL, Gore J, Cook D, Skinner J, *et al.*, Potential exposure of pollinators to neonicotinoid insecticides from the use of insecticide seed treatments in the mid-southern United States. *Environ Sci Technol* **48**(16): 9762–9769 (2014).
16 Krupke CH, Hunt GJ, Eitzer BD, Andino G and Given K, Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS One* **7**:e29268 (2012).
17 Dively GP and Kamel A, Insecticide residues in pollen and nectar of a cucurbit crop and their potential exposure to pollinators. *J Agric Food Chem* **60**:4449–4456 (2012).
18 Byrne FJ, Visscher PK, Leimkuehler B, Fischer D, Grafton-Cardwell EE and Morse JG, Determination of exposure levels of honey bees foraging on flowers of mature citrus trees previously treated with imidacloprid. *Pest Manag Sci* **70**:470–482 (2014).

19 Tosi S, Costa C, Vesco U, Quaglia G and Guido G, A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides. *Sci Total Environ* **615**:208–218 (2018).
20 Thompson H, Harrington P, Wilkins S, Pietravalle S, Sweet D, Jones A, Effects of neonicotinoid seed treatments on bumble bee colonies under field conditions. Food and Environment Research Agency (FERA) 2013.
21 Tong Z, Duan J, Wu Y, Liu Q, He Q, Shi Y, *et al.*, A survey of multiple pesticide residues in pollen and beebread collected in China. *Sci Total Environ* **640-641:** 1578–1586 (2018).
22 Henry M, Beguin M, Requier F, Rollin O, Odoux JF, Aupinel P, *et al.*, A common pesticide decreases foraging success and survival in honey bees. *Science* **336**(6079): 348–350 (2012).
23 Henry M, Cerrutti N, Aupinel P, Decourtye A, Gayrard M, Odoux J-F, *et al.*, Reconciling laboratory and field assessments of neonicotinoid toxicity to honey bees. *Proc R Soc B Biol Sci* **282**(1819): 20152110 (2015).
24 Tosi S, Burgio G and Nieh JC, A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. *Sci Rep* **7**:1201 (2017).
25 Stanley DA, Smith KE and Raine NE, Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide. *Sci Rep* **5**:16508 (2015).
26 Samuelson EE, Chen-Wishart ZP, Gill RJ and Leadbeater E, Effect of acute pesticide exposure on bee spatial working memory using an analogue of the radial-arm maze. *Sci Rep* **6**:38957 (2016).
27 Tison L, Holtz S, Adeoye A, Kalkan Ö, Irmisch NS, Lehmann N *et al.*, Effects of sublethal doses of thiacloprid and its formulation Calypso® on the learning and memory performance of honey bees. *J Exp Biol* **220**(20): 3695 (2017), 3705.
28 Decourtye A, Lacassie E and Pham-Delègue M-H, Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Manag Sci* **59**:269–278 (2003).
29 Decourtye A, Devillers J, Cluzeau S, Charreton M and Pham-Delègue M-H, Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicol Environ Safe* **57**:410–419 (2004).
30 Decourtye A, Devillers J, Genecque E, Menach KL, Budzinski H, Cluzeau S *et al.*, Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Arch Environ Contam Toxicol* **48**(2): 242–250 (2005).
31 Tan K, Chen W, Dong S, Liu X, Wang Y and Nieh JC, A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. *Sci Rep* **5**:10989 (2015).
32 Zhang E and Nieh JC, A neonicotinoid, imidacloprid, impairs honey bee aversive learning of simulated predation. *J Exp Biol* **218**:3199–3205 (2015).
33 Stanley DA, Russell AL, Morrison SJ, Rogers C and Raine NE, Investigating the impacts of field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability and colony growth. *J Appl Ecol* **53**:1440–1449 (2016).
34 Castle SJ, Byrne FJ, Bi JL and Toscano NC, Spatial and temporal distribution of imidacloprid and thiamethoxam in citrus and impact on Homalodisca coagulata populations. *Pest Manag Sci* **61**:75–84 (2005).
35 Stanley DA, Garratt MP, Wickens JB, Wickens VJ, Potts SG and Raine NE, Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees. *Nature* **528**:548–550 (2015).
36 OECD (Organization for Economic Cooperation and Development), *OECD Guidelines for the Testing of Chemicals*. Honeybees, acute oral toxicity test 213. OECD, Paris, France (1998).
37 Smith T and Jones VP, The flight mill. Available from http://entomology.tfrec.wsu.edu/VPJ_Lab/Flight-Mill. [2 May 2018].
38 Bitterman ME, Menzel R, Fietz A and Schäfer S, Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J Comp Psychol* **97**:107–119 (1983).
39 Li ZG, Li M, Huang JN, Ma CS, Xiao LC, Huang Q, *et al.*, Effects of sublethal concentrations of chlorpyrifos on olfactory learning and memory performances in two bee species, *Apis mellifera* and *Apis cerana*. *Sociobiology* **64**(2): 174–181 (2017).
40 McDonald JH, *Handbook of Biological Statistics*. Sparky House Publishing, Baltimore, MD (2009).
41 Croe Team R, *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria (2014).
42 Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team, nlme: Linear and nonlinear mixed effects models. R package version 31-118 (2014).
43 Bates D, Maechler M, Bolker B and Walker S, lme4: Linear mixed-effects models using Eigen and S4. R package version 11-7 (2014).
44 Zuur A, Ieno EN, Walker N, Saveliev AA and Smith GM, *Mixed Effects Models and Extensions in Ecology with R*. Springer Science & Business Media, Stanford (2009).
45 Tan K, Latty T, Dong S, Liu X, Wang C and Oldroyd BP, Individual honey bee (*Apis cerana*) foragers adjust their fuel load to match variability in forage reward. *Sci Rep* **5**:16418 (2015).
46 Brodschneider R, Riessberger-Gallé U and Crailsheim K, Flight performance of artificially reared honeybees (*Apis mellifera*). *Apidologie* **40**:441–449 (2009).
47 Palmer MJ, Moffat C, Saranzewa N, Harvey J, Wright GA and Connolly CN, Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. *Nat Commun* **4**:1634 (2013).
48 Suchail S, Guez D and Belzunces LP, Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environ Toxicol Chem* **20**:2482–2486 (2001).
49 Déglise P, Grünewald B and Gauthier M, The insecticide imidacloprid is a partial agonist of the nicotinic receptor of honeybee Kenyon cells. *Neurosci Lett* **321**:13–16 (2002).
50 Cutler GC and Rix RR, Can poisons stimulate bees? Appreciating the potential of hormesis in bee-pesticide research. *Pest Manag Sci* **71**:1368–1370 (2015).
51 Woods WA, Heinrich B and Stevenson RD, Honeybee flight metabolic rate: does it depend upon air temperature? *J Exp Biol* **208**:1161–1173 (2005).
52 Tison L, Hahn M-L, Holtz S, Rößner A, Greggers U, Bischoff G *et al.*, Honey bees' behavior is impaired by chronic exposure to the neonicotinoid thiacloprid in the field. *Environ Sci Technol* **50**(13): 7218–7227 (2016).
53 Tosi S and Nieh JC, A common neonicotinoid pesticide, thiamethoxam, alters honey bee activity, motor functions, and movement to light. *Sci Rep* **7**:15132 (2017).
54 Jin N, Klein S, Leimig F, Bischoff G and Menzel R, The neonicotinoid clothianidin interferes with navigation of the solitary bee *Osmia cornuta* in a laboratory test. *J Exp Biol* **218**:2821–2825 (2015).
55 Tomizawa M and Casida JE, Neonicotinoid insecticide toxicology: mechanisms of selective action. *Annu Rev Pharmacol Toxicol* **45**:247–268 (2005).
56 Fischer J, Muller T, Spatz AK, Greggers U, Grunewald B and Menzel R, Neonicotinoids interfere with specific components of navigation in honeybees. *PLoS One* **9**:e91364 (2014).
57 Arena M and Sgolastra F, A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology* **23**:324–334 (2014).

