

Early Detection of the Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) Infestation Using Olfactory Appetitive Learning of Honeybee, *Apis mellifera*

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ABSTRACT



Infestation of palm trees by the red palm weevil (RPW) *Rhynchophorus ferrugineus* in the tropics, Middle East, and North Africa represents a serious economic threat and has reached a critical level due to significant spread. The inability of early detection of this pest is the main obstacle against infestation management. Here, we investigated the potential use of the honeybees' olfaction for early detection of the odorant signature of the RPW's aggregation pheromone. We conditioned the Proboscis Extension Response (PER) of harnessed honeybee foragers using the RPW's aggregation pheromone as a conditioned stimulus in an appetitive Pavlovian learning protocol using either the synthetic pheromone or the natural pheromone released by groups of RPWs. To test the sensitivity of pheromone detection by bees, five groups of RPWs (25, 20, 15, 10, and 5 individuals each) were used to condition the bees. Medium- and long term- memory retention was evaluated after (4, 24, 48, and 72 hrs post-conditioning). The honeybees learned the odor of the RPW's pheromone both in its synthetic and its natural form. In the latter case, learning success increased with the number of weevils used as a pheromone source. The percentage of learners after six conditioning trials was 89%, 92%, 87%, 80%, 68%, and 49% for the control, 25, 20, 15, 10, and 5 RPW group sizes, respectively, and was positively correlated with RPW group size. Memory was retained for at least 24 h without a significant decay in all groups trained with different numbers of RPWs. Memory retention decreased significantly after 48 h. In conclusion, PER conditioning using RPW pheromone as a conditioned stimulus for subsequent detection of RPW infestations was valid up to a period shorter than 48 h for the 15 RPW group (27% of bees) and to 72 h for the 20 RPW group (25% of bees). The results show that honeybees could be used as promising bio-detectors for the red palm weevil. However, further research and field trials are needed to establish them as proven biological sensors.

Keywords: Honeybee, Olfactory conditioning, Palm trees, Pest bio-detectors, Red Palm Weevil.

INTRODUCTION

Date palms are not only important in economic and nutritional terms, but also in terms of the various medicinal effects of dates, which include antioxidant, antimutagenic, antibacterial, antifungal, antitumor, antiulcer, and immune-modulatory properties (Vayalil, 2002, Al Jaouni *et al.*, 2019, Zar Pasha *et al.*, 2022). In recent years, the date palm industry has suffered from severe infestations by the red palm weevil, *Rhynchophorus ferrugineus* (Olivier) (RPW). Established large colonies of RPW are able to kill even large healthy trees within a few months. Infestation with the red palm weevil (RPW) *Rhynchophorus ferrugineus* is currently threatening not only date palm trees but many other types of palms including oil, sugar, sago, palm-ya, royal, Washingtonia, and coconut palms.

Currently, a heavy infestation is seriously spreading in all the countries of the Middle East and North Africa, tropical Asia, and America (Dalbon *et al.*, 2019, Ferry, 2019). This pest is known to destroy entire grooves of palms (Al-Dosary *et al.*, 2016). It is estimated that about 30% of annual date palm production could be lost due to pests and diseases with RPW as a principal culprit (El-Juhany, 2010). In the Gulf region, statistics estimate the loss due to RPW alone to reach about 5% accounting for 26 million USD (El-Sabea *et*

al., 2009). In Egypt, the total loss of palm production reached ca. 4 billion Egyptian pounds (~ 400 million USD) between 1992 and 2000 (Abbas, 2019).

Early detection of RPW infestation is therefore critical for effective pest management, in particular, because symptoms are not visible until the palm tree is inevitably dying and falling (Kurdi *et al.*, 2021). As all the stages of the RPW life cycle are concealed inside the palm, early detection is hardly attainable (Soroker and Colazza, 2017). Yet, palm trees in early stages of infection may respond to chemical treatment via stem injection unlike those in advanced stages, where larvae have already caused extensive irreversible damage. The currently available methods for detecting infestation include visual inspection, chemical detection of volatile signatures produced by infested palm trees, detection of the sound produced by the feeding larvae, and thermal imaging to detect temperature increases in infested palms (Al-Dosary *et al.*, 2016, Jalinas *et al.*, 2019, Boulila *et al.*, 2023). Despite the great variety of detection techniques, none are capable of detecting an infestation early enough to save the palms before it is too late (Faleiro *et al.*, 2019).

In recent years, the enhanced olfactory capabilities of certain animals have been harnessed for disease and pest detection strategies. Distinguished animals enlisted for this purpose include dogs, honeybees, wasps,

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moths, ants, and the nematode *Caenorhabditis elegans* (Piqueret *et al.*, 2023). These animals' acute sense of smell has been utilized in the detection of a range of diseases including cancer, bacteriuria, *Clostridium difficile*, hypoglycemia, and tuberculosis (Edwards *et al.*, 2017; Namgong *et al.*, 2022). Pests can also be detected by certain animals. For instance, dogs can be trained to detect bark beetle-infested spruces through the beetles' pheromones (Vošvrková *et al.*, 2023). *Microplitis croceipes* wasps also can be conditioned to distinguish between odors of aflatoxigenic *Aspergillus flavus* fungal strains (Tertuliano *et al.*, 2005) and identify odors indicative of pathogenic fungal growth (Rains *et al.*, 2006).

Honeybees serve as established insect models for studying associative learning and memory. They have the ability to associate multiple events in their environment, particularly in the contexts of foraging and navigation. Their capability to connect environmental cues with anticipated outcomes is crucial for their efficiency in these contexts and ultimately for their survival. Research on honeybee learning has seen significant advancements through the utilization of classical Pavlovian conditioning. In this type of conditioning, hungry honeybees learn to associate a neutral stimulus (conditioned stimulus, CS) with a biologically relevant stimulus (unconditioned stimulus, US) such as a sucrose solution. During this conditioning protocol, when a honeybee's antennae come into contact with sucrose solution, it triggers an extension of the proboscis for feeding.

By pairing an odorant with the sucrose solution, honeybees form an association and exhibit a proboscis extension response (PER) to the odorant, anticipating the delivery of sucrose (Giurfa and Sandoz, 2012). The same principle is used by honeybees to learn and memorize odorants, allowing them to efficiently exploit the most profitable flowers in their complex natural habitats. The resulting memories vary depending on the conditioning protocol, such as the number of trials. However, recent studies have shown that even a single odor-rewarded experience may, in certain cases, lead to long-term memories lasting several days and stabilized via protein synthesis in the bee brain (Villar *et al.*, 2020).

The goal of this study was to determine whether honeybees and the olfactory PER training regimen in the lab could be used to effectively detect RPW infestation of palm trees at an early stage. The honeybees were trained to elicit PER, a sign of an RPW infestation, using both live RPW and synthetic RPW aggregation pheromones as the odor source. Along with measuring the early detection threshold that can be reached with this method, the study also sought to assess the minimum number of RPW individuals that honeybees could detect and the duration up to which the honeybees' memory of RPW's odor will last under these conditions, as a measure of applicability in future field investigations.

MATERIALS AND METHODS

RPW collection and rearing

Adult RPW were collected from infested palm trees in different farms in Sharkia and Ismailia governorates during the period from October 2020 to May 2022. They were kept in transparent plastic boxes (120 X 60 X 30 cm) in the laboratory under controlled conditions at 29-31°C and 35-45% RH. The lids of the plastic boxes were perforated (3 mm pores) for ventilation. Sugar-cane was used as a food source by the longitudinal splitting of 10 cm pieces of stem (Ahmed *et al.*, 2015).

Honeybee collection and maintenance

Honeybees were collected from the apiary located at the Faculty of Agriculture, Suez Canal University. Honey-bees leaving the hive at 8-10 am in the morning were collected at the hive entrance in small plastic jars (120 ml) with perforated lids during August 2021. Foragers were used in the experiments because they exhibit the highest appetitive motivation for sucrose (Scheiner *et al.*, 2001, Matsumoto *et al.*, 2013). Honeybees were taken immediately to the lab in shaded perforated boxes. In the lab, they were immobilized on ice for subsequent handling. Cooling time was kept to a minimum, as extended cooling could impair learning performance (Frost *et al.*, 2011). They were then harnessed in small plastic Eppendorf tubes that were cut at the tip to allow protrusion of the head and free movement of the proboscis and antennae. Honeybees were secured with tape and a droplet of wax on the back of their heads (Matsumoto *et al.*, 2012). Harnessed honeybees were then tested for intact PER responsiveness by stimulating their antennae with 50% sucrose. Non-responsive honeybee individuals were discarded, while responsive ones were fed 5 µl of 50% sucrose and kept resting for 3 hrs in an incubator at a temperature of 25±1°C and humidity of 60% before the experiments. Honeybees were fed twice a day throughout the experimental period, 12 hrs before and 30 min after conditioning or retention tests (Smith and Burden, 2014).

RPW aggregation pheromone

Synthetic RPW male aggregation pheromone (Ferrugineol 700 mg; 9:1 mixture of 4-methyl-5-nonanol and 4-methyl-5-nonanone) was purchased from the Plant Protection Research Institute in Dokki, Giza, Egypt. It is manufactured by Chim Tica International S.A. Company, Costa Rica, and is usually used in RPW pheromone traps (Faleiro *et al.*, 2003, Hussein *et al.*, 2014).

Absolute conditioning assays

Individual forager honeybees were subjected to a standard absolute olfactory PER conditioning protocol, where a single odorant (RPW aggregation pheromone) was paired with a sucrose solution. This was done to explore the potential for using the honeybees' olfactory abilities to detect red palm weevil (RPW) infestations. Sucrose solution (50%) served as the unconditioned stimulus (US).

The RPW pheromone was applied to a filter paper attached to a plastic syringe, which served as the odor cartridge. A red LED light was used to signify the start of each phase of the conditioning trials, positioned behind the honeybee's head outside of their visual field to prevent unintended conditioning. To prevent interference with olfactory learning, the odor was ventilated using a standard air ventilation system set at a moderate level to minimize mechanical disturbances from air currents (Matsumoto *et al.*, 2013).

Six conditioning trials were used with an inter-trial interval (ITI) of ten minutes. Each conditioning trial started with a 15 s familiarization period in which a honeybee was placed stationary in front of a clean air current delivered via a 20 ml plastic syringe directed at its antennae. This ensured that the air around the honeybee is clear of any other odor and familiarizes the honeybees with the stimulus delivery air current that they will be exposed to next. To maintain a steady constant rate of air current delivery throughout all sessions, the full 20 ml of the syringe was delivered steadily within 4 seconds. The odor was then presented for 4 s to the honeybee antennae. After an inter-stimulus interval (ISI) of 3 s from the onset of odor presentation, a 50% sugar reward was delivered via a moistened toothpick to the distal flagella of both antennae to elicit the reflexive PER. Bees were then allowed to lick the sucrose for 3 s. This ensured a 1 s overlap between CS and US. Honeybees remained in the setup for 15 s after stimulation offset and were then removed from the training platform to return to their numbered holding rack. For precise stimuli delivery, an acoustic signal was programmed to signal the onset and duration of stimulus delivery. The total duration of each trial was 36 s (Fig. 1).

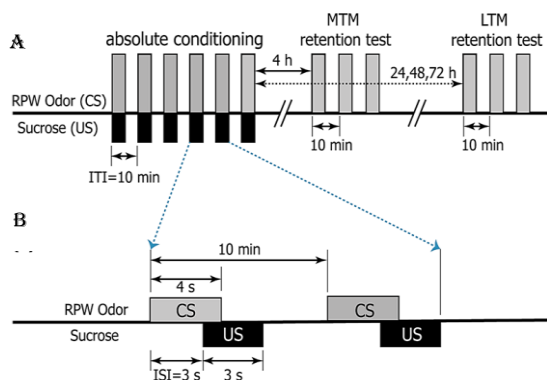


Figure (1): Experimental protocol of the absolute conditioning of honeybees by the RPW odor. A, the six conditioning trials were followed by four memory retention tests. B, a detailed conditioning trial showing that after 15 s of familiarization, the RPW odor (CS) was presented to the honeybees for 4 s. The last second of the RPW odor overlapped with the presentation of a sugar reward (US) for 3 s. Honeybees were allowed to lick the sugar reward (US) in the last 2 s without the RPW odor. ITI, is inter-trial interval; MTM, is medium-term memory and LTM, is long-term memory.

Control honeybees were conditioned and tested with the synthetic aggregation pheromone. Five treatment groups of honeybees were conditioned and tested via increasing intensities of the natural pheromone and

other odorants produced by different group sizes of live adult RPWs (5, 10, 15, 20, and 25 individuals per group). An aquarium aerator pump was used to extract a carrier airflow from the cage in which each of the five RPW groups had lived for at least two weeks and deliver the odor to the honeybees' antennae at a rate of about 7 ml/s (Smith and Burden, 2014).

Before conditioning started, the PER responsiveness of all honeybees was tested via antennal stimulation with 50% sucrose. Non-responsive honeybees were discarded while the responsive honeybees were used in the subsequent standard conditioning protocol (Felsenberg *et al.*, 2011). In this protocol, honeybees had to learn to associate the olfactory conditioned stimulus (CS) of the RPW aggregation pheromone (control) or odor of live RPW (treatment) with the unconditioned stimulus (US, 50% sucrose solution) as a reward.

Data were recorded on a binomial scale where full proboscis extension (PER), as a response to the stimulating odorant, was recorded as 1, while no or partial PER was recorded as 0. Meanwhile, learning success (LS) was determined based on the response in the last acquisition trial, where honeybees responding to the CS were considered Learners, while non-responding bees were considered Non-learners. Optimal learners (OL), was an additional third sub-category of learners in which we included bees that responded to the CS in all acquisition trials except in the first one where the response was forcefully random. A Learning score was calculated for each bee as the sum of all responses to the CS in the five acquisition trials (the first trial excluded).

Memory Retention Tests

Three unreinforced memory retention tests were performed after conditioning by presenting the conditioned RPW odor in the absence of US to assess the efficiency of honeybees' recall of the conditioned RPW odor. ITI in testing trials was the same as in conditioning trials. A PER response in a test was recorded if it occurred after odor onset and before odor offset. A positive response was recorded if the honeybee responded with a PER two or three times out of the three test trials, while a negative PER response was recorded if the honeybee didn't produce any PER response or only gave one PER in the three tests (Smith and Burden, 2014). Test for medium-term memory was carried out 4 hrs after conditioning, while long-term memory testing was carried out 24, 48, and 72 hrs after conditioning. A camera was used to record the honeybees' responses in addition to a red LED behind the bee to indicate odor onset and offset to facilitate the recording of responses.

Statistical Analysis

Acquisition and memory retention performances were analyzed using generalized linear mixed models (GLMM) for the binomially distributed PER responses of 0 and 1 which were linearized using a logistic logit link function. The models included the fixed effect of Treatment (RPW group size/odor intensity) and the random effect of individual honeybees *Bee_ID* to account for the repeated-measures design as each

single honeybee was measured six times during the course of acquisition trials and four times in the memory retention tests. Post hoc Tukey HSD was used to analyze pairwise comparisons after the GLMM analysis. JMP software (JMP, Version 17, SAS Institute Inc., Cary, NC, 2023) was used to perform these analyses.

Ethical considerations

All methods and procedures described in this study were performed by taking care not to harm the honeybees unnecessarily and to minimize the number of individuals used in the experiments. All procedures have been approved by the ethical committee of the Faculty of Science at Suez Canal University in Egypt (Certificate of Ethical Approval no. REC60/2020).

RESULTS

The current study first showed that honeybees can learn the odor of RPW’s aggregation pheromone. Then it demonstrated that the percentage of honeybees learning the odor is increased by the intensity of odor that is expressed by increasing the RPW group size. This could consequently help assess the minimum number of RPW individuals infecting a palm tree that can be practically detected at the earliest by trained honeybees at least in lab settings. The study finally assesses the honeybees’ memory retention of the RPW odor as a function of the RPW odor intensity/group size.

Honeybees successfully learned the odor of RPWs

Our data showed that honeybees can be conditioned to respond appetitively with reflexive PER to the olfactory stimulation of RPW’s aggregation pheromone. Control honeybees were trained to associate the RPW aggregation pheromone odor (CS) with a reward of sugar solution (US). The learning acquisition curve of control honeybees (Fig. 2) showed a continuous increase in the percentage of honeybees learning the association through six acquisition trials. The percentage of learners at the end of acquisition trials amounted to 89% (n = 36).

Increasing RPW group size enhanced the learning performance of honeybees

Learning acquisition results showed that raising the pheromone intensity by increasing the group size of RPW was positively correlated with the ability of honeybees to recognize and learn the odor. Acquisition performance for honeybees (n = 233) was analyzed using a repeated-measures binomial GLMM to model the learning performance as a function of RPW group size. Five groups of honeybees (n = 39, 39, 40, 40, 39) were conditioned to the olfactory stimulus produced by five levels of RPW group sizes of (25, 20, 15, 10, and 5 RPW individuals; namely RPW25, RPW20, RPW15, RPW10, RPW5, respectively) then their responses were fitted as a Treatment fixed effect factor compared to the control group (n = 36) which was conditioned by a synthetic aggregation pheromone. The model included the individual honeybees Bee-ID as random

effects to accommodate the repeated-measures design as each honeybee’s learning performance was measured six times in the six conditioning trials. The regression model was used to test if the RPW group size - indicative of pheromone intensity - significantly predicted the honeybees’ acquisition performance in the conditioning phase. The overall regression model was statistically significant (GLMM: Treatment: F5, 1358 = 31.64, $p < 0.0001$) indicating that group size/pheromone intensity significantly affected the honeybees acquisition performance in the conditioning phase (Fig. 2). *P-value* was obtained by the log-likelihood ratio tests of the full model with the Treatment effect against the null model without it. The GLMM model estimates of factors back transformed by a logistic function and their associated lower- and upper 95% confidence intervals, and group sizes are summarized in (Table 1).

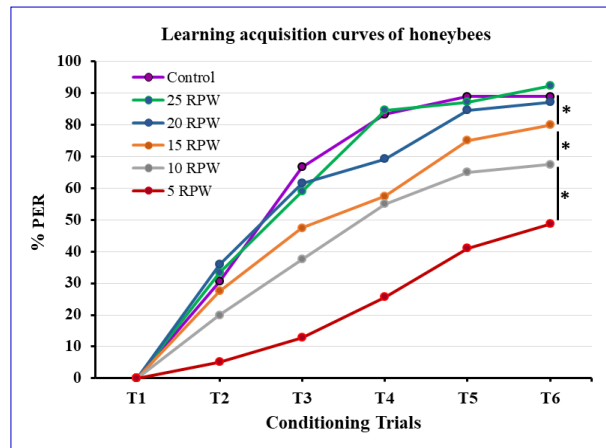


Figure (2): The percentage of successfully conditioned Extension Response (PER) responses of honeybees in relation to conditioning trials. The olfactory acquisition curves illustrate honeybees learning to associate the odor of the RPW aggregation pheromone with a sucrose reward over six acquisition trials. Six treatment conditions were tested, with synthetic pheromone used as a control and pheromone produced by different-sized groups of RPW for the five treatment groups. Statistical significance (*) at the $p \leq 0.05$ probability threshold was determined using binomial repeated-measures GLMM analysis. Group size (n) is displayed in Table (1).

Table (1): The GLMM model estimates of the olfactory acquisition fixed factor back transformed by a logistic function and their associated lower- and upper 95% confidence intervals (CI), and group sizes (n).

Treatments	n	Estimated fixed factor	Lower 95% CI	Upper 95% CI
Control	36	0.557	0.477	0.634
RPW25	39	0.575	0.498	0.649
RPW20	39	0.556	0.479	0.631
RPW15	40	0.377	0.307	0.453
RPW10	40	0.254	0.196	0.322
RPW5	39	0.113	0.077	0.164

The post hoc Tukey HSD multiple pairwise comparisons analysis after the GLMM model fitting revealed that there was no significant difference between the honeybees’ acquisition performance of the control

group and both RPW25 and RPW20 groups. However, honeybees' learning acquisition for the RPW10 and RPW5 dropped significantly compared to the control group. Multiple pairwise comparisons using Tukey HSD are shown in Table (2) with their corresponding t-ratios and probability values.

Learning score analysis

To analyze the overall performance of honeybees in the conditioning phases, a learning score was calculated for each experimental group that sums up all the 0s and 1s of PER responses across the six conditioning trial phases. As a count score with a Poisson distribution, we analysed it using a generalized linear model (GLM) to model the learning score of each treatment group as a function of its group size. With learning score fitted as a fixed factor, the GLM model was found statistically significant (GLM: Learn Score: $F_{5, 227} = 8.57, p \leq 0.0001$), indicating that the RPW group size can significantly predict the learning score of honeybees when conditioned using RPW odor. The GLM model estimates of the learning score factor back transformed by a logistic function and its associated lower- and upper 95% confidence intervals are shown in (Fig. 3).

Learning performance for learner categories

Although the above population-level analysis is important in showing the overall response pattern of honeybees' learning success to the RPW group size, it is a group-averaged analysis that hides the learning performance of individual types of learners (Gallistel et al., 2004). Therefore, the learning performance for the three distinct categories of honeybee learners were analyzed; learners (LR), optimal learners (OLR), and non-learners (NLR) using a GLM analysis since it can compare the percentage of success in blocks (learning categories) of a dichotomous binomial response variable (expressed as the number of honeybees classified in each of the learning categories divided by the total number of honeybees in the corresponding test group). A GLM was fit to analyze the effect of RPW group size as a proxy to pheromone intensity on the learning success of the previously defined three categories of honeybee learners LR, OLR, NLR using a logit link function.

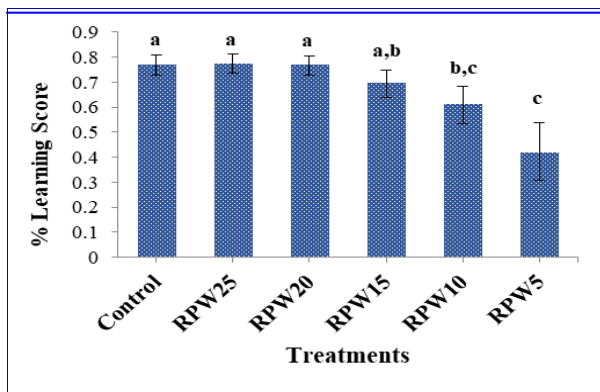


Figure (3): Learning score of honeybees as the sum of all their PER responses in the six acquisition trials for each treatment group as a function of RPW group size. The figure depicts the GLM estimates with their associated lower- and upper 95% confidence intervals. Data points with different alphabet letters are significantly different from each other at ($p \leq 0.05$) as shown by the relevant Tukey HSD multiple pairwise comparisons shown in the table.

Table (2): Pairwise comparisons of the relevant repeated-measures binomial GLMM olfactory acquisition estimates using Tukey HSD for the RPW group size fixed effects with their corresponding t ratios and probabilities.

Treatment	-Treat	t ratio	$p > t $
Control	RPW25	-0.39	0.9988
Control	RPW20	0.01	1.000
Control	RPW15	3.79	0.0021*
RPW25	RPW20	0.41	0.9985
RPW25	RPW15	4.28	0.0003*
RPW20	RPW15	3.87	0.0016*
RPW15	RPW10	2.93	0.0403*
RPW10	RPW5	3.96	0.0011*

* , Significant differences at $p \leq 0.05$ probability threshold. Group size (n) is 36, 39, 39, 40, 40 and 39 for Control, RPW25, RPW20, RPW15, RPW10 and RPW5, respectively as shown in Table (1).

The results of the whole model test showed that the RPW group size significantly affected the percentage of honeybees in the LR, OLR, and NLR categories ($\chi^2_{12} = 257.47, p \leq 0.0001$, Fig. 4A). The model revealed that the percentage of honeybees in LR category ($M = 0.86$, 95% CI [0.82, 0.89]) is 47% significantly higher than OL honeybees ($M = 0.39$, 95% CI [0.34, 0.44]) ($\chi^2_2 = 207.71, p \leq 0.0001$). More interestingly, the model revealed a significant interaction between the learning category and RPW group size ($\chi^2_{10} = 66.22, p \leq 0.0001$). More specifically, the decline of OLR percentage was more severe compared to LR percentage with the small RPW group sizes of RPW10 ($M = 0.35$, 95% CI [0.24, 0.48]) ($\chi^2_{10} = 5.46, p = 0.02$) and RPW5 ($M = 0.24$, 95% CI [0.16, 0.36]) ($\chi^2_{10} = 6.88, p = 0.009$), but not with the larger RPW group sizes or the control (Fig. 4B).

Memory retention tests

Memory retention and retrieval test results showed that reducing the pheromone intensity by decreasing the group size of RPW was correlated with a general reduction trend in the ability of honeybees to retain and retrieve memory of the odor was modulated by the RPW group size.

Treatment	-Treat	t-ratio	$p > t $
Control	RPW25	-0.18	1.0
Control	RPW20	0.05	1.0
Control	RPW15	2.12	0.279
Control	RPW10	3.78	0.003*
RPW25	RPW20	0.24	1.0
RPW25	RPW15	2.34	0.182
RPW25	RPW10	4	0.001*
RPW20	RPW15	2.11	0.287
RPW20	RPW10	3.78	0.003*
RPW15	RPW10	1.79	0.472
RPW15	RPW5	4.18	0.001*
RPW10	RPW5	2.68	0.084

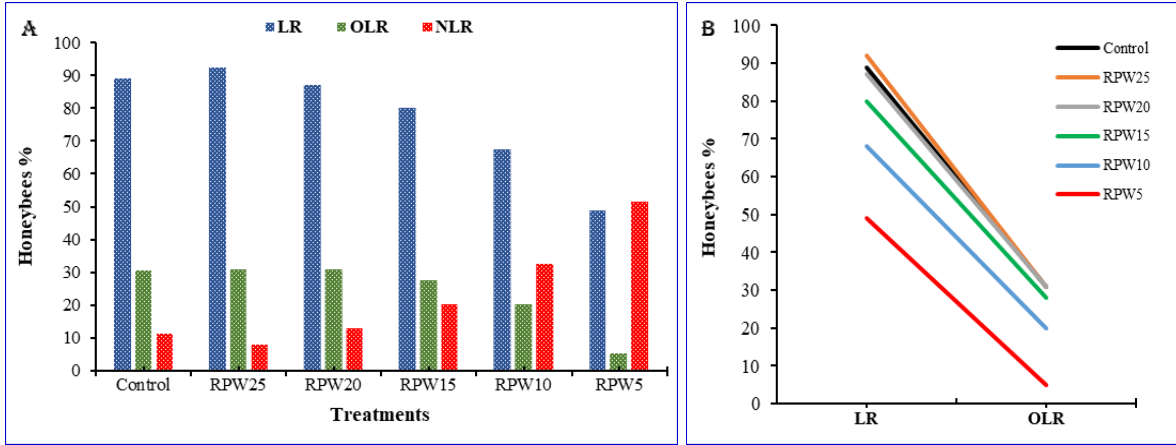


Figure (4): Classification of honeybees into three learning categories learners LR, optimal learners (OLR), and non-learners (NLR) after six conditioning trials in which the honeybees were trained to learn the association between the Red Palm Weevil (RPW) pheromone odor and a sugar reward. A, the data represents the percentage of honeybees in each category as a function of RPW group size. B, generalized linear models (GLM) analysis revealed a significant interaction between the learning category and RPW group size in which the decline in OLR percentage is more severe compared to LR honeybees as the RPW group size decreases.

Retention performance for honeybees revealed that the percentage of honeybees successfully retained and retrieved the learned odor of six treatments of RPW through four time points (Figure 5).

The binary response of memory test (0 = no PER, 1 = PER) for the RPW odor without sugar reward was fitted as a Treatment fixed effect factor with six levels (Control = synthetic pheromone + 5 levels of RPW group size treatments), while the MemoryTestAfter (MTA) was fitted as a fixed effect factor with four levels (4, 24, 48, and 72 hrs) after conditioning. The model included the individual honeybees *Bee_ID* as random effects to accommodate the repeated-measures design as each honeybee’s memory retention performance was measured at four different time points. The regression model was used to test if the RPW group size-indicative of pheromone intensity-significantly predicted the honeybees’ memory retention performance. An interaction term (Treatment X MTA) was included in the initial model but dropped due to insignificance (GLMM: Treatment X MTA: $F_{15, 797} = 0.39, p = 0.98$). The overall regression model for the fixed effects tests was statistically significant (GLMM: Treatment: $F_{5, 812} = 28.58, p \leq 0.0001$, MTA: $F_{3, 812} = 43.40, p \leq 0.0001$) indicating that both group size and the period after which memory was tested significantly affected the honeybees’ memory performance in the test phase. p -values were obtained by the log-likelihood ratio tests of the full model with the Treatment and MTA effects against the null model without it. The GLMM model estimates of factors back transformed by a logistic function and their associated lower- and upper 95% confidence intervals, and group sizes are summarized in (Table 3). Note that within some treatment groups, sample size decreased as the test progressed from one time point to the next because some honeybees died or became unresponsive and didn’t complete the test and were not included in the analysis. Multiple pairwise comparisons using Tukey HSD are shown in (Table 4) with their corresponding t -ratios and probability values.

The results showed that the honeybees in all treatment levels retained the memory for at least 24 hrs without significant decay except for the RPW5 group. Memory retention after 48 hrs differentiated the six honey-bees’ treatment groups into roughly three levels. The top memory retrievers (control and RPW25) remembered the odor with a similar pattern with about 30% of honeybees still able to retain the memory after three days without any reinforcement. Approximately 35% of the middle retrievers (RPW20 and RPW15) retained the memory well for 48 hrs, but not for 72 hrs.

Table (3): GLM model estimates of fixed factors (Treatment and MTA) back-transformed by a logistic function, with associated lower and upper 95% confidence intervals (CI) and group sizes (n).

Treatment	Mem Test hrs	n	Estimated fixed factor	Lower 95% CI	Upper 95% CI
Control	4	36	0.901	0.823	0.947
Control	24	34	0.832	0.722	0.904
Control	48	28	0.6	0.449	0.734
Control	72	24	0.279	0.166	0.43
RPW25	4	35	0.913	0.843	0.954
RPW25	24	31	0.851	0.747	0.917
RPW25	48	29	0.634	0.485	0.761
RPW25	72	25	0.309	0.187	0.466
RPW20	4	31	0.773	0.651	0.862
RPW20	24	31	0.649	0.506	0.769
RPW20	48	29	0.359	0.238	0.501
RPW20	72	27	0.127	0.07	0.22
RPW15	4	32	0.735	0.6	0.837
RPW15	24	28	0.601	0.452	0.734
RPW15	48	26	0.314	0.199	0.456
RPW15	72	24	0.106	0.054	0.198
RPW10	4	34	0.314	0.195	0.463
RPW10	24	32	0.199	0.113	0.325
RPW10	48	30	0.07	0.037	0.128
RPW10	72	28	0.019	0.009	0.04
RPW5	4	33	0.069	0.019	0.219
RPW5	24	33	0.038	0.01	0.141
RPW5	48	31	0.012	0.003	0.047
RPW5	72	21	0.003	0.001	0.013

Significant differences between these groups were observed at a probability level of $p \leq 0.05$.

Table (4): Pairwise comparisons of the relevant repeated-measures binomial GLMM estimates using Tukey HSD for within- and between-groups effects with their corresponding t-ratios and probabilities.

Treatment	Mem. Test hrs	-Treat	-Mem. Test hrs	t-ratio	$p > t $
Within Groups Comparisons					
Control	4	Control	24	2.59	0.603
Control	24	Control	48	5.20	0.001*
Control	48	Control	72	4.79	0.001*
RPW25	4	RPW25	24	2.59	0.603
RPW25	24	RPW25	48	5.20	0.001*
RPW25	48	RPW25	72	4.79	0.001*
RPW20	4	RPW20	24	2.59	0.603
RPW20	24	RPW20	48	5.20	0.001*
RPW20	48	RPW20	72	4.79	0.001*
RPW15	4	RPW15	24	2.59	0.603
RPW15	24	RPW15	48	5.20	0.001*
RPW15	48	RPW15	72	4.79	0.001*
RPW10	4	RPW10	24	2.59	0.603
RPW10	24	RPW10	48	5.20	0.001*
RPW10	48	RPW10	72	4.79	0.001*
RPW5	4	RPW5	24	2.59	0.603
RPW5	24	RPW5	48	5.20	0.001*
RPW5	48	RPW5	72	4.79	0.001*
Between Groups Comparisons					
Control	4	RPW25	4	-0.53	1
RPW20	4	RPW25	4	-4.26	0.005*
RPW15	4	RPW20	4	-0.79	1.0
RPW10	4	RPW15	4	-5.95	0.001*
RPW5	4	RPW10	4	-2.6	0.6
Control	24	RPW25	24	-0.53	1.0
RPW20	24	RPW25	24	-4.26	0.005*
RPW15	24	RPW20	24	-0.79	1.0
RPW10	24	RPW15	24	-5.95	0.001*
RPW5	24	RPW10	24	-2.6	0.6
Control	48	RPW25	48	-0.53	1.0
RPW20	48	RPW25	48	-4.26	0.005*
RPW15	48	RPW20	48	-0.79	1.0
RPW10	48	RPW15	48	-5.95	0.001*
RPW5	48	RPW10	48	-2.6	0.6
Control	72	RPW25	72	-0.53	1.00
RPW20	72	RPW25	72	-4.26	0.005*
RPW15	72	RPW20	72	-0.79	1.00
RPW10	72	RPW15	72	-5.95	0.001*
RPW5	72	RPW10	72	-2.6	0.6

* Significant at level $p \leq 0.005$ and $p \leq 0.001$.

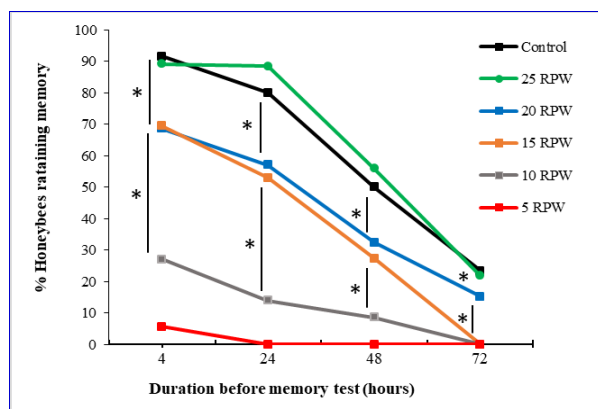


Figure (5): Memory retrieval of honeybees expressed as percentage of honeybees that successfully retained and retrieved the learned odor of six treatments of RPW over four time periods. No significant decrease in memory retrieval between 4 hrs and 24 hrs, but there was a significant reduction observed after 48 hrs and 72 hrs in all RPW treatments. Between groups-analysis, revealed that the honeybee groups could be classified into three levels based on memory retrieval: the top retrievers (control and RPW25), the middle retrievers (RPW20 and RPW15), and the worst retrievers (RPW10 and RPW5).

A small percentage (27% and 6%, respectively) of the lowest retrievers (RPW10 and RPW5) were able to retain the memory only for 4 h, but then lost it significantly after 24 hrs, especially in the RPW5 group, which completely lost that memory after 24 hrs (Fig. 5).

DISCUSSION

In this study, a promising novel technique for the early detection of RPW infestation in palm trees using honeybees is described. This is not the first attempt to use a living organism as a bio-detector for *R. ferrugineus* infestation of palms since Rottweiler and Golden Retriever dogs (*Canis familiaris* L.) were successfully tested in this regard (Suma *et al.*, 2014). It is critical to detect the infestation early since palms in advanced stages of infestation are irreversibly damaged and dying (Llácer *et al.*, 2009, OEPP/EPPO, 2020). Saving the palms is even more complicated recently because infestation is accelerated due to the prevailing climate warming and drought stress which might reduce the production of plant defensive compounds and facilitate pest infestation (Chávez-Arias *et al.*, 2022).

Some animals have advanced olfactory capabilities because their survival relies on scent detection in locating food, finding mates, hosts, and nests, avoiding predators, and navigating their environments. Many studies showed that these animals can be trained to detect target substances by producing identifiable responses in the presence of volatile compounds emanating from target materials. Dogs were successfully trained to identify and locate explosives, landmines, illegal drugs, disaster victims, and various other targets (Rains *et al.*, 2004, Browne *et al.*, 2006). Other animals including bees, wasps, moths, mice, rats, and pigs have also been successfully trained to detect target substances (Rains *et al.*, 2004, Tomberlin *et al.*, 2005, Rains *et al.*, 2008, Poling *et al.*, 2010, Leitch *et al.*, 2013). Honeybees were successfully trained to detect heroin and cocaine illicit drugs (Schott *et al.*, 2015) and in military studies detecting chemical smells of dead bodies and explosive devices containing 2,4-dinitrotoluene (2,4-DNT) at the level of parts per trillion (ppt) (Bromenshenk *et al.*, 2015). As a disease detector, honeybees were successfully trained to detect tuberculosis (Suckling and Sagar, 2011) and SARS-Cov-2 virus-infected mink samples using only 10 bees to score a success rate of 90% comparable to available antigen tests (Kontos *et al.*, 2022). Honeybees are good candidates to develop biosensors compared to other insects because adult honeybees may live for up to four months and can retain learned odors for several days up to their whole life if subjected to multiple conditioning trials (Müller, 2013).

In this study, the honeybees were able to successfully learn the association between the RPW pheromone odor (CS) and sucrose (US) with an acquisition profile similar to other ecologically relevant scents for the honeybees (Laska *et al.*, 1999, Guerrieri *et al.*, 2005, Aguiar *et al.*, 2018) or even odor fractions of

floral origins, for example the methyl p-anisate and methyl phenylacetate (Suckling and Sagar, 2011). Acquisition performance of the honeybees conditioned with the synthetic pheromone (control) was not significantly different from those conditioned via RPW25 and RPW20 groups. This supports the feasibility of using the honeybees for this application since they can be conditioned either with the synthetic pheromone or with live RPWs as both conditioning agents produce similar results. Despite theoretically, conditioning with the natural odor of live RPW should result in more specific conditioning compared to the chemically synthesized pheromone. The data shows that conditioning performance drops significantly for the RPW15 and RPW10 groups, to reach its lowest level for the RPW5 group in which less than half of the honeybees were successfully trained by the end of acquisition trials (Figs. 2 and 3). Although this is a low level of conditioning, some honeybees were still able to detect and learn the odor. It is worth noting that none of the honeybees responded with a positive PER in the first conditioning trial as expected. Because even if the specific honeybees recruited in our experiment had accidentally encountered the RPW pheromone in the field, it's very unlikely that this odor was associated with an appetitive reward.

To consider the individual differences between the types of learner honeybees, we compared the learning performance of three classes of learners: LR, OLR, and NLR. The data analysis showed a similar pattern of learning acquisition between the treatment groups. However, it revealed an interaction between the learning performance of the class of honeybee learners and the odor intensity represented by the RPW group size. In this interaction, the percentage of OLR declined more severely in the small group sizes of RPW compared to the control and the larger RPW groups (Fig. 4). Some previous studies showed that optimal learning occurs in honeybees in which exceptionally higher levels of Dopamine (DA) and to a lesser extent serotonin (5-HT) are secreted in their brains after conditioning (Raza *et al.*, 2022). In this study, we don't know if a high enough level of DA and/or 5-HT can be stimulated after the conditioning with the RPW aggregation pheromone. Therefore, one explanation for the decline in OLR in small group sizes could be that as the RPW group size decreases, it becomes even harder to be an optimal learner and probably achieve the required level of neurotransmitter excitation. However, further investigation of this relationship, especially in field conditions, can be used to identify the exact acceptable threshold of stimulation that would result in an adequate level of honeybee learning and its requirements for the detection of RPW in the field. Besides, honeybee foragers also differ in their sensitivity to odors, and therefore in their likelihood to show odor-mediated responses (Moreno *et al.*, 2022). Further analysis is required to measure the change in brain neurotransmitter levels after conditioning with the RPW pheromone.

Memory retention is vital for honeybees because, as central-place foragers, their survival depends on

collecting nectar and pollen from the flowers which have unreliable profitability fluctuation and are dispersed randomly. Different types and stages of memory are recognized in honeybees including short-term memory (STM), medium-term memory (MTM), and long-term memory (LTM). Each of them can be subdivided into finer stages (Menzel, 1999). Here, we focused on MTM and LTM as they are more relevant to the intended application of RPW detection. STM is functional within the range of several minutes, while MTM in a few hrs and LTM works in the range of days to months (Menzel, 1999, Eisenhardt, 2014).

Regarding memory, multiple conditioning trials are expected to lead to the formation of an LTM, more specifically late LTM, that can be retrieved up to 72 hrs later (Giurfa and Sandoz, 2012). In this study, the MTM memory test after 4 h showed that the control and RPW25 honeybee groups were able to efficiently retrieve their memories significantly higher than the other groups indicating their MTM is consolidated. MTM retrieval of the RPW20 and RPW15 was significantly lower but still functional. On the other hand, the RPW10 and RPW5 MTM (Fig. 5) were poor and indicated that the 5 and 10 RPW group sizes were not enough to evoke functional memory consolidation (Paoli and Galizia, 2021).

More importantly, in the LTM test, memory retrieval of the control and RPW25 were significantly higher at 24 hrs. Which according to Eisenhardt, 2014, might indicate that these memories must have been stabilized by the synthesis of new proteins through translation and transcription processes that must have contributed to neuronal structural changes. These memories decreased after 48 and 72 hrs but didn't completely diminish, which is similar to normal LTM in honeybees (Paoli and Galizia, 2021). LTM of RPW20 and RPW15 was significantly lower than the control and RPW25 but higher than RPW10 and RPW5. LTM retrieval in the RPW10 and RPW5 was poor and significantly the lowest indicating that the low RPW group size here was probably not enough to activate the transcription and translation pathways required for LTM. Overall, the LTM test showed that the minimum group size required for long-lasting memory retrieval is 20 RPWs that will last for at least 72 hrs. However, a 15 RPW had an LTM functional for 48 hrs and this can be probably extended if reinforcement was provided within that period (Müller, 2013). For this technique to be applicable in field detection, a small electronic device could be designed to automatically condition new honeybees by automatic release of the RPW aggregation pheromone, followed quickly by a small reward of sucrose.

Insects equipped with sophisticated olfaction detection are promising efficient bio-detectors as they have highly sensitive and selective olfactory capabilities. As insects, they are usually cheap to reproduce, and transport, and can be conditioned to various odors in a short time (Mitsuno *et al.*, 2020). Compared to canines, insect sniffers can be even more competitive considering the higher cost, time, and energy required for training and maintaining canines.

However, insects are still not accepted as proven biological sensors. For this reason, further studies are required to improve insect applicability in detecting odorants of interest. Therefore, we conducted this proof-of-concept (POC) laboratory study to demonstrate that honeybees can be used for early detection of palms infested with RPW. It shows that the design concept works, is feasible, and justifies a warranted field testing that might involve integration into an electronic detection system based on honeybees at its core. Similar models have been proposed before (Mitsuno *et al.*, 2020) in which an electronic odor detector is designed to harness the sensitive detection ability of whole living insects, their antennae, olfactory receptors (ORs), or just the odorant-binding proteins (OBPs). Although it is still early for this technology to be widely adopted, it is an important step towards a promising environmentally friendly detection technology that is highly sensitive and selective.

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الرصد المبكر للإصابة بسوسة النخيل الحمراء رينكوفوراص فيروجينيوس أوليفير باستخدام التعلّم الشمّي المشهّي في نحل العسل أبيس ميليفرا

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قسم علم الحيوان، كلية العلوم، جامعة قناة السويس، الإسماعيلية، مصر

الملخص العربي

تسبب إصابة أشجار النخيل بسوسة النخيل الحمراء، رينكوفوراص فيروجينيوس، بخسائر اقتصادية فادحة ولا سيّما بعد أن انتشرت بشكل واسع وصل إلى مستويات حرجة في المناطق الاستوائية وفي الشرق الأوسط وشمال إفريقيا. وتعدّ عدم إمكانية الاكتشاف المبكر للإصابة هي العائق الرئيسي أمام مقاومة هذه الآفة. وقد تم إجراء هذه الدراسة لاستكشاف إمكانية استخدام التعلّم الشمّي في نحل العسل في الكشف المبكر عن دلائل رائحة فيرمون التجمع الخاص بسوسة النخيل الحمراء. عملنا قمنا بإجراء تكيف لاستجابة مدّ الخرطوم PER conditioning في أفراد نحل العسل من طائفة جامعي الغذاء باستخدام فيرمون التجمع الخاص بسوسة النخيل الحمراء كمؤثر مكيف وذلك في إطار بروتوكول بافلوف التعليمي القياسي باستخدام فيرمون صناعي وكذلك باستخدام الفيرومون الطبيعي المختلط مع الروائح الطبيعية الصادرة عن مجموعات مختلفة في عدد أفرادها من السوس. ولتقييم مدى حساسية النحل في تعلم وتذكر رائحة الفيرومون تم تكيف واختبار خمس مجموعات من النحل كل منها حوالي 40 فرد وذلك باستخدام خمس مجموعات مختلفة العدد من سوسة النخيل الحمراء تتكون من 5، 10، 15، 20 أو 25 فرد في كل منها. تلى ذلك اختبار الذاكرة متوسطة المدى وطويلة المدى بعد التكيف بفترة 4، 24، 48، 72 ساعة. أظهرت النتائج قدرة نحل العسل على تعلم رصد رائحة فيرمون التجمع للسوس سواء الصناعي أو الطبيعي. كما زادت نسبة عدد أفراد النحل الذين تعلموا بنجاح رصد الرائحة طردياً مع زيادة عدد أفراد السوس في المجموعة. وقد جاءت نسبة أفراد النحل المتعلمين بعد ست مراحل تكيف إلى 89%، 92%، 87%، 80%، 68%، 49% للمجموعات: الضابطة، RPW25، RPW20، RPW15، RPW10، RPW5 على التوالي. أما اختبارات الذاكرة بدون أي تعزيز بالرائحة فقد أظهرت قدرة النحل على الاحتفاظ بالذاكرة واسترجاعها لفترة لا تقل عن 24 ساعة في كل المجموعات. فقد تمكنت 30% من أفراد النحل في المجموعة الضابطة وكذلك RPW25 من الاحتفاظ بالذاكرة بعد ثلاثة أيام. أما النحل في RPW20، RPW15 فقد تمكّن 35% منه من الاحتفاظ بذاكرة جيدة حتى 48 ساعة. إلا أن هذه الذاكرة أظهرت تراجعاً ملحوظاً بعد 48 ساعة، ولكن بشكل متباين بين المجموعات المختلفة، حيث وصل الحد الأدنى لنسبة أفراد النحل القادرين على تذكر الرائحة بعد 48 ساعة إلى 27% في المجموعة RPW15 وكذلك بعد 72 ساعة إلى 25% في المجموعة RPW20. أما المجموعتين RPW5، RPW10 فقد تمكنا من الاحتفاظ بذاكرة متوسطة المدى فقط لمدة 4 ساعات ولكن فقدا هذا الذاكرة بشكل ملحوظ بعد 24 ساعة. وبناءً على هذه النتائج يمكن استنتاج أن نحل العسل من الممكن أن يكون كاشف حيوي واعد في الكشف المبكر عن النخيل المصابة بسوسة النخيل الحمراء على الرغم من أن التطبيق مازال يتطلب العديد من الدراسات التجريبية والحقلية.