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Elucidating the Effects of Thiamethoxam Neonicotinoid on Honey Bee Learning Using the Proboscis Extension Response

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Elucidating the Effects of Thiamethoxam Neonicotinoid on Honey Bee Learning Using the Proboscis Extension Response

By

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Abstract

In this study, the effects of the neonicotinoid pesticide, thiamethoxam, are examined through the Proboscis Extension Response (PER) in honey bees (Apis mellifera). PER is a form of classical conditioning applied to honey bees through scent and reward association which quantifies learning rates. Results between groups treated with thiamethoxam did not differ significantly from untreated control groups. Potential reasons for these results are discussed. The method and experimental apparatus for testing the PER assay are also discussed.

Introduction

Insect pollinators are responsible for over 150 billion dollars of revenue in the American agricultural system and provide an essential service for the farming industry.[1–4] However, there has been a marked and sudden decrease in pollinator populations, specifically honey bees (*Apis mellifera*), across the United States and Europe in the past decade.[3,5] This decline has been attributed to a condition called Colony Collapse Disorder, or CCD. CCD is defined by vanEngelsdorp as the presence of three prevailing symptoms in a hive: (1) the rapid loss of adult worker bees from affected colonies as evidenced by weak or dead colonies with excess brood populations relative to adult bee populations; (2) a noticeable lack of dead worker bees both within and surrounding the affected hives; and (3) the delayed invasion of hive pests (e.g., small hive beetles and wax moths) and kleptoparasitism from neighboring honey bee colonies.[6] The specific causes of CCD are not fully known, but several primary contributors have been suggested.[2] Most agree that there is not a single culprit for CCD, rather, it is a combination of multiple stressors on a colony. These stressors include: *Nosema* microspores, viral pathogens,

varroa mites (*Varroa destructor*), tracheal mites (*Acarpis spp.*), small hive beetles (*Aethina tumida*), an insufficient food supply, and neonicotinoid pesticide use.[3] In recent years, particular interest has been directed to neonicotinoid pesticide use as a potential primary cause for CCD. [2,7]

Neonicotinoid pesticides have seen massive increases in their use in the past decade. Thiamethoxam, clothianidin, and imidacloprid are three of the most popularly used neonicotinoid pesticides in use today. They attack the nervous system of an insect by acting as an agonist to nicotinic acetylcholine receptors (nAChR's) which act as primary excitatory neurotransmitter sites.[8] Honey bees and other insect pollinators such as bumble bees (*Bombus terrestris*) are exposed to these chemicals on a regular basis in their habitat on industrial crop land in widely varying concentrations.[4,9–11] The concentration of each of these chemicals is usually such that a bee will not consume an amount matching or exceeding the LD50, which is the amount required to kill half of any population that consumes it, in the normal course of their life. Therefore, the concern with neonicotinoids is not necessarily with direct lethality of the chemicals, but with their sub-lethal effects.[12] These effects can cause an overall weakening in an individual bee's immune system, making it or an entire bee colony more susceptible to other stressors.

Older bees seem to be less affected than younger bees, but the older foragers bring back pollen and nectar to the hive where younger, more sensitive bees are exposed to the food tainted with neonicotinoids.[13] Some argue that that the negative effects of these compounds will be diluted by bee-collected pollen and nectar that is untreated, however, it has been shown

that, when given the choice, bees actually prefer collecting food that contains neonicotinoids, thus increasing the concentration. [14]

There is also evidence that shows that behaviors of both honey bees and bumble bees can be significantly affected by exposure to sub-lethal amounts of neonicotinoids. Reduced foraging, reduced homing success, and reduced short-term and long-term memory and increased mortality are all among the negative effects of chronic sub-lethal exposure to neonicotinoids. [2,7,15–21] Also recently discovered was that honey bee queens are significantly affected in both behavior and fecundity. [22,23] Many different assays are used to test the effects of neonicotinoids on bee behavior, but the focus of this study on honey bee memory promoted the selection of the proboscis extension response (PER) as the testing assay. PER is commonly used and accepted form of testing the learning rate and short-term memory formation in both honey bees and bumble bees.

PER relies on the principles of classical conditioning commonly taught in most introductory psychology courses in college. Similar to Pavlov's dog example, there is an unconditioned stimulus (food) that, when presented, causes the dog to salivate in an unconditioned response. At the same time of the presentation of the unconditioned stimulus, a conditioned stimulus is presented (a bell) that is soon associated with the unconditioned stimulus, the food. After all stimuli and responses have ceased, the conditioned stimulus is repeated without the presence of food. If the dog started to salivate, a conditioned response had been elicited and association was successful. If not, the dog had not learned or remembered the association of the bell and the food. From this test, the memory creation ability of the dog could be quantified. The procedure is similar for honey bees. Instead of dog

food, sucrose solution (sugar water) is presented, and instead of a bell, a scent is used. A bees' unconditioned response to sucrose is to extend its mouthparts (proboscis) in a visible way. Later, when the scent is presented after learning trials, the bee will extend its' proboscis in the absence of sucrose solution which signifies a learning association and memory formation and retention. [24]

Since the PER assay relies on honey bee memory formation and retention, our hypothesis was that honey bees treated with thiamethoxam would show decreased learning rates from the control group which was not treated with the neonicotinoid in question. Due to the negative effects of thiamethoxam on honey bee learning and memory retention shown in other studies previously mentioned, this hypothesis seemed reasonable and worthwhile to test.

Materials and Methods

Developing a reliable method of conducting PER conditioning was more challenging than expected. Methods varied greatly from paper to paper in original research preparing for the project. Elements were tried from these papers to see if results similar to theirs could be attained. Some methods seemed to work well for a few trials, but were not consistent for as long a period of time to confirm reliability. An article by Brian Smith proved to be especially helpful in troubleshooting many issues we faced. [24] One of the first problems recognized was the venting of the scent out of the training arena when each bee was not supposed to be exposed to it. Contaminations of some of the instruments with residual scent as well as incomplete sealing of the ventilation system we developed were both major issues that had to be addressed by better control of the location of the scent, wearing disposable gloves while

handling the scent, and only opening the source bottle of the scent to soak the filter paper strips used in the air lines outside the lab to allow any excess scent to evaporate or be vented away. These changes ensured that the presence of the scent was being controlled manually using the sample contained within the airline instead of there being an ambient presence at all times.

The other primary problem at first was the amount of sucrose being delivered to the bee as well as the method of delivery. Toothpicks soaked in sucrose solution were used at first, but the wood of the toothpick seemed to have an aversive taste when it was presented. We then used a syringe containing the sucrose solution with a 26-guage needle to deliver the reward via a droplet pushed out of the tip. This method seemed to work well at first as the bees seemed to be learning at the rate they should be for the first few trials, but then learning rates began to decrease as the bees would not extend their proboscis for the reward. We attributed this to satiation of the bee. As it was already satiated, it would not extend even if it had learned to associated the scent with the sucrose reward. Therefore, it was determined that we had to come up with a method that controlled the amount of sucrose made accessible to the bee that was also reliable in its delivery.

Two different methods were proposed as solutions for this problem. The first was to deliver the reward using a micropipette using extremely controlled amounts. However, when bringing the droplet over the head of the bee to touch the antennae and signal the presence of sucrose, the droplet would often stick to the antennae or another part of the head and never make it to the mouthparts, thus missing the time window for presentation and breaking an essential part of the conditioning process. The second method tried that ended up working best

was to soak a cotton bud (Q-tip) in the sucrose solution. This allowed the reward to touch the antennae, signaling the bee, then present to the extended proboscis for only one second to limit the amount of sucrose being consumed. This method proved to be reliable in both amount delivered as well as timely delivery, therefore this method was chosen for the experiment.

Once these confounding factors were remedied, the learning rates of the bees consistently matched or exceeded those of other published studies.[13,25] Data collection could begin at this point. Data collection took approximately two weeks for four people to complete working in shifts several hours a day.

Beekeeping

The hive used to collect bees for this research was provided by East Tennessee State University as part of the university apiary used for all honey bee research on campus. One hive was dedicated to this project for its duration. Bees were trained to come to a feeding station of 50% v/v sucrose solution using anise (licorice) scent.

Bee collection/transport

Bees were collected from the feeding station described above to ensure that all bees collected were foragers. Since the research was conducted during November, there were almost no other sources of sucrose for the bees, so this was an effective way to collect the specific type of bee that was needed.

Bees were collected using collection tubes constructed in-lab. These tubes consisted of a clear plastic tube of 1 inch diameter with one end covered by plastic mesh and affixed using hot

glue while the other end was covered with a plastic cap. The open end of the tube was placed over a bee at the feeding station. The bee would then either fly or walk up into the tube and the cap was placed over the open end.

Once all bees were collected, the filled collection tubes were placed in a plastic container with no lid to allow gas exchange. The top of the container was then covered with paper towels to shield from sunlight during transport. The bees were then placed in the floorboard of a car with the A/C running on high during the 10 minutes from the bee field to lab to prevent heat shock in the bees.

Ice treatment

Honey bees are ectothermic, so their metabolism slows drastically when exposed to cold. To safely handle the bees for harnessing, they were placed in their collection tubes and then down into a plastic container full of ice until the ice level reached just below the top of the tube, allowing for gas exchange. After about 4 minutes, the bee would be motionless and safe to handle for harnessing. After harnessing, the bees would become active again within a couple of minutes and showed no sign of adverse effects as they were never in direct contact with the ice.

Harnessing/feeding

Bees were harnessed in harnesses adapted from examples of other previous research.[26] Harnesses consisted of .38 special or .357 magnum empty and clean bullet casings and duct tape strips.(Figure 1) Both calibers could be used because they have the same inner diameter of casing. These were ideal to use because of their weight, (which kept them from falling over

easily) durability, and ease of cleaning. A cut was made into the side of the casing about half way down from the neck and half way into the casing, then another cut was made from the top of the casing down to that point and the section was removed. This provided an accessible area to place the bee. The bees were immobilized using the ice treatment described previously then picked up with soft tweezers to prevent damage to the bee.

Once placed in the casing, a strip of duct tape about 2-3mm wide and 2.5cm long was placed in over the top of the casing in between the head and thorax of the bee, so the head of the bee was held just over the lip of the casing and the mouthparts were free to move and extend. Another slightly wider strip of duct tape was wrapped around the casing to hold the back of the bee and help prevent escape. Once secured, the bee could become accustomed to the harness for about 20 minutes, then fed 30% sucrose solution using a syringe and 26-gauge needle until satiated. The control group received the sucrose solution just described, while the experimental group received a 30% sucrose solution laced with 25ng/mL of Thiamethoxam. Only bees that ate at least a full drop (~0.05mL) of solution were tested to ensure sufficient treatment with Thiamethoxam.

Starvation

After being caught, immobilized, harnessed, and fed to ensure a baseline satiation level, the bees were placed in vented plastic containers with damp paper towels in the bottom to retain moisture. The containers were placed in a cool dark space where the bees were starved for 24 hours before testing to ensure the bees would be motivated to respond to the PER testing.

Apparatus

The experimental apparatus used for testing was designed based off an idea seen in another paper found during initial research.[27] The body of the apparatus was made of ¼ inch thick Spectar™ plastic sheets from Eastman Chemical Company bonded together with epoxy. The apparatus consisted of a 12-chambered system making a dodecagonal prism shape with a 4-inch hole in the middle for a venting hose. (Figure 2B) A 4-inch diameter PVC coupler was glued to the opening and a 3-inch PVC pipe with a 3-inch to 4-inch enlarger was inserted inside so that the lip of the enlarger rested on the 4-inch coupler, creating a fairly effective seal that still allowed the system to be rotated. Each inner edge of the Spectar™ chambers was covered with foam weather stripping. The inner 3-inch PVC pipe had a vertical cut 1 inch wide and 3 inches tall and covered with weather stripping so that as the system was rotated to each of the twelve cells, the weather stripping from the inner PVC lined up with that of the cell, creating a seal and preventing non-ambient airflow or scent to leak into the chambers not being tested as well as improve the suction and evacuation of the scent from the testing chamber. In front of the testing chamber, the airflow was directed in a constant stream using 3/16 inch Tygon™ flexible plastic tubing zip-tied to a PVC stabilization structure (Figure 1C).

The scent was introduced using filter paper soaked with 10µL of 1-Hexanol in a secondary tube controlled using a 2-way gang valve pictured above. When the scent was to be activated, the secondary tube was opened, immediately followed by the closing of the primary line to direct all air flow through the secondary tube containing the scent. This allowed for an uninterrupted and consistent airflow. To shut off the scent delivery, the primary line was reopened and then the secondary line was closed.

Pre-testing

Before testing began, a discrimination trial was conducted to ensure that the bees to be tested were motivated to be tested. This was done by touching the antennae of each bee with a droplet of 5% sucrose solution. If a PER was elicited, then then bee would be used, if not, then it was excluded from testing.

Testing

After the bees had been pre-tested to ensure their motivation to participate based on hunger level, they were placed in each cell of the apparatus and airflow was started. They were separated into four groups: experimental paired, experimental unpaired, control paired and control unpaired. The order and timing of the trials performed were modeled after the PER process described by Matsumoto (Figure 3).[26] The procedure for the experimental group is as follows: For the first trial, 20 seconds of unscented airflow was applied, then 5 seconds of airflow with the scent added, then 5 more seconds of scented air with a sucrose reward given for 1 second during the 5 second interval. (Figure 3) During this period, the cotton swab soaked with a 50% sucrose solution was held behind the head of the bee and downstream from the airflow by a couple of cm to prevent the bee seeing or smelling the sucrose before the appropriate time. At the time when the sucrose reward was to be presented, the soaked cotton swab was brought forward over the head of the bee touching the antennae and moving down to the front of the face in one motion to make the reward accessible to the extended proboscis of the bee. After the scent and reward period, the bee was exposed to 20 seconds of unscented air to allow it to solidify the memory of the association. This completed one trial.

After the first trial, the bee was rewarded immediately if it extended its proboscis during any point where the scent was applied, but only for the 1 second reward period. This was repeated for a total of 5 association trials. The time for each bee during a trial was 50 seconds, so after turning the turntable to the next bee, the total time per bee per trial was just under a minute. A total turn of the system took between 11-12 minutes. Therefore, the inter-trial time was 11-12 minutes for each bee. For the control group, the bees were exposed to the same amount of scent and sucrose reward, but never paired together. During a scent trial, the scent was introduced, but no sucrose reward was presented, only a dry cotton swab. During a sucrose trial, no scent was introduced, but the sucrose reward was given at the normal time. The trials were completed in a random order to prevent bees from anticipating patterns instead of associating the scent with the reward. The order and timing of the trials performed were modeled after the PER process described by Matsumoto (Figure 3).[26]

Retention

After Testing, the bees were left for 2 hours in the same storage method described earlier, then tested for retention. This was done by placing the bees back in the apparatus in their individual cells and repeating an experimental trial as described before, except without any sucrose reward. At the point where scent was added, a novel odor (Geraniol) was used to see if the bees would respond to an odor they had not been paired with. 10 minutes later, another trial was completed with the original paired scent (1-Hexanol) to determine if the bee had retained the association formed before.

Disposal

After all testing was completed, the bees were placed in a freezer for at least 20 minutes to ensure euthanasia. Releasing the bees was considered, but the duct tape harnesses used were too sticky to remove the bees from without causing substantial damage to the wings of the bee. After being in the freezer for 20 minutes or more, they were taken out and removed from the casings and disposed of. The casings were then cleaned with water and Dawn© soap before being used again.

Results

The proportion of bees positively responding (eliciting a PER response) is shown versus the learning trial number (Figure 4). In learning trial one, the bees were exposed to the scent for the first time, then it was paired with the sucrose reward. The expected proportion for this trial was zero, or close to zero since no previous pairing had occurred. The untreated control group that was fed only 30% sucrose solution contained 31 individual bees and had an average proportion of 0.065, or 6.5%. The experimental treated group also contained 31 individual bees and had an average proportion of 0.097, or 9.7%.

By trial two, all the bees had been exposed to the scent paired with the sucrose reward, so they had an opportunity to form the association between the two in short-term memory, therefore the proportion of responding bees was expected to go up. In trial 2, the responding proportion in both groups did rise substantially, to 77.4% in controls, and 74.2% in experimental, as expected. From there, the proportion continued to rise. In trial 3, both groups had the exact same proportion responding: 90.3%. Trial 4 saw a slight divergence in the scores, with the control group dropping slightly to 87.1% and the experimental continuing a slight rise

to 93.5%. Trial five showed the opposite divergence of trial 4, with controls rising to 93.5% and experimental bees dropping slightly to 87.1%.

The results for unpaired learning are shown in Figure 5. In the unpaired trials, the sucrose reward was never presented at the same time as the scent. Instead, they were alternated randomly as shown in Figure 3. Each of the trials displayed is where the scent was introduced without the sucrose reward. Since there is no immediate pairing of the two factors, the proportion responding is expected to be zero, or very close to it. The untreated control group contained 31 individual bees, while the experimental group treated with thiamethoxam had 29 individual bees.

In trial 1, the control group had a responding proportion of 0.267, or 26.7%, and the experimental group had a responding proportion of 0.118, or 11.8%. In trial 2, controls dropped to 20% responding and experimental bees dropped to 8.8%. In trial 3, controls dropped further to 13.3% and remained constant until completion. The experimental group rose slightly to 11.8% and remained constant until completion as well. The overall ANOVA data analysis for paired and unpaired learning gave an F-value of 64.71 and a P-value of <0.0001, indicating a significant difference in some samples in the data set. However, no significant difference was found between control and experimental groups in paired learning according to a Tukey test. Also, no significant difference was found between control and experimental unpaired learning according to another Tukey test. There were significant differences found between paired and unpaired testing ($P<0.01$), indicating that the PER pairing method was working.

The retention tests performed consisted of an exposure to a novel scent two hours after PER conditioning, followed by the original scent used for training earlier ten minutes later. The bees were not expected to respond to the novel odor, but were expected to respond to the scent used for training earlier. The control and experimental unpaired groups were not expected to respond significantly, or at least equally, to either scent since no reward association had been performed.

Figure 6 shows the results of retention tests for the novel scent presentation. The control paired group, containing 27 bees, had a response proportion of 0.185, or 18.5% to the novel scent while the experimental paired group, containing 25 bees, had a response proportion of 0.280, or 28.0% to the novel scent. The response proportion for the control unpaired (24 bees) to the novel scent was 0.217, or 21.7%, while the response proportion for the experimental unpaired (26 bees) was 0.038, or 3.8%. ANOVA testing gave a P-value of 0.144 and an F-value of 1.84 for this set of data, therefore the null hypothesis (no significant difference in the samples of the data set) was unable to be rejected confidently.

Figure 7 shows the retention test results for the original scent used in training. The control paired group had a response proportion of 0.815, or 81.5% to the original scent used for training, while the experimental paired group had a response proportion of 0.800, or 80.0%. The response proportion for the control unpaired was 0.130, or 13.0% to the scent used during trials while the response proportion for the experimental unpaired was 0.038, or 3.8%. ANOVA testing gave a p-value of <0.0001 and an f-value of 38.08, indicating a significant difference found in the samples of the data set. However, the difference found between the control and experimental groups was determined to be insignificant according to an additional Tukey HSD

test. There was also no significant difference found between the control and experimental unpaired groups according to a Tukey HSD test. There was, however, a significant difference found between paired and unpaired groups according to a Tukey HSD test, indicating that the pairing procedure was working.

To ensure the potency of the thiamethoxam used in this experiment, mortality rates of bees were compared. Four groups of bees were treated with varying levels of thiamethoxam and one group was left untreated as a control. The four experimental groups were fed with 30% v/v sucrose laced with 25, 50, 75, and 100 ng/ml thiamethoxam while the controls were fed an untreated 30% v/v sucrose solution. The bees were then stored as described previously and kept over a 42-hour period. Figure 8 shows the resulting mortality rates that were measured every six hours during that 42-hour period. The Y-axis shows the proportion of bees still surviving at the time checked relative to the original number of bees in that group. The X-axis shows the times that the bees were checked. As expected, the groups treated with increasing amounts of thiamethoxam died at a faster rate than those with lower amounts or no treatment at all.

Discussion

Developing a reliable PER assay procedure and apparatus proved to be one of the most difficult parts of this experiment. After many modifications to the apparatus and conditioning procedure, our results started to appear similar to or better than other current research using neonicotinoids in a PER setting. In 2015, Imidacloprid was tested with a PER assay with response proportions starting at 0% for trial 1, then up to 40-60% by trial 2, then tapering off a

little above 60% for the rest of the trials. [13] Matsumoto, who we modeled several aspects of the PER procedure after, showed responses of 0%, 40%, 75%, 80%, and 90% for untreated bees. [26] Overall, our PER scores seemed to be in the range that others were achieving, showing that we had developed a reliable method for conducting the PER assay.

Once data collection was complete, the data showed no significant difference in learning rates between the control groups and the groups treated with thiamethoxam. This result was surprising since other studies seemed to show decreased short-term memory in bees exposed to neonicotinoids. This data seemed to refute our original hypothesis that the learning rates of treated groups would be significantly lower than the control groups.

In further review of the literature after data collection, a study was found that discussed the process of neonicotinoid clearance from honey bees, including data for thiamethoxam. This study showed that honey bees can clear at least 2.2 ng per day, and that effects on behavior were directly correlated to the neonicotinoid residues in the system at the time of testing.[28] Since the amount of thiamethoxam the bees were exposed to in this study was between 1.25-3.75 ng per bee based on how many droplets of treated sucrose a bee consumed before satiation, most, if not all of, the thiamethoxam in the system of the bee would have been cleared at the time of testing 24 hours later. The level of exposure that honey bees in the field encounter varies widely. Levels as low as 2.5 ng/bee all the way up to 41.1 ng/bee have been reported, so the level of thiamethoxam administered in this experiment is well within that range.[4] However, we have no reason to believe that the particular hive used for this experiment was exposed to a significant amount of neonicotinoids before the intentional administration of thiamethoxam in this experiment, therefore the effects would only be acute.

Several studies stress that the negative effects of neonicotinoids on hives is due to chronic exposure rather than acute exposure, so the short time and small amount of exposure in this study would not be enough to make a difference.

At first glance, the results from this experiment seem to show that thiamethoxam has no effect on bee learning, but after understanding the rate of metabolism in honey bees when it comes to thiamethoxam, they make perfect sense. In the future, studies may be completed in the same manner as performed in this experiment, but with potentially higher doses of thiamethoxam as well as a shortened period of time where the honey bees were left in their harnesses before testing. This would allow for a better picture of how thiamethoxam would affect honey bee behavior while the amount of thiamethoxam they would normally be exposed to in the field was still in their system. Understanding the broader scope of thiamethoxam's negative effects on honey bee health, and therefore hive health, warrant further examination of the chronic effects of neonicotinoids like thiamethoxam on pollinator health worldwide.

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Figures

Figure 1

Figure 1: Harnessing Styles

A) Side view of modified .38 caliber casing for harnessing. Note neck strap securing bee to casing and horizontal back strap to keep abdomen stationary. B) Front view of harness. Note that mouthparts including proboscis are free to move.

Figure 2

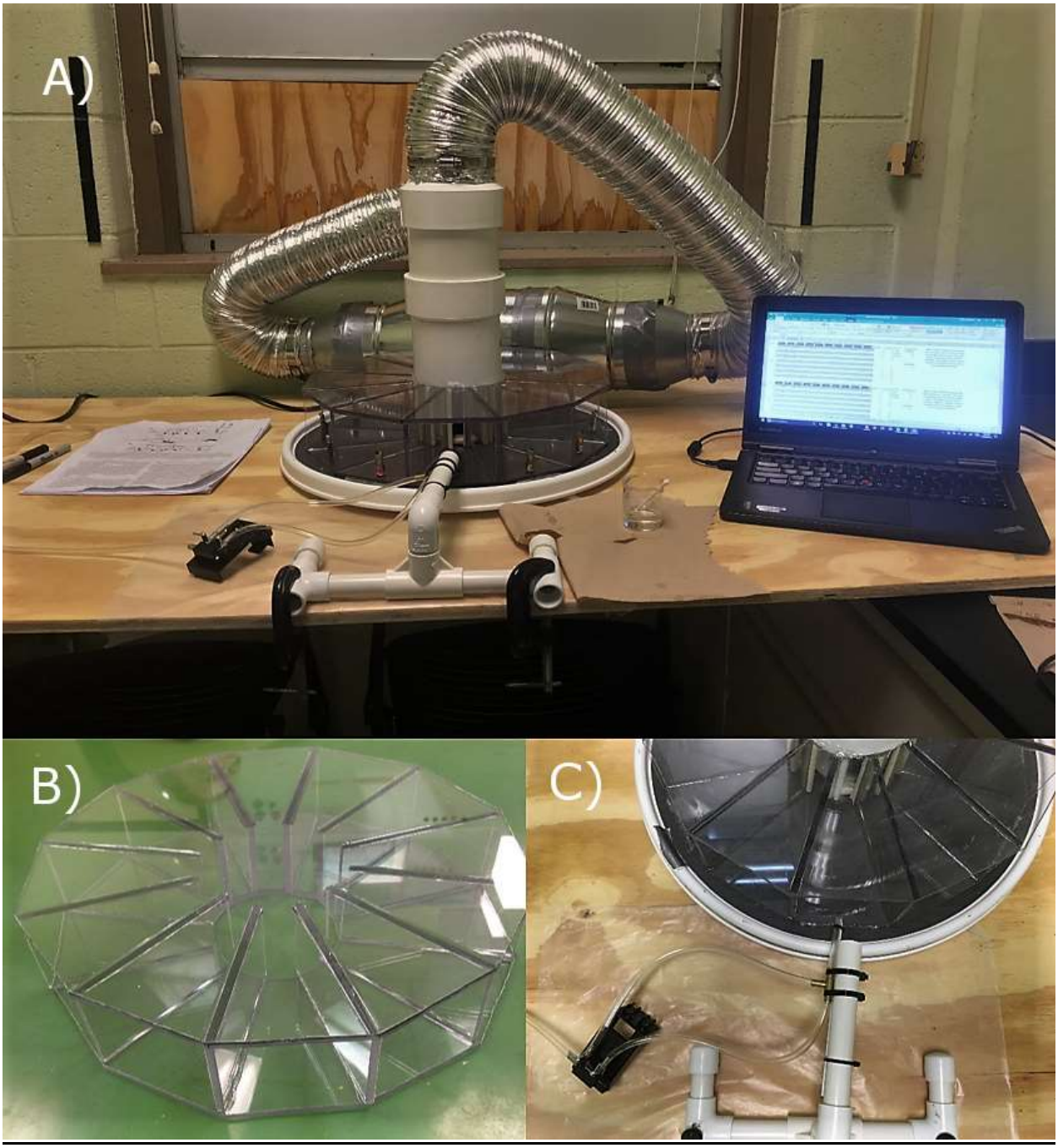

Figure 2: Experimental Apparatus Component Overview

A) Complete apparatus overview including airflow and ventilation systems. B) Early stage of airflow cell construction. C) Top view of air and scent control and delivery system.

Figure 3

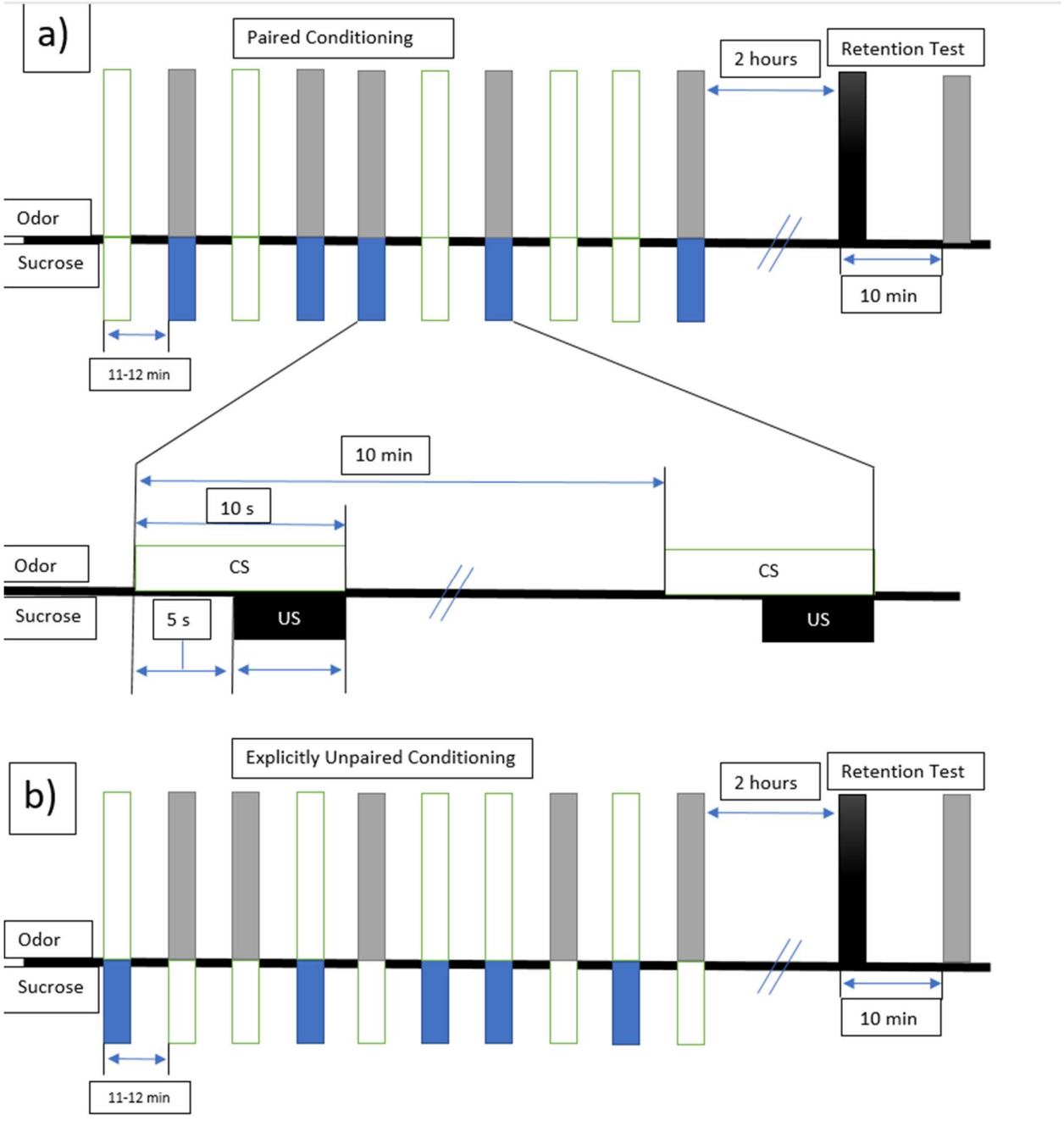

Figure 3: Diagram of conditioning procedure

A) Paired conditioning procedure. Unshaded boxes represent blank trials. Exploded view of one trial shown below. Retention test starts with novel scent, then original. B) Unpaired conditioning. Unshaded boxes represent lack of stimulus. Retention test starts with novel scent, then original. Note that sucrose and scent are never presented at the same time.

Figure 4

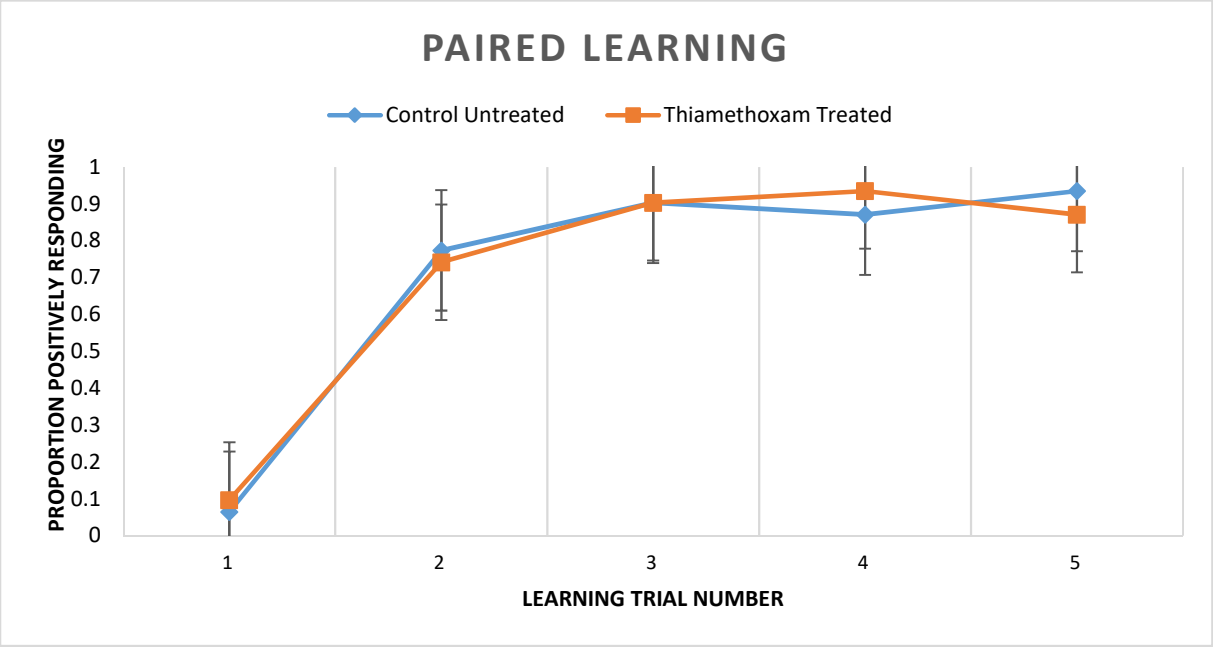

Figure 4: Paired Conditioning Results for Both Control and Experimental Groups

Control groups denoted by blue lines with diamond markers for each trial. Experimental groups denoted by orange lines with square markers at each trial. Y-axis shows the proportion of bees eliciting a PER response (extending proboscis) versus x-axis showing trial number.

Figure 5

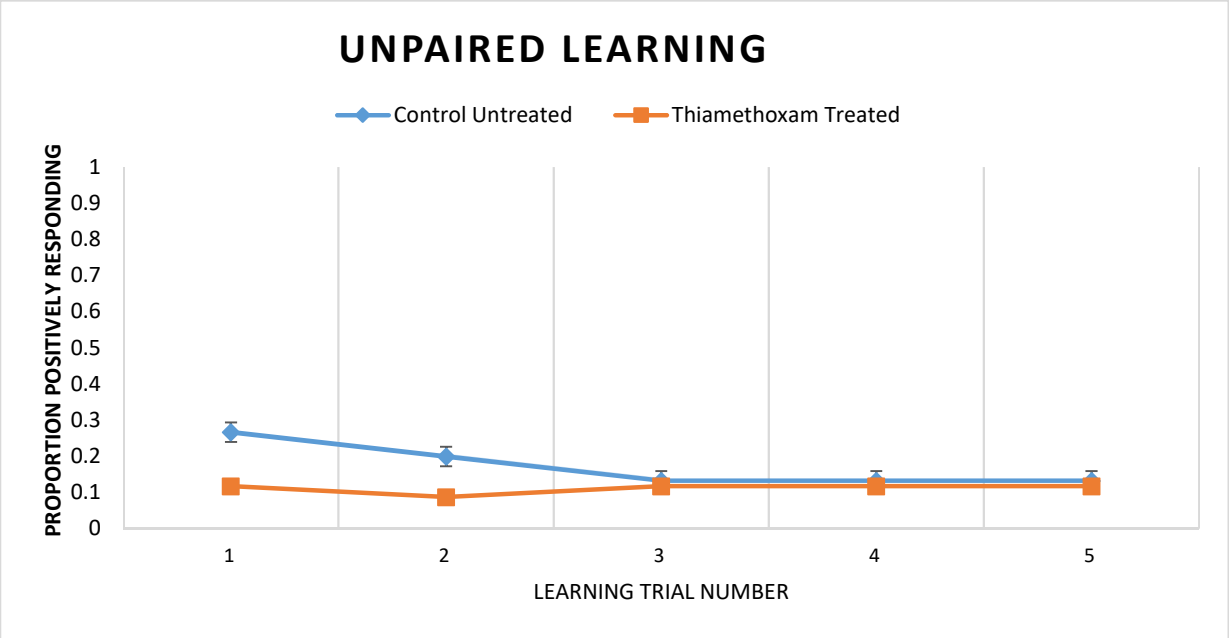

Figure 5: Unpaired Conditioning Results for Both Control and Experimental Groups

Control groups denoted by blue lines with diamond markers for each trial. Experimental groups denoted by orange lines with square markers at each trial. Y-axis shows the proportion of bees eliciting a PER response (extending proboscis) versus x-axis showing trial number.

Figure 6

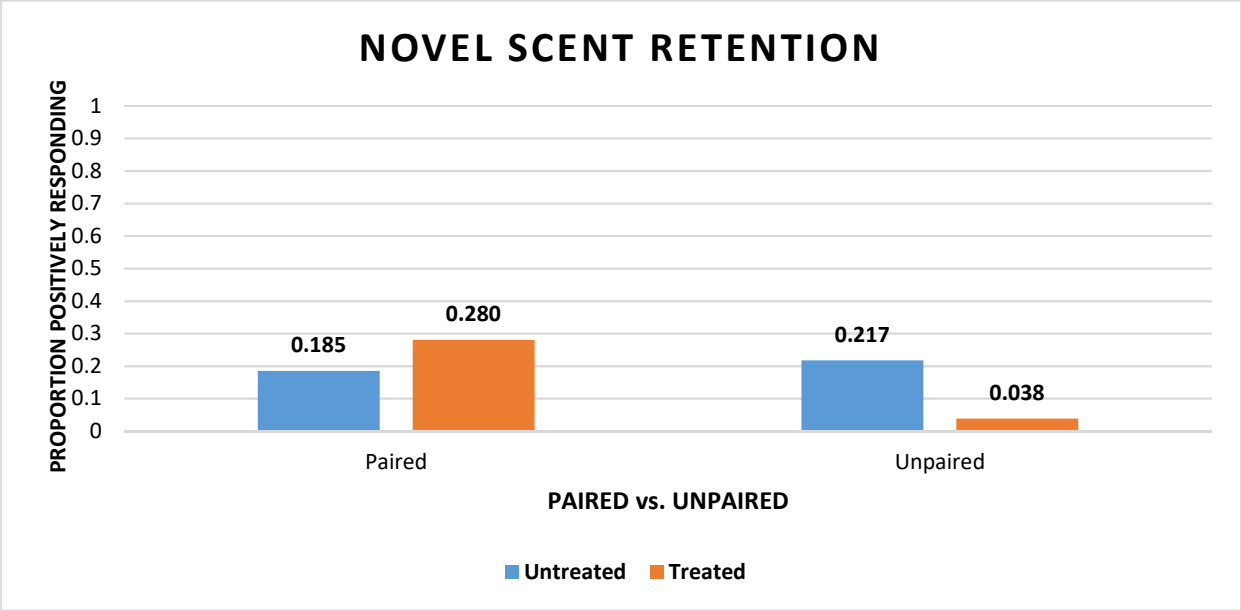

Figure 6: Retention Test for Novel Scent

The left side of the graph shows the results for paired groups exposed to the novel scent during retention testing. The blue bar on the left side shows the proportion of untreated control bees extending their proboscis upon the presentation of the novel scent, while the orange bar immediately to its right shows the treated bees response. The right hand side of the graph shows the results for unpaired control groups retention testing. The blue bar on the left side shows the proportion of untreated control bees extending their proboscis upon the presentation of the novel scent, while the orange bar immediately to its right shows the reponse of the treated bees.

Figure 7

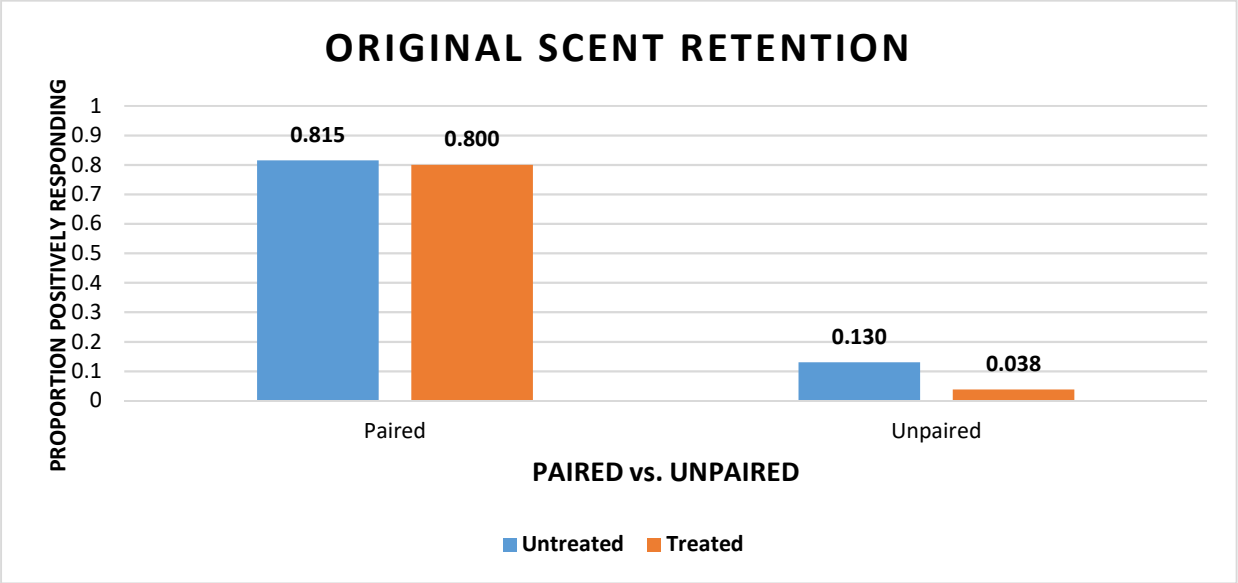

Figure 7: Retention Test for Original Scent

The left side of the graph shows the results for paired groups exposed to the original scent during retention testing. The blue bar on the left side shows the proportion of untreated control bees extending their proboscis upon the presentation of the original scent, while the orange bar immediately to its right shows the treated bees. The right hand side of the graph shows the results for unpaired groups retention testing. The blue bar on the left side shows the proportion of untreated control bees extending their proboscis upon the presentation of the original scent, while the orange bar immediately to its right shows the treated bee response.

Figure 8

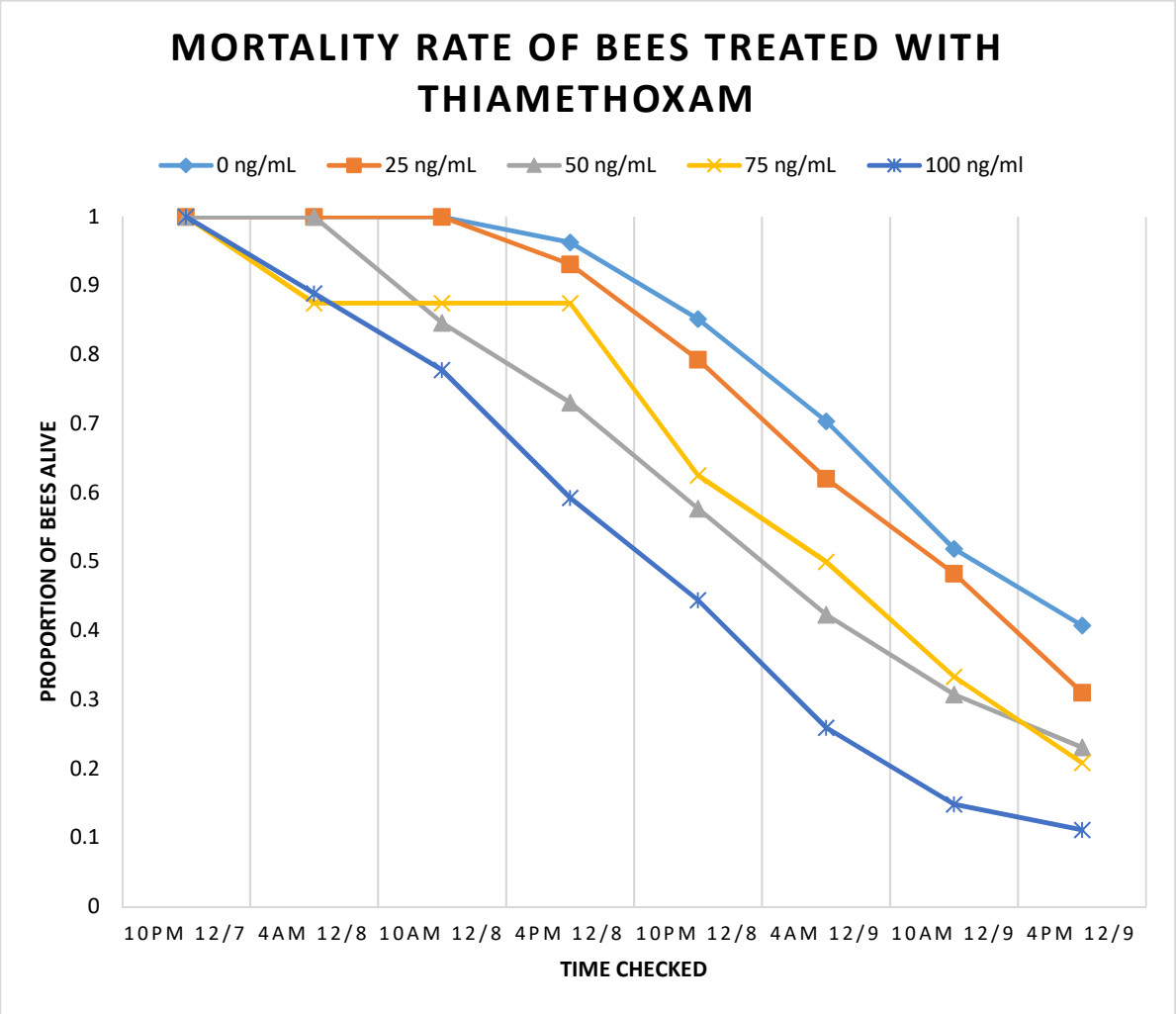

Figure 8: Mortality Rate Comparison for Varying Levels of Thiamethoxam

The y-axis displays the proportion of bees still alive relative to the original amount of bees in each group. The x-axis shows the time that the bees were checked(every 6 hours). Five different groups are represented containing: an untreated control group, and groups treated with 25, 50, 75, and 100 ng/mL thiamethoxam. The untreated control group is denoted by a dark blue line with diamonds at each data point. The 25ng/mL group is denoted by an orange line with squares at each data point. The 50ng/mL group is denoted by a gray line with triangles at each data point. The 75ng/mL group is denoted by a yellow line with an X at each data point. The 100ng/mL group is denoted by a light blue line with starbursts at each data point.