UNIVERSITY OF CALIFORNIA SAN DIEGO

The effects of thiamethoxam on *Apis mellifera* visual learning and behavior

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

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2019

The thesis of Joshua Cobi Ludicke is approved and it acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2019

DEDCIATION

To those that helped me along the way and those I lost along the way.

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ACKNOWLEDGMENTS

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ABSTRACT OF THE THESIS

The effects of thiamethoxam on *Apis mellifera* visual learning and behavior

by

Joshua Cobi Ludicke

Master of Science in Biology

University of California San Diego, 2019

Professor James C. Nieh, Chair

Learning is important for both honey bee fitness and the pollination services they provide. Neonicotinoid pesticides impair learning, fitness, colony health, and pollination. However, most studies on learning have only looked at the effect of neonicotinoid pesticides on olfactory learning. We found that bees fed field realistic doses of 0.8 ng/bee and 1.34 ng/bee of the neonicotinoid pesticide, thiamethoxam had increased abnormal behaviors such as falling. In addition, the doses decreased the time they took to complete each learning trial and took less time to make decisions, suggesting hyperactivity. However, there was no effect of these thiamethoxam doses on bee learning or memory. We used an adaptation of a T-maze bioassay from Han et al. (2010) and simulated flower color with blue and yellow colored lights to

classically condition bees. By using a previously developed bioassay we hope that our methods are easily replicable and can be improved upon easily. Our results suggest that more studies should be conducted on the effects of pesticides in visual learning in *A. mellifera* and other related species.

Introduction

With the widespread use of neonicotinoids, a class of insecticides that block nicotinic acetylcholine receptors (Tosi et al. 2017) and over-stimulate cholinergic neurons (Johnson et al. 2010), concern is growing about the effects of such pesticides on key pollinators such as the western honeybee (*Apis mellifera)* (Gallai et al. 2009). Honey bees can provide important pollination services for natural and agricultural ecosystems (Quarles 2011). Their efficacy as pollinators is enhanced by their visual and olfactory memory because bees can learn the appearance and odors of rewarding food, thereby facilitating their search for the same floral species throughout the landscape (Dukas & Visscher 1994). Honey bees have an excellent visual and olfactory memory (Pahl et al. 2007; Gross et al. 2009), and can learn complex visual patterns (Srinivasan 2010) and odors (Takeda 1961). However, multiple studies have now demonstrated that neonicotinoid pesticides can harm honey bee learning and foraging (Decourtye et al. 2013; Han et al., 2010), impairing colony fitness and likely reducing their ability to pollinate (Lundin, Ola, et al. 2015).

Neonicotinoids can significantly impair *A. mellifera* olfactory learning (Decourtye et al. 2005) and memory (Williamson & Wright 2013). Exposure to even very small, field-realistic doses of imidacloprid can harm olfactory learning in *A. mellifera* (Yang, En-Cheng, et al. 2012). Some studies have shown that neonicotinoids can harm short-term memory formation in foragers, while leaving long-term memory unaffected (Wright et al. 2015). However, other studies show that both short-term and long-term memory can be harmed (Williamson & Wright 2013).

Unlike studies on olfactory learning, relatively few studies have examined the effects of neonicotinoids on honey bee visual learning. A homing study of free flying bees dosed with

neonicotinoids impairmed the ability of bees to landmark and return to their hive, suggesting visual learning impairment (Fisher et al. 2014). Han et. al (2010) reported that honey bees had impaired visual learning in a visual color maze when they were fed pollen containing imidacloprid. The relative lack of studies on how pesticides affect visual learning may arise because honey bee visual learning is more robust when conducted with unrestrained, freely flying bees (Avarguès-Weber et al. 2016). However, laboratory assays of visual learning are desirable because they facilitate testing larger numbers of bees under highly controlled conditions that do not depend upon favorable weather (Avarguès-Weber et al. 2016). For example, the development and implementation of risk assessment protocols by pesticide regulatory agencies would be facilitated with a lab assay.

We focused on thiamethoxam (TMX), a neonicotinoid, because it is used on a wide range of crops and is the second most used neonicotinoid in the United States (Elbert et al. 2008). The major breakdown product of TMX, clothianidin, is also highly toxic to insects (Nauen et al. 2003), which contributes to the long-term environmental hazards of this pesticide (Kah et al. 2018). In fact, concerns about the effects of neonicotinoids on beneficial pollinators has led the European Union to ban all neonicotinoids on plants if this use could impact pollinators (European Commission 2018).

Our goals were therefore to refine the assay of Han et al. (2010) to test the effects of a different neonicotinoid, thiamethoxam, on honey bee visual learning. We also wished to add a more detailed assay of bee behavior, because it is known that thiamethoxam can impair honey bee locomotion (Tosi & Nieh 2017) and other nicotinic acetylcholine receptor agonists can increase the number of abnormal bee behaviors (Tosi & Nieh 2019).

Materials and Methods

Study site and colonies

Experiments were conducted with 8 healthy honey bee colonies at the apiary at the Biology Field Station (32°53'07.9"N 117°13'55.1"W), University of California, San Diego. Bees were tested in a dark, temperature-controlled room (30°C and 40% relative humidity). We used an elevated room temperature (and corresponding lower humidity) because our preliminary experiments showed that bees tended to have increased motivation to feed under these conditions.

Visual learning apparatus

We modified a T-maze bioassay (Han et al. 2010) developed to test the effects of pesticides on *A. mellifera* visual learning and behavior. We used LED lights as the visual stimulus instead of filter paper (Han et al. 2010) due to LEDs having the ability to be calibrated to the same light intensity. Bees were randomly selected for reward conditioning to either blue or yellow light. The maze consisted of clear plastic tubes (FORMUFIT P001FGP-UV-5 schedule 40 clear PVC pipe, furniture grade, 2.54 cm outer diameter, 2.5 cm inner diameter, and matching clear F001TEE-UV Tee fittings). Lights were mounted on a breadboard with 12 blue and 12 yellow lights alternated along the board on the left and identically on the right arm of the maze. Each side had a switch that controlled the colors illuminated, allowing the experimenter to control which color was displayed on each side. In each trial, one side was yellow, and one side was blue; these colors were randomly determined at the start of the conditioning trails. The intensity of these lights (CO-RODE Amazon store; yellow: 589-591 nm; blue: 460-465 nm) was adjusted until they were 120 lux, as measured with a digital illuminance meter (DrMeter,

LX1220B) for blue and yellow light shining through the tube (to account for the absorption of light by the tube).

Forager bees were collected, fed 2.0 M sucrose to satiation, and then incubated overnight to bring them to a similar hunger stage (Avarguès-Weber et al. 2016) in an incubator that had a temperature of 32.5°C and 60% humidity. Bees were then selected at random and given either 2uL of 50% sucrose solution or a 50% sucrose solution containing TMX (concentrations given below). These bees were then incubated in the dark (32.5°C and 60% humidity) for 1 h (Tosi et al. 2017). Mortality was recorded after the starvation period.

Pesticide doses

We exposed bees to an acute dose of 0.8 ng TMX/bee (lower dose) or 1.34 ng TMX/bee (higher dose). The lower and higher dose experiments were run sequentially. Both doses are sublethal and field realistic (Tosi & Nieh 2017; Tosi et al. 2017; Henry et al. 2012). The European Food Safety Authority (EFSA) estimated that foragers can consume up to 1.80 ng TMX/bee in 1 h of foraging for nectar (10% sugar w/w, oilseed rape contaminated with 15 ppb of TMX (EFSA 2012, Tosi and Nieh 2017). In addition, foragers, can imbibe up to 6.66 ng TMX/bee/day while collecting nectar from TMX seed-treated plants such as oilseed rape containing 5 ppb of TMX (EFSA 2012). Our higher dose of 1.34 n TMX/bee is therefore closer to a worst-case scenario, whereas the lower dose of 0.8 ng TMX/bee represents a more likely field-realistic exposure.

We prepared a TMX stock solution with Milli-Q water and analytical grade 99.3% purity TMX (CAS#153719-23-43, Sigma Aldrich 37924-100MG-R). This solution was kept in darkness (Eppendorf tubes covered with aluminum foil), frozen, and defrosted at 1.6°C when used. Serial dilutions were made with Milli-Q water and reagent grade 50% sucrose solution. We

also used Milli-Q water to make a 50% sucrose solution control. All researchers were blind to the solution being used. The identity of solutions used in each experiment was only revealed at the end of all testing.

Maze learning procedure

To help improve learning, we used a conditioning pre-trial (Dobrin & Fahrbach 2012) in which bees were introduced to the entrance of the apparatus under red light. The conditioned stimulus (CS, yellow or blue light) was turned on at the end of the T-maze on either the left or right side. Han et al. (2010) did not test bees by training them to yellow because bees have a strong preference for yellow (also shown by Zhang et al. 1996). They trained bees to associate blue light with reward (as compared to a non-rewarded yellow light) to demonstrate that bees could associate a *non-preferred* color with a reward. Our study deviated from this protocol and conditioned bees to both yellow and blue as we were specifically examining the full effects of neonicotinoids on color learning. Per bee, we randomly selected which color would be the CS. Only the CS was on during the pre-trial. When the bee reached the maze arm with the CS, she was rewarded with a 2.0 M sucrose solution for 3 s of feeding, recaptured in the same vial she was released in, and returned to the dark. We used a 10 min intertrial interval for each individual bee.

We then trained bees with six conditioning trials. When the bee entered the T-maze apparatus, both blue and yellow lights were turned on, at opposite ends of the maze (Figure 1). One color was the neutral stimulus (NS) that would become the conditioned stimulus (CS); the other was the NS that would become the unrewarded stimulus (US). The side for the CS was randomly chosen for the first trial and then alternated for subsequent trials. A bee was only

rewarded if it reached the end of the maze arm of the correct CS color. If a bee did not make a choice within 5 min, then trial was scored as having failed and the bee was released and rewarded at the correct color and side.

Bees that did not feed for two trials or complete the maze within the 5 min time period twice in a row were excluded from completing the experiment. In addition, bees that completed less than two of their trials were excluded from our analysis of learning because they demonstrated little motivation to accept the reward or to complete the learning experiment (Takeda 1961; Bitterman et al. 1983). However, pesticide could have contributed to these failures, and we therefore analyzed failure data separately.

One hour after these learning trials, we performed a memory retention test. To perform this trial (trial 7), another T-maze apparatus was connected to the end of the right side of a T-maze by a 31 cm tube. The bee, to demonstrate its learning ability at its previous six trials, had to complete this double T-maze apparatus within the same period (Han et al. 2010).

Abnormal behaviors

Several abnormal bee behaviors were scored and totaled throughout the duration of the experiment. Falling behavior, which is also observed in Tosi & Nieh's (2017) work with TMX, was observed during the 5 min time interval and was scored for each fall a bee took from the upper half of the inside of the tube. To be scored, the bee had to fall on its back. Falling could be scored multiple times in a single trial if the bee were to upright itself again (Table 1).

Abnormal behavior was another behavior that was observed during the experiment. This behavior included hyperactivity, non-responsiveness, paralysis, and rolling (bee not falling but rolling over onto its back trying to right itself). Abnormal behavior was only scored once per trial as it was often a behavior that encompassed the whole period.

The last behavior we considered was trembling behavior (Tosi & Nieh 2017). Trembling included stumbling, tripping, and rapid vibration of the bee while walking. Trembling was only scored once per trial as it was often an ongoing behavior during each trial. All three behaviors could be scored in a trial as they often coincided with one another.

Statistics

We used JMP v. 13.0 statistical software and ran a Repeated Measures Mixed Model (REML algorithm) to test the effects of pesticide on three different aspects: learning, abnormal behaviors, or time to complete each trial. We log transformed the time to complete each learning trial. For each model, we used the following fixed effects (trial and dose) and random effects (colony and individual bee). For all models, we tested for the interaction trial x dose, but then eliminated it if it was not significant. We initially ran a separate model for each color because Han et al. (2010) found that learning only occurred to blue, not yellow. To look at the effect on dose on learning more simply, we focused on the sixth and final learning trial. In this model we considered the effect of dose and color only. This model was not an REML algorithm.

For the analysis of abnormal behaviors, we pooled the data from both colors because there was no effect of color (as expected) on abnormal behaviors. In analyzing the effect of dose on abnormal behaviors more concisely, we used a model to examine the effects of dose on the totaled abnormal behaviors for each bee over the six conditioning trials. We ran LS Mean Contrast Tests as a post hoc analysis that compared each dose to the control.

We only included bees that completed all trials (this excluded 53 bees from the analysis). We did not exclude bees based upon their learning performance, rather we excluded them based on whether a bee was able to complete all its trials. We used Tukey Honestly Significant

Difference (HSD) post-hoc tests to make corrected pairwise comparisons. To determine if pesticide affected the proportion of bees that successfully completed their trials or died during trials, we ran a Fisher's Exact 2x2 test (https://graphpad.com/quickcalcs/contingency1.cfm) and compared control bees with pesticide treated bees (low and high doses pooled).

Results

In total, we used eight colonies and 108 bees with 29 bees in the 0.8 ng/bee trials (low dose), 24 bees in the 1.34 ng/bee trials (high dose), and 55 bees in control experiments after exclusion of trial completion. Temperature and humidity respectively averaged 29.6°C ± 1.8°C and 43.2% ± 8.3% (Mean ± SD). Pesticide exposure did not affect the proportion of bees that completed their learning trials: 69% of pesticide-treated and 71% of control bees completed their trials (Fisher's Exact 2x2 test: P=0.733). Our pesticide treatments were sublethal. Pesticide exposure did not alter the proportion of bees that died during a trial: 1% of pesticide and control bees died during the trials (Fisher's Exact 2x2 test: P=1.0)

Bee visual learning

When blue was the rewarded color, bees learned to associate color with reward as conditioning trials progressed (trial effect: $F_{6,293}$ =3.45, P<0.003, Figure 2). There was no significant effect of pesticide dose on bee choice ($F_{2,43}$ =0.21, P=0.81, Figure 2), and there was a non-significant interaction of trial x dose effect ($F_{12,293}$=1.66, P=0.073, Figure 2). However, control bees showed significantly higher learning in trial 6 than in trial 1 (Tukey HSD test, P<0.05) for control bees. There were no significant differences in learning between pesticide (any dose) and control bees at any learning or memory trial.

When yellow was the rewarded color, bees did not learn to associate color with reward as conditioning trials progressed (trial effect: $F_{6,317}$=1.38, P=0.221, Figure 3). There was also no significant effect of dose ($F_{2,45}$=2.05, P=0.14, Figure 3) or the interaction trial x dose ($F_{12,317}$ =1.22, P=0.269, Figure 3).

When the memory test (trial 7) was analyzed independently of all conditioning trials (trials 1-6), it did not show a significant difference between doses for blue ($F_{2,1}$=1.87, P=0.166) or yellow ($F_{2,0}$=0.67, P=0.519). When analyzing the sixth and final conditioning trial, there were no significant effects of color ($F_{1,96}$=2.84, P=0.095) and color crossed with dose ($F_{2,5}$=0.78, P=0.513). However, there was a significant effect of dose ($F_{2,58}$=4.33, P=0.018; Figure 6A) such that high dosed showed significantly less correct choices than control and low dose bees (Tukey HSD test, P<0.05).

Abnormal behaviors

Pesticide increased the number of all abnormal behaviors with falling being the most prevalent (Figure 4B). There were significant effects of dose ($F_{2,102}$=4.17, P=0.018) and trial ($F_{6,629}$=11.82, P<0.0001) and on abnormal behaviors (Figure 4A). There was also a significant interaction of trial x dose ($F_{12,629}$ =2.44, P=0.0042). There were more abnormal behaviors in high and low dose trials 1 than in control trial 1. In addition, there were significantly more abnormal behaviors in trial 1 then in trials 2-6, and significantly different abnormal behaviors in trials 2-3, than trial 6 (Tukey HSD test, P<0.05). Colony accounted for 13.37% of total model variance.

In looking at abnormal behaviors more concisely, we found there was a significant effect of dose on the sum of the abnormal behaviors over all conditioning trials ($F_{2,99}$=3.88, P=0.024)

with both low dose ($F_{1,103}$=3.94, P=0.0498; Figure 6B) and high dose ($F_{1,103}$=5.13, P=0.0256; Figure 6B) demonstrating a significant difference from controls (LS Means Contrast P<0.05).

Time to completion

When blue was the rewarding color, the time each bee took to complete a trial was significantly associated with trial ($F_{5,245}$=2.56, P=0.028). In addition, there was a significant effect of dose ($F_{2,16}$=4.33, P=0.032, Figure 5A) and no significant interaction between trial x dose ($F_{10,245}$=1.77, P=0.0663, Figure 5A). Low dose and control bees significantly differed, however more specifically, control trial 2 took significantly longer to complete than low dose trial 4 (Tukey HSD test, P<0.05). Colony accounted for <1% of blue model variance.

When yellow was the rewarding color, there was a significant effect of trial ($F_{5,263}$=4.59, P=0.0005, Figure5B) and dose ($F_{5,52}$=3.25, P=0.0467). There was no significant effect of trial x dose interaction ($F_{10,263}$=1.20, P=0.293). Trial 1 took significantly longer than trials 4, 5 and 6 (Tukey HSD test, P<0.05). Control dose bees took significantly longer than high dose bees (Tukey HSD test, P<0.05). Colony accounted for 6.27% of yellow model variance.

Table 1. Describes the various monitored activities of honeybees while in the apparatus during the trial period.

Monitored Activity	**Description**
Falling behavior	Whether or not a bee fell on its back in the apparatus. Can be counted multiple times in a single trial.
Trembling behavior	Recorded only once during a trial. Includes stumbling, tripping or rapid vibration of the bee while walking. Scored once per trial.
Other abnormal behaviors	Included hyperactivity, non-responsiveness, paralysis, and rolling. Scored once per trial.

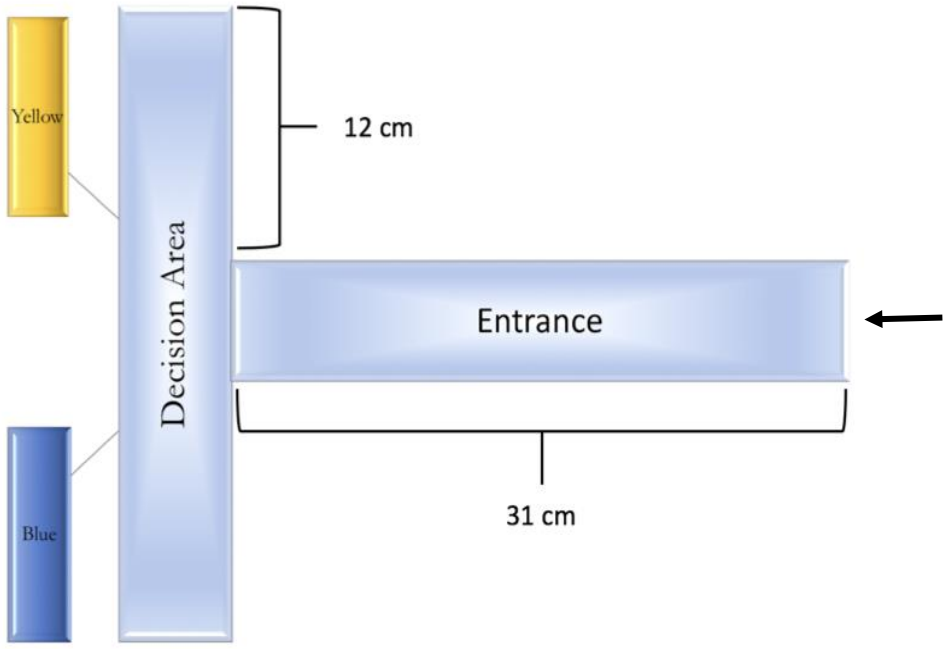


Figure 1. The single tube T-Maze modified from Han et al. (2010) to condition bees.

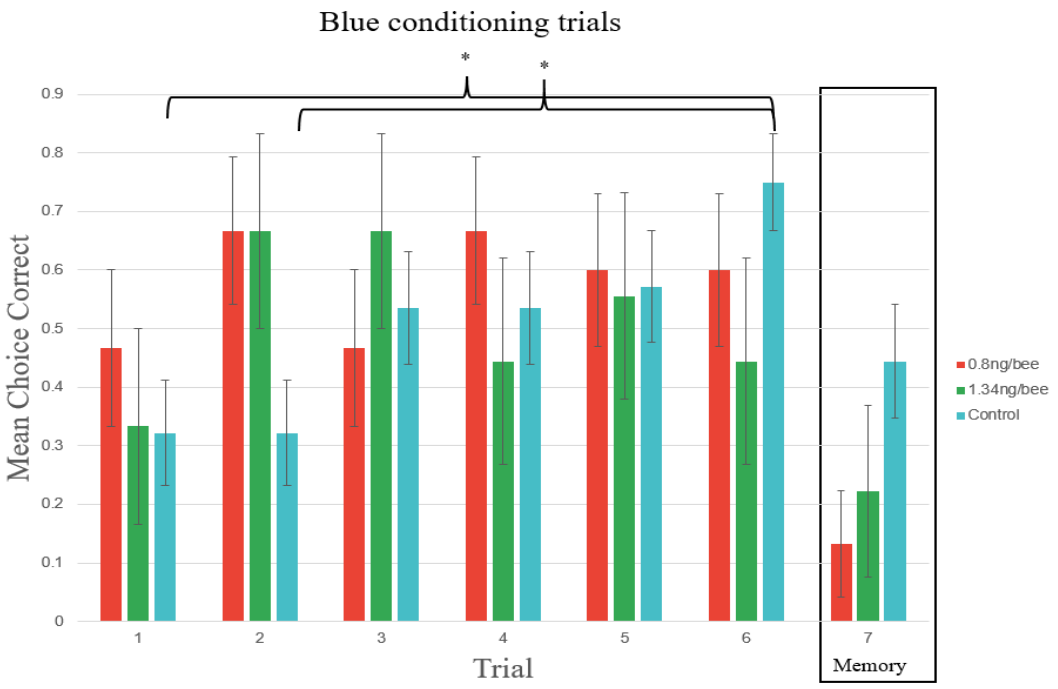


Figure 2. Average percentage of correct choices of all blue trained bees for each trial excluding the pretrial. Error bars are SE. Brackets with stars represent significant differences between trials (Tukey HSD test, $P<0.05$). Memory test not included in pairwise differences.

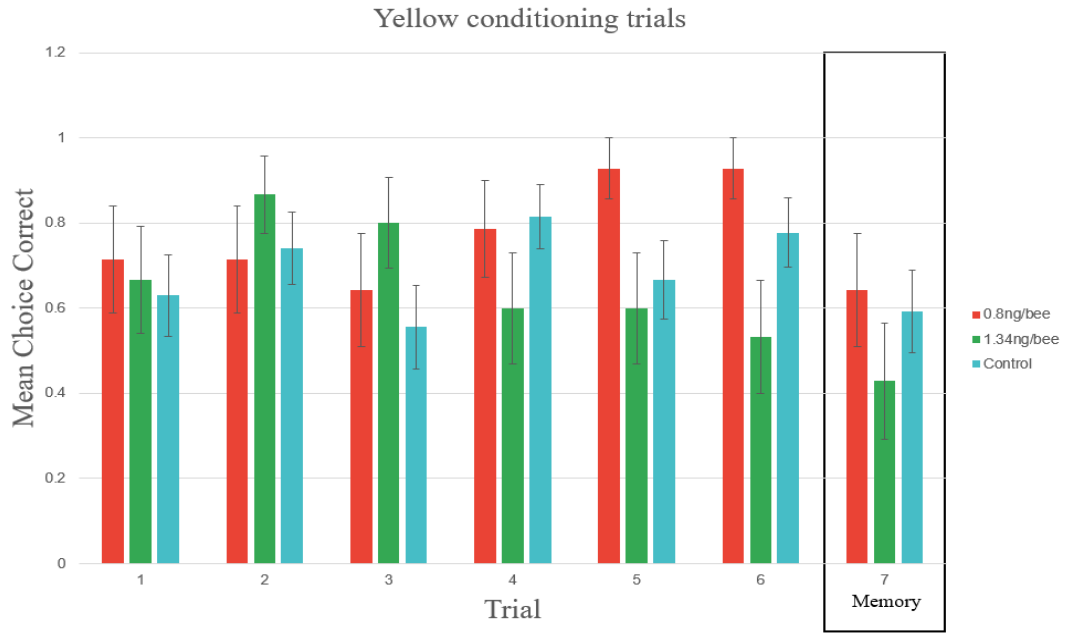
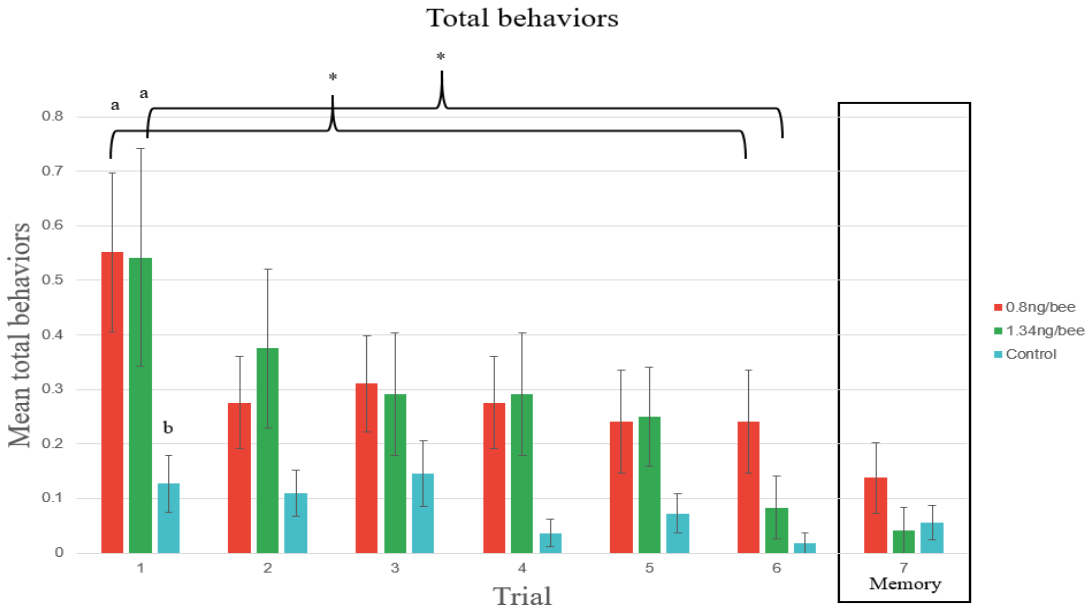


Figure 3. Average percentage of correct choices of all yellow trained bees for each trial excluding the pretrial. Error bars are SE. There were no significant pairwise differences.

A)



B)

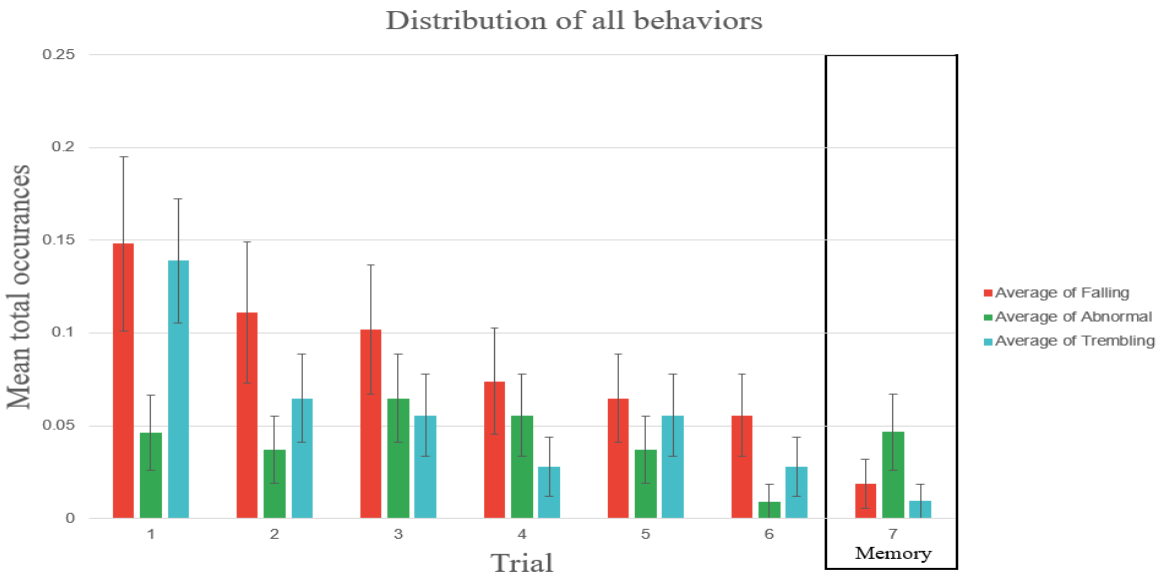
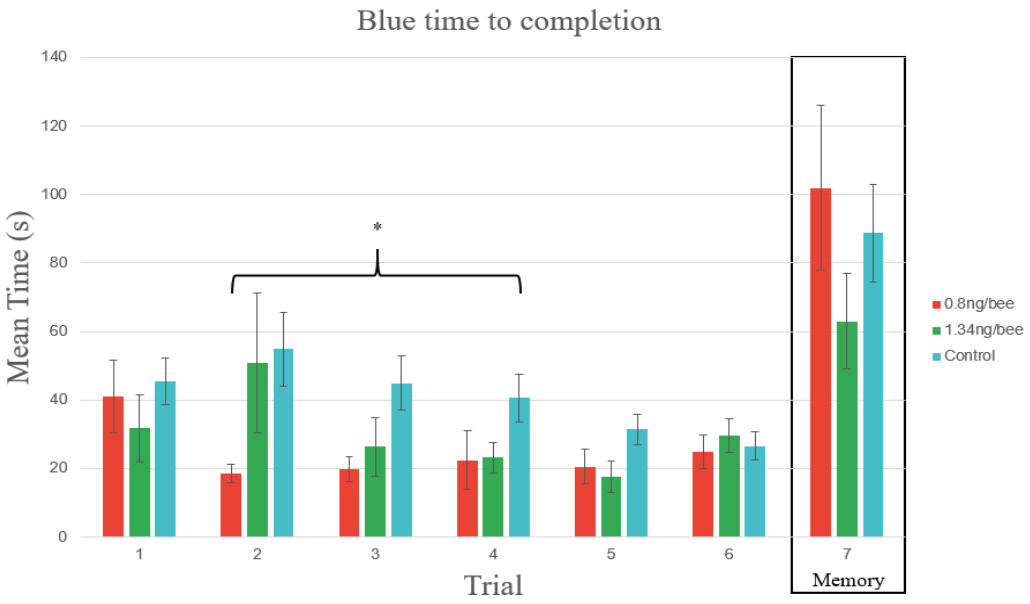


Figure 4. The distribution of abnormal behaviors over trials. Error bars SE. Different letters represent significant differences within same trial. Brackets with stars represent differences between different trials (Tukey HSD test, $P<0.05$). (A) Total behaviors divided by pesticide dose. (B) Different types of abnormal behaviors over trials, no pairwise comparison (all doses pooled).

A)



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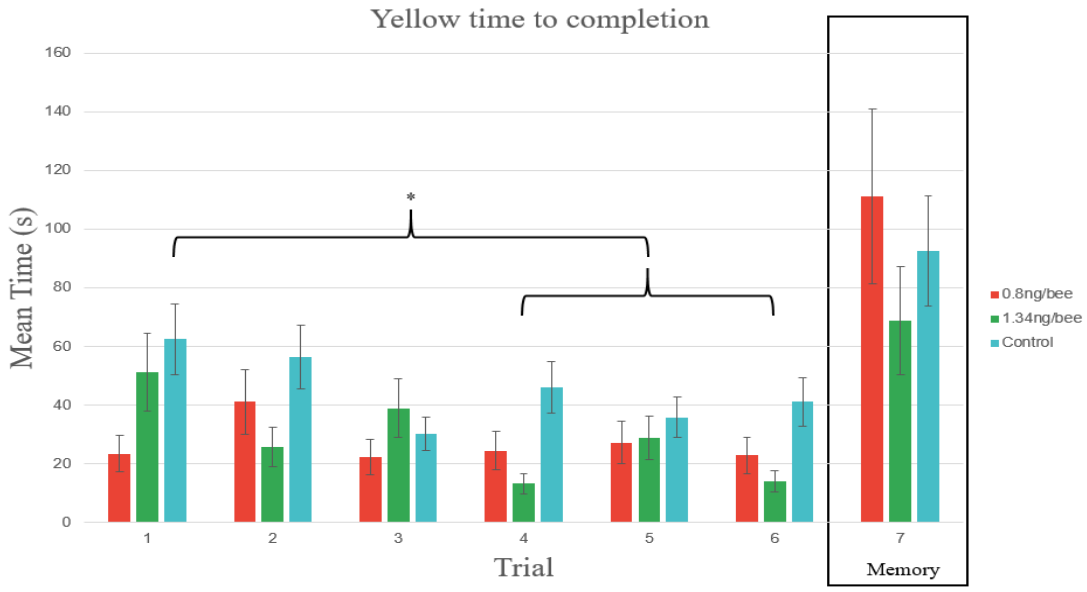
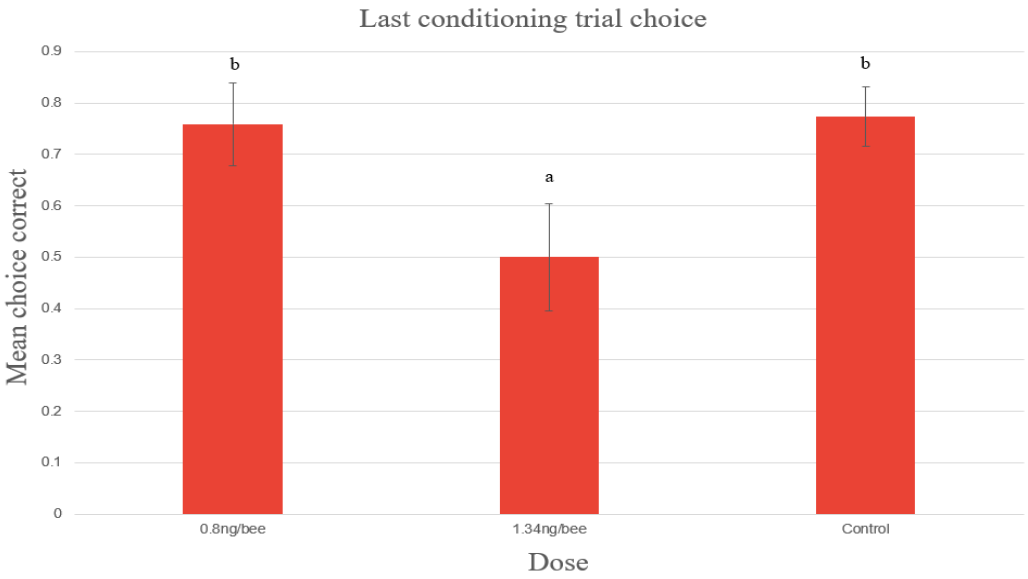


Figure 5. Average time bees took to complete a trial learning blue for all doses. (A) Average time bees took to complete a learning trial for yellow (B). Error bars are SE. Brackets with stars represent significant differences (Tukey HSD test, $P<0.05$).

A)



B)

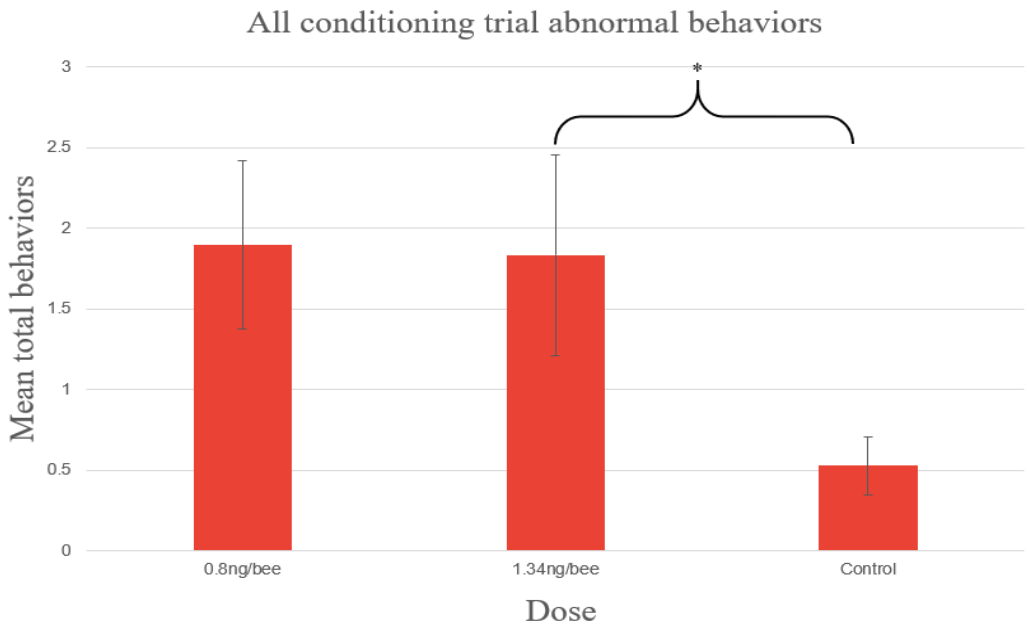


Figure 6. More simplified analyses of visual learning and abnormal behaviors. Error bars are SE. (A) Average of choices in the last conditioning trial of low dose and high dose bees. Different letters represent significant differences in data (Tukey HSD test, $P<0.05$). (B) Average of all abnormal behaviors over the 6 conditioning trials. Brackets with stars represent significant differences.

Discussion

Visual Learning

We found that bees learned to associate blue light with a reward. Control bees demonstrated an improvement from trials 1 to 6 (Figure 2), while acutely dosed bees did not show a significant difference in performance (Figure 2). There was no significant effect of TMX dose. Bees did not exhibit any significant learning with yellow light as the reward, as expected (see below, Han et al. 2010), and there was also no effect of TMX dose on learning. However, we found strong, significant effects of TMX on bee behavior and the speed at which they completed each trial. As TMX dose increased, the number of abnormal behaviors significantly increased, particularly falling behavior. Abnormal behaviors occurred at a significantly higher rate in earlier than later trials, perhaps reflecting changing effects of the pesticide over time. Bees also completed earlier trials more rapidly than later trials, perhaps reflecting a hyperactive state that can be induced by TMX (Tosi and Nieh 2017).

One of our goals was to improve the visual learning assay developed by Han et al. (2010) for lab use. In general, visual learning with restrained bees or bees maintained within the lab is difficult. With restrained bees, Dobrin and Fahrbach (2012) were able to achieve maximum learning at around 40% after five trials. However, if only bees that showed good learning are included, about 70% of bees learned after five trials. We did not filter our data by learners, but rather by bees that did not complete all of their learning trials which accounts for all bees, not just “good” learners. For blue light, we achieved approximately 75% learning with control bees in trial 6.

We did not find evidence that TMX impairs visual learning, although TMX can reduce honey bee olfactory short-term memory (Wright et al. 2015), and neonicotinoids can harm visual learning (Han et al. 2010). The high level of variance in our learning data made it more difficult for us to detect a TMX effect. However, neonicotinoids may alter visual learning differently from olfactory learning. For example, neonicotinoids such as imidacloprid, thiamethoxam, and clothianidin did not impair the ability of free flying bumblebees to form visual associations (Muth & Leonard 2019). We used 108 bees from eight colonies in our study, a fairly large sample size, but given the high variation shown by bees in the T-maze, even larger sample sizes may be needed to detect pesticide effects on learning.

In our study, we wished to compare bees trained to either blue or yellow light. The failure of our bees to learn the yellow light is not surprising because the strong preference of bees for yellow appears in their first trial, in which approximately 73% of bees chose the yellow maze arm (Han et al. 2010). Thus, bees could not actually demonstrate learning (increased choice for yellow light) given that the highest rate of choice for yellow light (90%) was only marginally higher (a non-significant 17% "increase"). In contrast, bees in the blue light rewarded trials showed an increase from 45% entering the blue arm of the maze (approximately chance level) in the first trial to 75% blue choice in the final learning trial (a significant 30% increase).

Behavior

Bees fed TMX at low and higher doses had significantly more abnormal behaviors than control bees. In our study, we noticed that TMX appeared to harm the ability of bees to walk. Multiple bees spent more time on their backs. Similarly, Williamson & Wright (2014) reported seeing this as "upside down" behavior in bees fed TMX in sugar solution. Bees exhibited higher

levels of abnormal behaviors in the initial trial, perhaps reflecting the changing effects of TMX over time. Tosi & Nieh (2017) showed that TMX treated bees had more abnormal behaviors than control bees 60 min after acute exposure. Tosi et al. (2016) found that the effects of TMX on bee behavior changes over time. Within 1 h of an acute dose, bees showed excitation and increased flight duration, distance, and velocity. However, chronic exposure (1 or 2 days) to TMX decreased flight duration, distance, and velocity.

Time

Overall, bees dosed with TMX completed their trials significantly more rapidly than control bees, regardless of color. High TMX dose bees trained to yellow were significantly faster than control bees trained to yellow. The increased speed for TMX treated bees is similar to the findings of Tosi & Nieh (2017). TMX dosed bees took less time to reach the top of a vertical phototaxis arena, perhaps because of hyperactivity due to TMX (Tosi & Nieh 2017). For blue trained bees, it is relevant to consider speed accuracy tradeoffs, since control bees showed learning, but pesticide-treated bees did not. In *Bombus terrestris*, foraging bees sacrifice the accuracy of their destination for speed and vice versa (Chittka et al. 2003). In *A. mellifera*, foragers made more accurate choices when they spent more time making a foraging choice (Burns & Dyer 2008). Our TMX-treated bees were faster, but arguably less accurate since they showed no learning (Figure 2). Thus, the hyperactivity caused by TMX may have contributed to lower choice accuracy, which we measured as poorer learning. We decided it would be best to use the data we collected to run tests as a starting point for future researchers to investigate the effects of neonicotinoids on speed-accuracy (Dyer & Chittka 2004).

To look at the potential time accuracy tradeoff, we conducted multiple analyses. First, we conducted a repeated measures analysis using a nominal logistic regression to look at all factors including trial and time on bee choice. We first split this analysis by rewarded color then combined both colors. No models had any significant effects. Next, we looked at the effect of total time over all conditioning trials and dose on the number of correct choices a bee made, this was not a repeated measures analysis. Again, we first analyzed each color separately then pooled the data together. No models were significant. In addition, we pooled both pesticide doses as a single factor and followed the same models as above. Again, no models were significant. Thus, our data did not support the existence of a speed accuracy tradeoff. It is possible that this potential link exists between neonicotinoids and a speed-accuracy tradeoff, but it would require more data or a different experimental approach.

Summary

Although we found no clear overall effect of TMX on bee color learning, it is interesting that blue-trained control bees showed learning, but blue-trained pesticide treated bees did not, when the learning of each of these groups was separately analyzed (Figure 2). High variance in our data likely contributed to these findings. We hoped to modify and improve the Han et al. (2010) lab-based assay. Indeed, the learning (up to 80%) exhibited by control bees to blue light, was higher than the maximum learning shown by control bees (65% in Han et al, 2010). Our method therefore advances the techniques for testing visual lab-based learning (Avargues-Weber and Mota, 2016). However, further improvement is necessary to reduce learning variation before this assay can become sufficiently reliable for standard risk assessments. We did confirm that field-realistic doses of TMX increase the number of abnormal behaviors and the speed of

locomotion, inducing apparent hyperactivity. These results are concerning and suggest that additional studies testing the effects of TMX should be conducted on multiple bee species because visual learning plays an important role in all pollinating bees.

References

Avargues-Weber, A., Mota, T. 2016. Advances and limitations of visual conditioning protocols in harnessed bees. Journal of Physiology-Paris. DOI: 10.1016/j.jphysparis.2016.12.006

Bitterman, M. E., Menzel, R., Fietz, A., & Schäfer, S. 1983. Classical conditioning of proboscis extension in honeybees (Apis mellifera). Journal of Comparative Psychology. 97(2), 107-119. doi: http://dx.doi.org/10.1037/0735-7036.97.2.107

Burns, J.G., Dyer, A.G. 2008. Diversity of speed-accuracy strategies benefits social insects. Current Biology. 18(20):R953-R954. https://doi.org/10.1016/j.cub.2008.08.028

Chittka, L., Dyer, A.G., Bock, F., Dornhaus, A. 2003. Bees trade off foraging speed for accuracy. Nature. 424:388.

Decourtye, A., Henry, M., Desneux, N. 2013. Overhaul pesticide testing on bees. Nature 497:188.

Decourtye, A., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S., Pham-Delegue, M.H. 2005. Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee Apis mellifera. Arch Environ Contam Toxicol. 48(2):242-50.

Dobrin SE, Fahrbach SE 2012. Visual Associative Learning in Restrained Honey Bees with Intact Antennae. PLoS ONE. 7(6): e37666. https://doi.org/10.1371/journal.pone.0037666

Dukas, Reuven & Visscher, P. Kirk. 1994. Lifetime learning by foraging honeybees. Animal Behaviour 48:1007-1012. https://doi.org/10.1006/anbe.1994.1333

Dyer, A.G., Chittka, L. 2004. Bumblebees (B*ombus terrestris*) sacrifice foraging speed to solve difficult colour discrimination tasks. Journal of Comparative Physiology A. 190(9):759-763.

EFSA. 2012. Statement on the findings in recent studies investigating sub-lethal effects in bees of some neonicotinoids in consideration of the uses currently authorised in Europe. *EFSA J.* **10**, 1–27.

Elbert, A., Haas, M., Springer, B., Thielert, W. and Nauen, R. 2008. Applied aspects of neonicotinoid uses in crop protection. Pest. Manag. Sci., 64: 1099-1105. doi:10.1002/ps.1616

Erica Zhang, James C. Nieh. 2015. The neonicotinoid imidacloprid impairs honey bee aversive learning of simulated predation. Journal of Experimental Biology. 218: 3199-3205; doi: 10.1242/jeb.127472

European Commission. (2018). Neonicotinoids. Retrieved from https://ec.europa.eu/food/plant/pesticides/approval_active_substances/approval_renewal/neonicotinoids_en

Fischer, J., Muller, T., Spatz, A., Greggers, U., Grunewald, B., Menzel, R. 2014. Neonicotinoids Interefere with Soecific Components of Navigation in Honeybees. PLoS ONE. 9(3):e91364. https://doi.org/10.1371/journal.pone.0091364

Galen P. Dively and Alaa Kamel. 2012. Insecticide Residues in Pollen and Nectar of a Cucurbit Crop and Their Potential Exposure to Pollinators. Journal of Agricultural and Food Chemistry 60 (18), 4449-4456 DOI: 10.1021/jf205393x

Gross, H.J., Pahl, M., Si, A., Zhu, H., Tautz, J., Zhang, S. 2009. Number-based visual generalisation in the honeybee PLoS ONE, 4 (1), art. no. e4263.

Han, P., Niu, C. Y., Lei, C. L., Cui, J. J., & Desneux, N. 2010. Use of an innovative T-tube maze assay and the proboscis extension response assay to assess sublethal effects of GM products and pesticides on learning capacity of the honey bee Apis mellifera L. *Ecotoxicology (London, England)*, *19*(8), 1612–1619. doi:10.1007/s10646-010-0546-4

Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A. 2012. A Common Pesticide Decreases Foraging Success and Survival in Honey Bees. Science. 336:6079:348-350. DOI: 10.1126/science.1215039

Kah, M., Walch, H., Hofmann, T. 2018. Environmental fate of nanopesticides: durability, sorption and photodegradation of nanoformulated clothianidin. Environmental Science: Nano. 5:882-889. DOI: 10.1039/C8EN00038G

Lundin, O., Rundlof, M., Smith, H.G., Fries, I., Bommarco, R. 2015. Neonicotinoid Insecticides and Their Impacts on Bees: A Systematic Review of Research Approaches and Identification of Knowledge Gaps. PLoS One. 10(8):e0136928. doi: 10.1371/journal.pone.0136928

Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., Haettenschwiler, E., Walti, M. 2001. The discovery of thiamethoxam: a second-generation neonicotinoid. Pest Manag. Sci. 57(2):165-176.

Mandyam V. Srinivasan. 2010. Honey Bees as a Model for Vision, Perception, and Cognition Annual Review of Entomology. 55:1, 267-284.

Matsuda, K., Shimomura, M., Ihara, M. Akamatsu, M., Sattelle, D.B. 2005. Neonicotinoids show selective and diverse actions on their nicotinic receptor targets: electrophysiology, molecular biology, and receptor modeling studies. Biosci. Biotechnol. Biochem. 69(8):1442-1452.

Muth, F. Leonard, A.S. 2019. A neonicotinoid pesticide impairs foraging, but not learning, in free-flying bumblebees. Scientific Reports. 9:4764. doi:10.1038/s41598-019-39701-5

R. Nauen, U. Ebbinghaus-Kintscher, V.L. Salgado, M. Kaussmann. 2003. Thiamethoxam is a neonicotinoid precursor converted to clothianidin in insects and plants. Pestic. Biochem. Physiol. 76(2):55-69, doi: 10.1016/s0048-3575(03)00065-8

Gallai., N., Salles, J., Settele, J., Vaissière, Benard E. 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics 68:3:810-821 (2009). DOI: https://doi.org/10.1016/j.ecolecon.2008.06.014

Pahl M, Zhu H, Pix W, Tautz J, Zhang SW. 2007. Circadian timed episodic-like memory - a bee knows what to do when, and also where. Journal of Experimental Biology. 210: 3559–3567.

Pierre, J., Mesquida, J., Marilleau, R., Pham-Delègue, M. H. & Renard, M. 1999. Nectar secretion in winter oilseed rape, Brassica napus -Quantitative and qualitative variability among 71 genotypes. Plant Breed. 118:471–476.

Reed M. Johnson, Marion D. Ellis, Christopher A. Mullin, Maryann Frazier. 2010. Pesticides and honey bee toxicity – USA. Apidologie. 41(3):312-331 DOI: 10.1051/apido/2010018

Saija, P., Dave, G. 2016. Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees. Proc. R. Soc. 283:20160246. DOI: http://doi.org/10.1098/rspb.2016.0246

Sally M. Williamson, Geraldine A. Wright. 2013. Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. Journal of Experimental Biology. 216:1799-1807. doi: 10.1242/jeb.083931.

Schneider, C. W., Tautz, J., Grünewald, B., Fuchs, S. 2012. RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of Apis mellifera. PLoS One. 7:e30023, doi: 10.1371/journal.pone.0030023

Takeda. 1961. Classically Conditioned response in the honey bee. Journal of Insect Physiology. 6(3):168-179.

Tan, K., Chen, W., Dong, S., Liu, X., Wang, Y., Nieh, J.C. 2015. A neonicotinoid impairs olfactory learning in Asian honey bees (*Apis cerana*) exposed as larvae or as adults. Sci Rep. 5:10989. doi:10.1038/srep10989

Tison, L., Robner, A., Gerschewski, S., Menzel, R. 2019. The neonicotinoid clothianidin impairs memory processing in honey bees. Ecotoxicology and Environmental Safety. 180:139-145. DOI: https://doi.org/10.1016/j.ecoenv.2019.05.007

Tosi, S., Burgio, G., Nieh, J.C. 2017. A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability. Scientific Reports. 7:1201.

Tosi, S., Nieh, J.C. 2017. A common neonicotinoid pesticide, thiamethoxam, alters honey bee activity, motor functions, and movement to light. Scientific Reports. 7:15132. DOI: 10.1038/s41598-017-15308-6

Quarles, William. 2011 Pesticides and Honey Bee Death and Decline. The IPM Practitioner. 33: ½.

Williamson, S., Willis, S., Wright, G. 2014. Exposure to neonicotinoids influences the motor function of adult worker honeybees. Ecotoxicology. 23(8):1409-1418.

Wright, G., Softely, S., Earnshaw, H. 2014. Low doses of neonicotinoid pesticides in food rewards impair short-term olfactory memory in foraging-age honeybees. Scientific Reports. 5:15322.

Yang, E.C., Chuang, Y.C., Chen, Y.L., Chang, L.H. 2008. Abnormal Foraging Behavior Induced by Sublethal Dosage of Imidacloprid in the Honey Bee (Hymenoptera: Apidae). Journal of Economic Entomology. 101:6(1):1743-1748. DOI; https://doi.org/10.1603/0022-0493-101.6.1743