LA-UR- 08-7100

Approved for public release; distribution is unlimited.

Title: HONEY BEES (Apis mellifera) AS EXPOLOSIVES DETECTORS: EXPLORING PROBOSCIS EXTENSION REFLEX CONDITIONED RESPONSE TO TRINITROTOLULENE (TNT)

Author(s): Timothy Haarmann, Robert M. Wingo, Kirsten J. Taylor-McCabe

Intended for: Apidologie

Los Alamos
NATIONAL LABORATORY
EST. 1943

HONEY BEES (*Apis mellifera*) AS EXPOLOSIVES DETECTORS: EXPLORING PROBOSCIS EXTENSION REFLEX CONDITIONED RESPONSE TO TRINITROTOLULENE (TNT)

Timothy Haarmann[a], Robert M. Wingo[b*], Kirsten J. Taylor-McCabe[a^]

[a] Los Alamos National Laboratory, Biosciences Division, MS M887, Los Alamos NM, 87545
[b] Los Alamos National Laboratory, Chemistry Division, MS J964, Los Alamos NM, 87545
[*] Telephone (505) 665-0216, FAX (505) 665-3024, wingo@lanl.gov
[^] Telephone (505) 667-5019, FAX (505) 665-3024, kjmccab@lanl.gov

Abstract–We examined honey bee's associative learning response to conditioning with trinitrotolulene (TNT) vapor concentrations generated at three temperatures and their ability to be reconditioned after a 24 h period. We used classical conditioning of the proboscis extension (PER) in honey bees using TNT vapors as the conditioned stimulus and sucrose as the unconditioned stimulus. We conducted fifteen experimental trials with an explosives vapor generator set at 43°C, 25°C and 5°C, producing three concentrations of explosives (1070 ppt, 57 ppt, and 11 ppt). Our objective was to test the honey bee's ability to exhibit a conditioned response to TNT vapors at all three concentrations by comparing the mean percentage of honey bees successfully exhibiting a conditioned response within each temperature group. Furthermore, we conducted eight experimental trials to test the honey bee's ability to retain their ability to exhibit a conditioned response to TNT after 24h period by comparing the mean percentage of honey bees with a conditioned response TNT on the first day compared to the percentage of honey bees with a conditioned response to TNT on the second day. Results indicate that there was no significant difference between the mean percentage of honey bees with a conditioned response to TNT vapors between three temperature groups. There was a significant difference between the percentage of honey bees exhibiting conditioned response on the first day of training compared to the percentage of honey bees exhibiting conditioned response 24 h after training. Our experimental results indicate that honey bees can be trained to exhibit a conditioned response to a range of TNT concentrations via PER. However, it appears that the honey bee's ability to retain the conditioned response to TNT vapors after 24h significantly decreases.

Apis mellifera **/ TNT / proboscis extension response / high explosives detection/ associative learning**

1. INTRODUCTION

As part of a larger project to explore the novel use of honey bees as a potential explosives detection tool, we examined the associative learning response in honey bees to conditioning with trinitrotolulene (TNT). One of the first steps in demonstrating the feasibility of using honey bees as explosives detectors was to test the honey bee's olfaction detection of explosives vapors and explore their ability to exhibit a conditioned response to commonly used high explosives, in this case TNT. To achieve this , we used classical conditioning of the proboscis extension (PER) in honey bees using TNT vapors as the conditioned stimulus (CS) and sucrose as the unconditioned stimulus (US) (Abramson et al. 1997, Masterman et al. 2001, Wright et al. 2002, Wright et al. 2004).

The honey bees ability to detect high explosives was studied using associative learning techniques, more specifically the PER experimental paradigm. Associative learning in honey bees has been well studied and documented (Bitterman et al. 1983, Hammer and Menzel 1995, Faber et al 1999, Menzel and Giurfa 2001). This experimental paradigm is relatively straight forward; when a honey bee's antennae are stimulated using a sucrose solution (US) the honey bee will extend her proboscis. If a sucrose reward is coupled with the presentation of an odor (CS) the honey bee will begin to associate the odor with the reward. Hence, honey bees can be conditioned to respond, through proboscis extension reflex, to the presence of the odor alone.

While much scientific research has used the PER paradigm to study a variety of applications, from learning behavior to neural pathways (Chandra et al. 1998, Komischke et al. 2002, Ray et al. 1997), to date we are unaware of published experiments specifically designed to test honey bees ability to detect the presence of explosives at various concentrations and over a 24 h period of time.

The primary objectives of our study were to (1) train distinct groups of honey bees on three concentrations of TNT vapors and compare the number of bees that exhibit the conditioned response between the three groups (2) investigate the percentages of bees that manifest a conditioned response to TNT vapors on the day of training to the percentage of bees manifesting a conditioned response 24 h after training.

2. MATERIALS AND METHODS

Experiments were conducted in 2005 using Italian honey bees (*Apis mellifera lingustica*) kept in standard Langstroth equipment and maintained in Apiaries at Los Alamos National Laboratory, NM. The day before each experiment, approximately 100 bees were collected at 3 pm from a colony using a modified vacuum cleaner with an insect collection tube. Foraging bees were collected from the entrance of the hive. The bees were transferred from the collection tube into holding cages where they were fed with a 1.8 M sucrose solution. Cages were placed in a dark cabinet at room temperature for 2 h allowing the bees to freely feed. The sucrose was removed after approximately 2 h and bees were left in the cage for 15 h until the next day's experiments.

To reduce the stress on the bees, and to make handling of the bees easier, the bees were immobilized prior to the experiments by cooling them in a refrigerator (4°C) for 15 min. After which, 30 bees were mounted in plastic holders (the size of a drinking straw) so that their antennae and mouthparts could move freely. The bees were restrained in the holder using tape (Figure 1). To acclimate the bees to the airflow of the TNT odorant delivery system, the bees were left for 30 min in front of a fan delivering an airflow similar to the one used for the explosives odor delivery.

Prior to starting the olfactory conditioning procedure, bees were screened for ability to exhibit an unconditioned response by touching the antennae with 1.8 M sucrose solution. Bees that fail to exhibit the reflex were not used in subsequent experiments. Standard methods for training honey bees using differential PER conditioning were followed (Bitterman et al., 1983). Only bees that showed the unconditioned response (PER following the application of 1.8 M sucrose solution to the antennae) and that did not respond to the mechanical air flow stimulus were used. A device that delivered a continuous airflow was used for odorant application.

Each conditioning trial lasted for 31 s which consisted of 20 s of air flow using activated charcoal filtered laboratory air at 1.3 L/min provided by a Syntech Stimulus Controller, 6 s of explosives vapor diffused into the filtered laboratory air into total flow rate of 1.3L/min for the (conditioned stimulus) and 10 s of activated charcoal filtered laboratory air at 1.3 L/min. All gas flows were measured using an Aalborg Mass Flow Meter 30 times and averaged. Stimulus controller flows were stable by +/- 2% the stated values. During the 6 s of explosives vapor presentation, but after the first 3 s, the reward (unconditioned stimulus-CS) was delivered. Bees that demonstrated a spontaneous response to the first presentation of the CS were eliminated from the experiments. A total of four conditioning trials were performed with a 15 to 25 min inter-trial duration. The conditioned proboscis extension responses occurring within the 6 s of the stimulus delivery for the conditioning trials was rewarded and recorded. Separate experimental trials, using new bees each time, were conducted with the vapor generator set at 3 distinct temperatures (43°C, 25°C and 5°C), thus producing three distinct concentrations of TNT vapors (1070 ppt, 57 ppt, and 11 ppt). To test the bee's ability to retain the training out to 24 h, when possible, the trained bees were fed at 5 pm, kept in the dark cabinet at room temperature for 24 h after original conditioning rounds and re-tested for the ability to demonstrate conditioned PER.

During all the trials, the conditioned stimulus was presented using a Syntech Stimulus Controller interfaced with a custom designed explosives vapor diffusion generator. We followed the published protocol on making and using a diffusion vapor generator ASTM Method F2069-00 "Standard Practice for Evaluation of Explosives Vapor Detectors". The explosive (100-200 mg) resided in large test tube to which a ground stopper was attached that had two Teflon tubes connected at the top of the stopper via barbed fittings. The tubes allowed air flow across the top of the test tube into which explosives vapor diffused. The test tube resided in a large thermal ballast comprised of a copper cylinder 10" in length and 3" in diameter, heated or cooled via a water bath/coil around the bottom ½ of the copper cylinder and via a heating blanket on the top ½ of the cylinder and controlled with Gemini Temperature controller with T-type thermocouple interfaced to the ballast. Cooling of the copper was by the water bath/coil, heating was accomplished with the heating blanket/Gemini controller and the entire assembly was insulated. The ballast had a thermocouple inserted to monitor temperature near the glass test tube/explosives sample and 12-18 h of thermal equilibrium was allowed prior to initial vapor pulse. The explosives vapor was fed into the continuously flowing air stream via solenoid valves allowing gas flow across the top of the test tube during actuation. The vapor generator was pulsed until stable explosives vapor output was achieved as measured by a portable Ion Trap Mobility Spectrometer (ITMS, GE VaporTracerII) interfaced with a Dell Precision M90 laptop workstation. The generator was pulsed on a timed sequence for the duration of the experiments to maintain a stable and repeatable explosives vapor concentration.

A response curve for explosives vapor produced by the generator, when measured by the ITMS operated in vapor mode, was developed by pipetting known masses of explosives dissolved in acetone onto a desorption trap that is inserted the ITMS and operated in particle mode. A response curve from the signals generated on the ITMS from known masses of explosives, was used to determine vapor phase concentrations (mass/volume) of TNT produced by the diffusion vapor generator (using flow rate of gas exiting the vapor generator and integrated over time using ITMS response to a given HE mass).

The vapor diffused into an air flow of 200 mL/min which merged into the main stream of air (1.3 L/min) and the main flow was flow compensated for the addition of the vapor flow to ensure constant vapor velocity and flow rate (1.3 L/min). The device sat within a portable chemical hood to prevent a build up of volatiles.

2.1 Statistical Methods

We used a one-way Analysis of Variance (ANOVA) to compare the mean percentages of bees conditioned at the three separate temperatures. We used a non-parametric Wilcoxon Signed Ranks Test to compare the mean percentages of bees exhibiting a conditioned response on the day of training to 24 h after training.

3. RESULTS

3.1 TNT Concentration

Table I and Figure 2 demonstrate the results of the experiments to condition the bees with three concentrations of TNT vapors generated at three distinct temperatures. We compared the mean percentages of bees conditioned at three concentrations of TNT using an ANOVA. There was no significant difference between the percentage of bees exhibiting a conditioned response to TNT vapors between any of the three groups ($P = 0.984$, $df = 2$, $F = 0.016$).

3.2 Time

Table II and Figure 3 demonstrate the results of the comparison between the mean percentage of bees exhibiting a conditioned response to TNT vapor on the day of training and the mean percentage of bees exhibiting a conditioned response after 24 h for all temperature experiments. We compared the mean percentages using a Wilcoxon signed rank test. There was a significant difference between the percentage of bees exhibiting conditioned response on day 1 compared to 24 h ($P = 0.017$, $Z = -2.383$)

4. DISCUSSION

4.1 TNT Temperature/Concentration

The mean percentage of honey bees that exhibited the conditioned response to TNT vapor between the groups of honey bees trained at the three TNT vapor concentrations were remarkably similar: 63.78 (± 22.63), 65.38 (± 20.94), and 61.76 (± 22.83). While the lowest percentage (61.76%) of honey bees exhibiting PER response was seen in the 5 °C group, presumably because of the lower ppt concentration of TNT molecules, the same was not true with the high temperature/concentration group of honey bees (63.78%). The actual difference in these percentages is rather small and the standard deviation large enough, that the statistical analysis indicated, as expected, no significant difference between all three groups. One might expect that a higher percentage of honey bees would exhibit a PER response with the higher temperature/concentration of TNT, but this did not hold true for our experimental set.

We chose this particular temperature range because in an attempt to create TNT vapor concentrations that might typically be encountered in temperate climates. Our results indicated that a consistent percentage of honey bees were able to exhibit PER at a varied temperature/concentration regime. As part of the larger project to explore the use of honey bees as an explosives detection tool, we plan to expose and train honey bees to a variety of explosives that are commonly found in terrorist scenarios.

During our experiments, we noted that not all honey bees were trainable; however, those that were trained had high retention rates during training. The results from our experiments indicated that not all honey bees trained equally and there was a difference seen amongst individual honey bees. The honey bees collected from our experiments were foragers of varying ages and likely varying states of health and vigor, which contributed to some of the variability seen in training rates. Furthermore, it is apparent during training that not all honey bees train equally and thus several training rounds are required to increase the total number of trained individuals. During our experiments, we experienced that once a honey bee was trained, she typically remained trained throughout the subsequent training rounds. However, at the end of four rounds, roughly 62% were trained leaving roughly 38% that never trained successfully. This "drop out" rate will need to be taken into account during training of honey bees should they eventually be applied as explosives detectors. We would highly recommend further experiments to determine if this trend is repeatable for other explosives.

4.2 Time

There is a higher percentage of honey bees exhibiting a PER response on the day of training than after a 24 h period. There are several possible explanations for this trend. It could be concluded that the honey bees are losing their acquired training and are exhibiting some memory loss. Or, it may simply be a result of stress from being strapped in a harness for 24 h rather than memory loss itself. Other research has shown that honey bees have the ability to retain their memory past 24 h (Meller et al. 1995, Menzel 2001). Hence, memory loss is likely not the cause of the significant difference between the two groups. It is more likely a result of the honey bees being unduly stressed. This result does shed light on the potential application of honey bees in an explosives detection tool and it would be critical to find out if this trend is repeatable with other explosives. We did not attempt to analyze the cause of this loss percentage drop after 24 h but it might be useful to investigate this aspect in the future.

More experiments are planned using a variety of other explosives including, among others, C-4 plastic explosive and propellant. Additionally, we plan to test honey bees using "interferents" to assess the false positive rates of detection. These interferents are items or scents that commonly cause false positives in explosives detection technologies or canines. As part of these future experiments, we plan to cross-test honey bees using a variety of explosives. In other words, test the ability of honey bees trained on TNT to detect other explosives for which they have not honey been trained. We also plan on training and testing honey bees on the ability to be trained on multiple explosives and attempt to understand the limitations or advantages to this type of multiple explosives training protocol. Based on our experiments with TNT, it is our belief that there is a potential that honey bees could be used as a future tool for explosives detection. However, much research is still needed to fully understand the limitations of their capabilities and how best to utilize honey bees for this unique application.

ACKNOWLEDGEMENT

Funding for this project was provided by the Defense Advanced Research Projects Agency. We are grateful to Dr. Morley Stone who functioned as Program Manager for these experiments and provided useful guidance and insight. We also would like to acknowledge Incentinel Inc. for their help and collaboration on this project. Bee training support was provided by Rhonda Robinson, Sheri Sherwood and Sarah Mauer.

Detailed Abstract– As part of a larger project to explore the use of honey bees as potential explosives detectors, we examined the associative learning response in honey bees to conditioning with trinitrotolulene (TNT). The first step in demonstrating the feasibility of using honey bees as detectors was

to test the honey bees ability to detect and exhibit a conditioned response to a high explosive, in this case TNT. To achieve this, we used classical conditioning of the proboscis extension (PER) in honey bees using TNT vapors as the conditioned stimulus and sucrose as the unconditioned stimulus.

The primary objectives of our study were to (1) compare the percentages of honey bees that exhibited the conditioned response between groups that were conditioned using high explosives vapors generated at three disperate temperatures (2) investigate the percentages of honey bees that can be retrained after a 24 h period and were still capable of detecting and responding to TNT.

We conducted fifteen experimental trials with an explosives vapor generator set at 43°C, 25°C and 5°C, thus producing three concentrations of explosives (1070 ppm, 57 ppm, and 11 ppm). We wanted to test the honey bee's ability to exhibit a conditioned response to all three concentrations by comparing the mean percentage of honey bees successfully exhibiting a conditioned response within each temperature group. Furthermore, we conducted eight experimental trials to test the honey bee's ability to retain their ability to exhibit a conditioned response to TNT after 24h period by comparing the mean percentage of honey bees with a conditioned response TNT on the first day compared to the percentage of honey bees with a conditioned response to TNT on the second day.

Results indicated that there was no significant difference between the mean percentage of honey bees with a conditioned response to TNT within the three temperatures groups. There was a significant difference between the percentage of honey bees exhibiting conditioned response on day one compared to 24 h. From our experiments, it appears that honey bees can be trained to exhibit a conditioned response to a range of TNT concentrations using

REFERENCES CITED

Abramson, CI; Abramson, CI; Aquino, IS; Aquino, IS; Silva, MC; Silva, MC; Price, JM; Price, JM, 1997. Learning in the Africanized honey bee: Apis mellifera L. Physiology and Behavior; 1997; v.62, no.3, p.657-674.

ASTM Method F2069-00 Standard Practice for Evaluation of Explosives Vapor Detectors

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

Bromenshenk J.J., Henderson C.B., Smith G.C., 2003. Biological systems. In: Jacqueline MacDonald, J., Lockwood, J.R., McFee, J., Altshuler, T., Broach, T., Carin, L., Rappaport, C., Scott, W.R., Weaver, R. (Eds.), Alternatives for Landmine Detection. RAND, pp. 273--284.

Chandra, S; Smith, BH. 1998. An analysis of synthetic processing of odor mixtures in the honeybee (Apis mellifera). Journal of Experimental Biology; Nov., 1998; v.201, no.22, p.3113-3121.

Faber, T; Joerges, J; Menzel, R. 1999, Associative learning modifies neural representations of odors in the insect brain, Nature Neuroscience; Jan., 1999; v.2, no.1, p.74-78.

Hammer, M; Menzel, R. 1995, Learning and Memory in the Honeybee. Journal of Neuroscience; v.15, no.3 PART 1, p.1617-1630

Komischke, B., Giurfa, M., Lachnit, H., Malun, D., 2002. Successive olfactory reversal learning in honey bees. Learning and Memory 9(3), 122—129.

Masterman, R., Ross, R., Mesce, K., Spivak, M., 2001. Olfactory and behavioral response thresholds to odors of diseased brood differ between hygienic and non-hygienic honey bees (*Apis mellifera L.*). Journal of Comparitive Physiology A 187, 441—452.

Meller, V. and Davis, R. 1996, Biochemistry of Inscet Learning: Lessons from Bees ad Flies. Insect Biochemistry and Molecular Biology; Vol. 26, No.4, pp. 327-335.

Menzel, R. 2001, Searching for the Memory Trace in a Mini-Brain, the Honeybee, Learning & Memory; 8:53-62.

Menzel, R; Giurfa, M. 2001, Cognitive architecture of a mini-brain: the honeybee, TRENDS IN COGNITIVE SCIENCES; FEB 2001; v.5, no.2, p.62-71.

Ray S., Ferneyhough B., 1997. The effects of age on olfactory learning and memory in the honey bee *Apis mellifera*. Learning and Memory 8; 789--793.

Wright, GA; Skinner, BD; Smith, BH, 2002, Ability of honeybee, Apis mellifera, to detect and discriminate odors of varieties of canola (Brassica rapa and Brassica napus) and snapdragon flowers (Antirrhinum majus), Journal of Chemical Ecology; April, 2002; v.28, no.4, p.721-740.

Wright, GA; Smith, BH, 2004. Different thresholds for detection and discrimination of odors in the honey bee (Apis mellifera). CHEMICAL SENSES; FEB 2004; v.29, no.2, p.127-135.

Table I. Mean Percentage of Trained Honey Bees Exhibiting PER to TNT Vapors (Generated at Three Temperatures) after Four Conditioning Rounds.

Temperature (°C)	N		% Exhibiting PER
43	79		72.15
	36		44.44
	40		87.5
	29		79.31
	31		35.48
		Mean	**63.78 ± 22.63**
25	22		77.27
	55		41.82
	26		38.46
	20		85.00
	17		64.71
	20		85.00
		Mean	**65.38 ± 20.94**
5	25		84.00
	29		75.86
	26		53.85
	27		33.33
		Mean	**61.76 ± 22.83**

Table II. Percentage of Bees Exhibiting PER to TNT Vapors on the Day of Training and After 24 h.

Temperature (°C)	N		% Exhibiting PER on Day 1		% Exhibiting PER after 24h
43	29		79.31		55.56
43	31		35.48		44.44
43	79		72.15		26.76
25	22		77.27		10.00
15	29		75.86		50.00
5	26		53.85		19.05
5	27		33.33		20.00
5	29		75.86		50.00
		Mean	**62.89 ± 19.29**	***Mean***	**34.48 ± 17.45**

Figure 1: Harnessed Honey bee exhibiting the Proboscis Extension Reflex (PER).

Figure 2. Percent of Honey Bees, Within Three Temperature Groups, that Exhibited PER to TNT Vapors

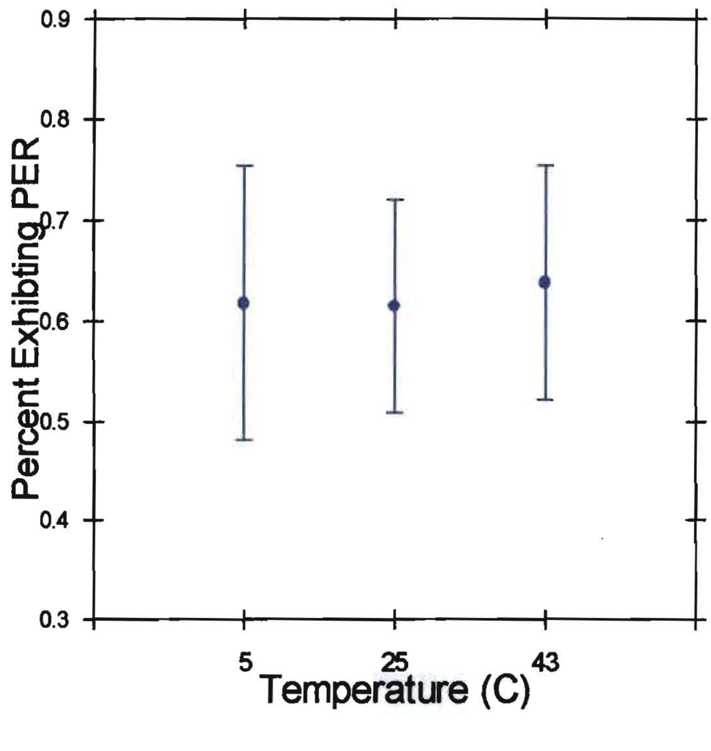
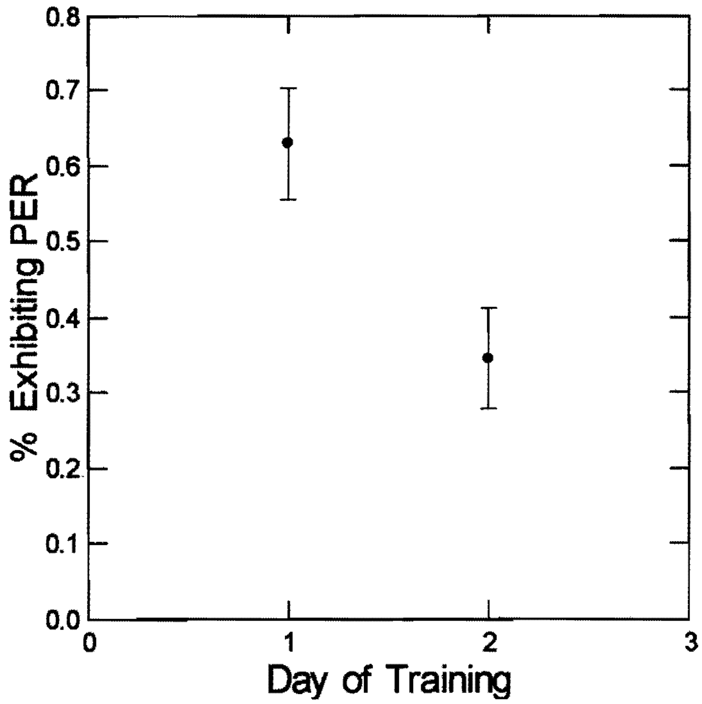


Figure 3. Percentage of Honey Bees Exhibiting PER to TNT Vapors on the Day of Training and after 24 h.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
% Exhibiting PER
0
1
2
3
Day of Training