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A balanced omega-3/6 fatty acid diet can reduce the harms of pesticide exposure for honey bee olfactory learning

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A balanced omega-3/6 fatty acid diet can reduce the harms of pesticide exposure for honey bee olfactory learning

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

in

Biology

by

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2021

The thesis of Frank Paul Loduca is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

A balanced omega-3/6 fatty acid diet can reduce the harms of pesticide exposure for honey bee olfactory learning

by

Frank Paul Loduca

Master of Science in Biology

University of California San Diego, 2021

Professor James Nieh, Chair

Among the stressors honey bees are exposed to in agricultural settings, poor diet and pesticide exposure are common and concerning. We tested if a balanced omega-3/6 fatty acid substitute pollen diet could increase the resistance of honey bees to the harms of pesticide exposure. We raised bees on either a substitute pollen diet balanced in omega 3 and 6 fatty acids (1:1 ratio) or imbalanced (1:5) for two weeks before providing them with either 10 nM thiamethoxam (TMX) in 2.0 M sucrose solution or a control. Using a Proboscis Extension Response (PER) learning assay, we classically extend their proboscises to odors associated with punishment, as expected but did learn odors associated with reward. Bees fed an imbalanced diet and TMX had significantly poorer learning in the sixth learning trial and significantly poorer

memory when tested 1 h after this final learning trial. However, bees fed the balanced diet were resistant to the effects of TMX and had normal learning in the sixth trial and normal 1 h memory. Bees fed the balanced diet and exposed to TMX had higher survival than bees fed the imbalanced diet and TMX, suggesting that a balanced diet helped bees exposed to TMX. However, the survival data is complex and may suggest hormesis, in which a short, low dose of pesticide can increase insect survival. Our results suggest that a balanced omega-3/6 fatty acid diet can increase the resistance of honey bees to TMX, providing hope for enhancing the resistance of honey bee colonies.

Introduction

Honey bees provide vital pollination services for agriculture and natural ecosystems (Aebi et al. 2012). However, for multiple years, managed bee populations have faced annual winters declines of over 30%, and require increasingly intensive treatments to support their health (Buchmann, and Nabhan 1996, Staveley et al. 2013, Rhodes 2018.) Although the global population of managed honey bees is being sustained with increasingly intensive treatments, such management adds to the cost of maintaining colonies for agriculture, consequently has major implications for crops that depend upon bee pollination (Gallai, et al., 2008), and results in the double-edge sword of honey bee colonies increasingly receiving chemical treatments to sustain their health as researchers discover multiple interacting effects between bee medications, mite suppression treatments, and agrochemical exposure (Johnson et al., 2013).

Several factors contribute to the poor health of managed honey bees: diseases, parasites (particularly *Varroa* mites), poor management practices, agrochemicals, and inferior nutrition (Van Engelsdorp, et al., 2009). Many of these factors stem from agriculture and changes in land use. Agrarian monocultures can expose bees (particularly honey bees rented to pollinate crops) to agrochemicals and can limit the kinds of pollen that bees have access to (Colwell et al. 2017), leading to imbalanced diets. In ecosystems with diverse floral resources, honey bee colonies can compensate for nutritional deficits by recruiting foragers to collect diverse pollens with nutrients that balance colony needs (Hendriksma et al., 2016). However, monocultures and the loss of foraging habitat can contribute to dietary imbalances (Paudel et al. 2015). Omega-3 and omega-6 fatty acid imbalances are a common problem because the ratios of these fatty acids can be orders of magnitude different in agricultural settings than in natural ones (Arien et al., 2015).

Omega fatty acids play a major role in health, particularly cognitive health, of diverse animals including vertebrates and invertebrates. Dietary omega-3 fatty acids are essential for mice, humans, and *Drosophila* because none of these organisms can synthesize such fatty acids (Shen, et al. 2010). Omega-3 deficient mice suffered a 51% reduction in brain docosahexaenoic acid (DHA), an important structural omega-3 fatty acid in the brain, and, as a result, had decreased spatial learning and spent more time and made more errors in a tunnel escape assay (Fedorova, Irina, et al., 2007). Mice whose diet was supplemented with omega-3 fatty acids performed better later in life, retained better cognitive abilities, and had less grey matter loss in the brain (Cutuli, et al. 2020). Similarly, these fatty acids are important for neuronal function (Cho, et al. 2014) and learning (Arman et al. 2020) in *Drosophila*. Despite such common functionalities, there are some differences. For example, insects use far fewer long chain omega-3 fatty acids compared than mammals (Stanley-Samuelson et al. 1996).

Honey bees are also dependent on omega-3 fatty acids for proper development and cognition. European honey bees (*Apis mellifera ligustica*) deprived of dietary omega-3 fatty acids had impaired olfactory learning compared to bees fed a balanced omega-3/6 diet (Arien et al., 2015). Bees fed the imbalanced diet were also immune compromised because they were more susceptible to disease and less resistant to parasites (Arien et al., 2015). These bees additionally had shrunken hypopharyngeal glands, which nurse bees use to produce brood food, causing a food deficiency that further impairs colony fitness (Arien, et al., 2015). A balanced fatty acid diet is thus important for honey bee colony health and fitness.

Pesticide exposure is another important factor underlying colony declines (Van Engelsdorp, et al., 2009). Depending on the pesticide type and dose, honey bees either had a decreased ability or complete inability to learn and differentiate between different scents

associated with food (Mustard et al., 2020). Furthermore, honey bee visual learning also decreased when exposed to trace levels of pesticides (Colin et al, 2020). Pesticides also increased vulnerability to a common honey bee parasite, *Varroa destructor* (Morfin et al, 2020). A meta-analysis found that both field realistic and artificially high levels of pesticide negatively affected learning in chronic and acute exposures to the pesticides (Siviter et al, 2018).

Thiamethoxam (TMX) is a good model for examining pesticide effects on honey bees. Since 2012, TMX has been among the three most prevalent neonicotinoid pesticides used in the United States and is deployed on a variety of crops that bees forage from (Bass et al. 2015). TMX is also highly toxic because its primary degradation product, clothianidin, is also highly toxic to insects, leading to two waves of toxicity when ingested (Nauen et al. 2013) and contributing to long term environmental impacts (Kah et al. 2018). These properties combined with its common usage make TMX a good candidate pesticide for a study that seeks a practical solution for neonicotinoid pesticide exposure in honey bees.

Nutrition is well studied and improves colony fitness in several ways (Huang, 2012 and Arien et al., 2015), but the interactions between nutrition and agrochemicals are mostly unexplored. These interactions are worth considering because agrochemicals (Muth and Leonard, 2019) and nutrition (Arien et al., 2015) often affect bee cognitive functions. Specifically, nutrition can increase the resistance of bees to the negative effects of pesticides. Bees fed *Salix* pollen were better able to overcome pesticide effects as compared to bees fed a diet of *Brassicae* and *Quercus* pollens (Barascou et al., 2021). Some controlled studies addressing the interactions between pesticides and nutrition exist. Not all studies have demonstrated that diet can increase resistance to pesticides (Moreira et al. 2021) showed that protein supplementation can increase sealed brood area in colonies but that hemolymph protein content and resistance to the pesticide

fipronil did not change (Moreira et al. 2021). However, there are multiple factors that play a role in pesticide effects (pesticide concentration, frequency and time of exposure) and thus more studies are needed (Naggar and Baer, 2019). The kinds of nutrients provided play a role. For these reasons, we studied the effects and interactions of nutritional and agrochemical stress (thiamethoxam exposure) on the survival and learning of honey bees.

Materials and Methods

Study site and colonies

This study was conducted at the Biology Field Station Apiary at UCSD with 13 colonies from September of 2020 to May of 2021. All colonies were in good condition, as determined by standard inspection techniques.

Substitute pollen diets

Two substitute pollen diets with omega 6:3 ratios of 1:1 and 5:1, henceforth the balanced and imbalanced diet respectively, was created using commercial soy flour, corn and flax oils as protein sources. Honey was included in the diet as a phagostimulant. Fatty acid methyl esters (FAME) gas chromatography analysis was used to determine diet composition. This analysis led us to use ratios of 39.34 % soy flour, 2.93% corn oil, 0.64% flax oil, and 57.08% honey for the omega-3 fatty acids and 39.34 % soy flour, 1.07% corn oil, 2.50% flax oil, and 57.08% honey for the omega-6 fatty acids. We used a C17 quantification standard. The diet includes 7.55 mg/g linoleic acid and 1.17 mg/g alpha-linolenic acids for a total ratio of 6.45 omega-6:3. Based on previous preparations and literature, these oils are expected to be 92% fatty acids. Researchers

were blinded to pollen treatments. We will refer to bees fed a balanced omega-3/6 fatty acid diet as “balanced” and those fed the imbalanced diet as “imbalanced”.

Sucrose & pesticide solution preparation

Stock 2 M sucrose solution was prepared from boiled deionized water and laboratory grade granulated sugar. The solution was stirred until fully dissolved and stored in refrigerated glass beakers. To prepare pesticide solution for PER tests, a stock solution of 100 mM thiamethoxam (CAS#153719-23-43, Sigma Aldrich 37924-100 MG-R) in ultrapure water was created. This stock was diluted to make a final concentration of 10 nM thiamethoxam in 2.0 M sucrose for later experiments. We used 10 nM of TMX as a field realistic dose based on the findings of previous literature examining the pesticide load of pesticide treated plant, in both seed and leaf sprayed plants (reviewed in Kessler et al. 2015).

Researchers were blinded to the sucrose treatments. We will refer to bees fed pesticide as “TMX” and those fed pure sucrose with no pesticide as “control”. We therefore had four different treatments: balanced-TMX, balanced-control, imbalanced-TMX, and imbalanced-control.

Capturing procedure & cage maintenance

To capture bees, outdoor colonies were briefly smoked and then opened. A hive tool was used to uncap brood cells and check the age of larvae. Brood frames were selected for larvae near emergence, with black, nearly fully-formed heads. Frames captured from colonies were incubated at 30-34° C and 50-60% RH (relative humidity) for 24 h. After 24 h had elapsed, bees were brushed from their frame with a bee brush into a container with vegetable oil applied to the

walls so that they could not escape. Bees were taken from this container and placed in clear plastic cages (each 11 × 9 × 11 cm, length × width × height, 25 bees per cage). We used standard in vitro methods for maintaining adult bees in cages (Williams et al., 2013). Four cages were used per trial, with two cages per substitute pollen diet. Each cage was randomly assigned a different diet, which was placed inside a single labelled Eppendorf tube containing 1.5 g of diet. Diets were kept at -20 °C and the defrosted the day before use.

Bees were given *ad libitum* access to their respective diets and stock 2.0 M sucrose solution. Sucrose solutions were given in 5 mL syringes with the tips cut off to avoid the formation of a bubble that could restrict access. Syringes were suspended from a hole in the top of each cage (one syringe per cage) and filled with 5 mL of their respective solutions and refilled as needed. All cages received pure 2.0 M sucrose solution with no TMX for the first 14 days. Then, at the end of day 14, each cage was given only one kind of sucrose treatment (control or TMX) for 24h. After this 24 h, we removed these sucrose treatments and again provided all cages only with pure 2.0 M sucrose solution with no TMX

Dead bees were counted and removed twice per week. To do this, a metal spatula was slid underneath the door of the cage, and the dead bees were carefully moved to the front. The door to the cage was then briefly opened to create a 1 cm gap so the dead bees could be removed. During this process, we also removed the sucrose syringe from the top of the cage, briefly covering the hole with tape for enough time to weigh and record the mass using a ME-T Analytical balance. The substitute protein diet (contained inside a small 2.5 ml centrifuge tube) was also removed during this processed and weighed.

To measure and correct for evaporation in the sucrose syringes, one cage of each sucrose treatment was used as an evaporation control, and was weighed on the same days as the other

syringes. The mass lost from this syringe was approximately 1.5% over three days period for both the TMX and control sucrose solutions. Evaporative losses of 1% per day are typical and thus, these results fall within standard parameters (Bell et al., 2020). Sucrose consumption was corrected for this loss. Due to essentially no substitute pollen diet consumption after several days and small even increases in substitute pollen diet tube masses due to bees defecating on the tubes, there were inconsistencies in substitute pollen diet masses (calculated per surviving bee per day) and we therefore did not analyze these data. However, bees in all cages clearly consumed the substitute pollen diets within the first two weeks.

Experiment 1: Bee survival

Bees were captured as described above and allowed to age for two weeks in their cages until they were approximately the age of forager bees. Although 21 days is generally considered the average age of foraging, there is variation and bees as young as 14 days can forage and exhibit olfactory learning (Winston, 1991). After 14 days, the pure sucrose solution in each cage was replaced with either a control solution (2.0 M pure sucrose) or 10 nM thiamethoxam in 2.0 M sucrose. Bees were monitored continually until they were removed for the PER test detailed in experiment two or died. All bees removed for the PER experiments were censored in our survival analyses. Survival monitoring continued until all bees in all cages had died.

Experiment 2: Bee learning and memory

Harnessing

We trained and tested learning and memory 24 h after bees were exposed to TMX or the control sucrose solution. Bees were removed from cages and cold-anesthetized by placing them

individually inside small plastic vials placed inside crushed ice until we detected very little movement, usually about 2-3 min. Once unconscious, bees were carefully placed head first into a 1 ml Eppendorf tube that had its tip removed. A notched piece of drinking straw was place into the Eppendorf tube between the bee and her wings. This harnessing prevents the bee from escaping the tube but allows her head access to the outside of the tube (Tan et al. 2017).

Sucrose Responsiveness

Bees were tested for responsiveness to sucrose solution before being used in the learning trials to exclude bees unmotivated to feed. To test responsiveness, a small toothpick was dipped into a vial containing 2.0 M control sucrose and the toothpick was lightly touched to the bees' antennae to check for proboscis extension. Bees that extended their proboscis were not allowed to feed from the toothpick to maintain their hunger levels. Testing for responsiveness continued until five bees from each treatment responded to the sucrose or no bees remained in a cage. Bees that responded were used for the remainder of the procedure, regardless of future responsiveness. Any bee that responded and was tested for learning or that did not respond were not returned to cages after testing because the cold anesthetization was an artificial stressor that was not a focus of our experiment. After the PER trials, bees were euthanized by freezing.

Learning trials

We conducted differential learning assays in which bees needed to associate one odor with a reward (CS+) and another odor with a punishment (CS-), based upon the design of Mustard et al. (2020). Learning to discriminate between different floral odors occurs naturally in bees and is an important part of their foraging ecology because they often need to distinguish

between rewarding and unrewarding floral resources with different odors (Mustard et al., 2020). Harnessed bees were placed on a stand. Two PFTE lined silicon tubes facing the bee were attached to a cylinder containing a piece of Kimwipe saturated with 2 μL of pure odorant (either hexanol or decanol). The odorants were refreshed at the start of each trial. An aquarium pump (Active Aqua air pump, Hydrofarm model AAPPA25L) was attached to each cylinder containing odorant, with an electronic apparatus attached that controlled airflow through each cylinder using two momentary-on push button switches. The odors were associated with either a positive stimulus or a negative stimulus, but the pairings were randomized at the start of each trial.

For the learning trials, the positive stimulus was 3 s of exposure to the associated odor (CS+) before an additional 3 s of a sucrose reward combined with continued exposure to the odor (US and CS+). If the bee was kept in a cage that received 10 nM TMX sucrose solution, their reward also contained 10 nM TMX. Bees were next exposed to the "punished" stimulus, a different odor (CS-) and an aversive 10mM quinine solution. Bees are exposed to each odor six times in pseudorandomized fashion (randomly generated, but both stimuli must be given exactly six times and no more than three of any given stimuli in a row), so that the next stimulus could not be reliably predicted by the bee. The intertrial interval was 5 min. During each exposure, we recorded if bees extended their proboscises in either the initial three seconds with no reward or punishment (evidence of learning), and in the three seconds after the reward or punishment was administered (evidence of responsiveness for the US). After all trials were conducted, bees were exposed to two unrewarded trials with no presentation of sucrose solution or quinine solution. In these trials, they were tested with the CS+ and CS- (presentation order alternated between bees). The first for memory was 5 min after the learning/punishment procedure concluded, and the second unrewarded trial was 1 h later to test memory.

Statistics

To determine the significance of the different treatments on trial completion rates of bees (defined as the number of bees the survived to complete the full set of PER trials), we used two-tailed Fischer's Exact 2x2 tests (http://vassarstats.net/fisher2x3.html) to compare completion rates across both pesticide and diet treatments.

We used JMP v. 16.0 statistical software for all other tests. To analyze the effects of treatments on bee survival, we ran survival analyses with censoring. We made multiple pairwise comparisons between the four different treatments and applied Dunn-Sidak tests to correct for potential Type I statistical error, reporting our results as "DS" if they were still significant after correction.

For our sucrose consumption data, we ran Repeated-Measures Mixed Models (REML algorithm) with cage identity as the repeated measure (a random effect). Fixed effects were time, treatment, and their interaction. Since this interaction was non-significant ($p \geq 0.05$), we removed it and ran the model again.

To analyze the effects of pesticide exposure and substitute pollen diet on honey bee learning, we ran Repeated Measures Mixed Models (REML algorithm). Because we used ≥82 bees per treatment, we were able to use this method of analysis on PER data (Matsumoto et al., 2012). Across all treatments, a total of 26 bees that did not complete their learning trials were excluded (7% of all bees). In all models, colony, and bee identity (the repeated measure) were included as random effects and diet, pesticide, trial number, and their interactions were included as fixed effects. We ran a separate model for the punished trials.

We also ran a Mixed Model (non-repeated-measures) using only data from the final learning trial (6^{th} trial) because this last trial had the highest level of learning for all groups. In this trial, colony was a random effect and diet, pesticide, trial number, and their interactions were included as fixed effects. We ran two models, one per CS+ odor. To simplify our analyses, we only examined CS+ odor in this model. We used Tukey's Honestly Significant Difference (HSD) tests were used to make corrected, all-pairwise comparisons.

The memory models contained only one measurement per bee, and thus we did not conduct a repeated measures analysis. We included only bees that displayed learning in the last three rewarded trials. We ran Mixed Models (REML algorithm) with colony as a random effect and treatment as a fixed effect. We did not run CS+ odor in this model because odor was not significant in the learning model. Tukey's HSD tests were used for all pairwise comparisons.

Results

Experiment 1: Bee survival

A total of 1539 bees from 13 colonies were used for the survival trials. Of these 1539 bees, 1196 were not tested for learning and were simply monitored for their survival (306 bees were balanced-control, 323 bees were balanced-TMX, 293were imbalanced-control and 274 were imbalanced-TMX).

There was no significant effect of substitute pollen diet in the first two weeks, the time before the pesticide or sucrose control treatments were fed to bees (Wilcoxon Chi-square=0.16, 1 df, p=0.69, **Fig. 1A**). However, there were significant differences between the treatments (Wilcoxon Chi-square=40.21, 3 df, p<0.0001) after the introduction of pesticide.

There was no significant difference between the balanced-TMX and imbalanced-control groups (Wilcoxon Chi-square=1.790, 1 df, p=0.1743). Likewise, the imbalanced-control and imbalanced-pesticide groups were not significantly different (Wilcoxon Chi-square=0.8149, 1 df, p=.3667), with both groups providing intermediate levels of survival (**Fig. 1B**).

A key result is that the *balanced-TMX group had significantly better survival than the imbalanced-TMX group* (Wilcoxon Chi-square=7.395, 1 df, p=0.0065, DS, **Fig. 1B**), suggesting that a balanced diet helped bees exposed to TMX.

However, somewhat surprisingly, the balanced-control group had lower survival as compared to the balanced-TMX (Wilcoxon Chi-square=39.53, 1 df, p<0.0001, DS), as compared to the imbalanced-control group (Wilcoxon Chi-square=14.89, 1df, p=0.0001, DS), and as compared to the imbalanced-TMX group (Wilcoxon Chi-square=7.286, 1df, p=0.0070, DS, **Fig. 1B**).

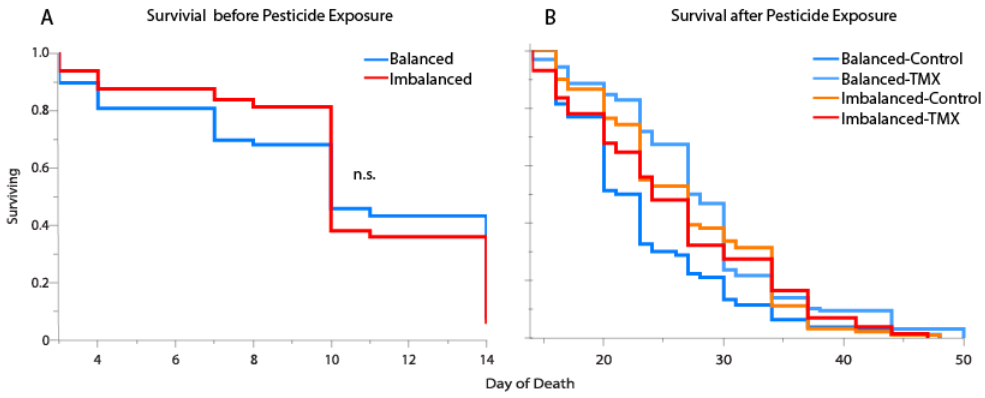

Figure 1. Effects of substitute pollen diet and TMX on survival. (A) Survival before pesticide exposure divided by diet. Diet did not significantly impact survival before the introduction of pesticide (p=0.16). (B) Survival data after 24 of pesticide exposure beginning on day 15 (the origin of this plot). After this 24 of exposure, all cages were fed only pure 2.0 sucrose solution with no TMX. The balanced-TMX did significantly better than the imbalanced-TMX group (p=0.0065, DS). However, the control-balanced group did significantly worse than the balanced-TMX (p<0.0001, DS), imbalanced-control (p=0.0001, DS) and imbalanced-TMX (p=0.0070, DS) groups. All other pairwise comparisons were insignificant (p>0.05)

Experiment 1: Bee consumption

We calculated the sucrose consumption per surviving bee per day. There was a significant effect of treatment ($F_{3,547}$=3.27, p=0.02), but no significant effect of day ($F_{1,547}$=0.22, p=0.63) or the interaction of treatment x day ($F_{3,544}$=0.06, p=0.98). The imbalanced-control bees had significantly higher sucrose consumption than the balanced-control bees (Tukey HSD test, p<0.05). There was no significant difference between the sucrose consumption of bees fed TMX, regardless of whether they consumed the balanced or imbalanced diet (Tukey HSD test, p>0.05).

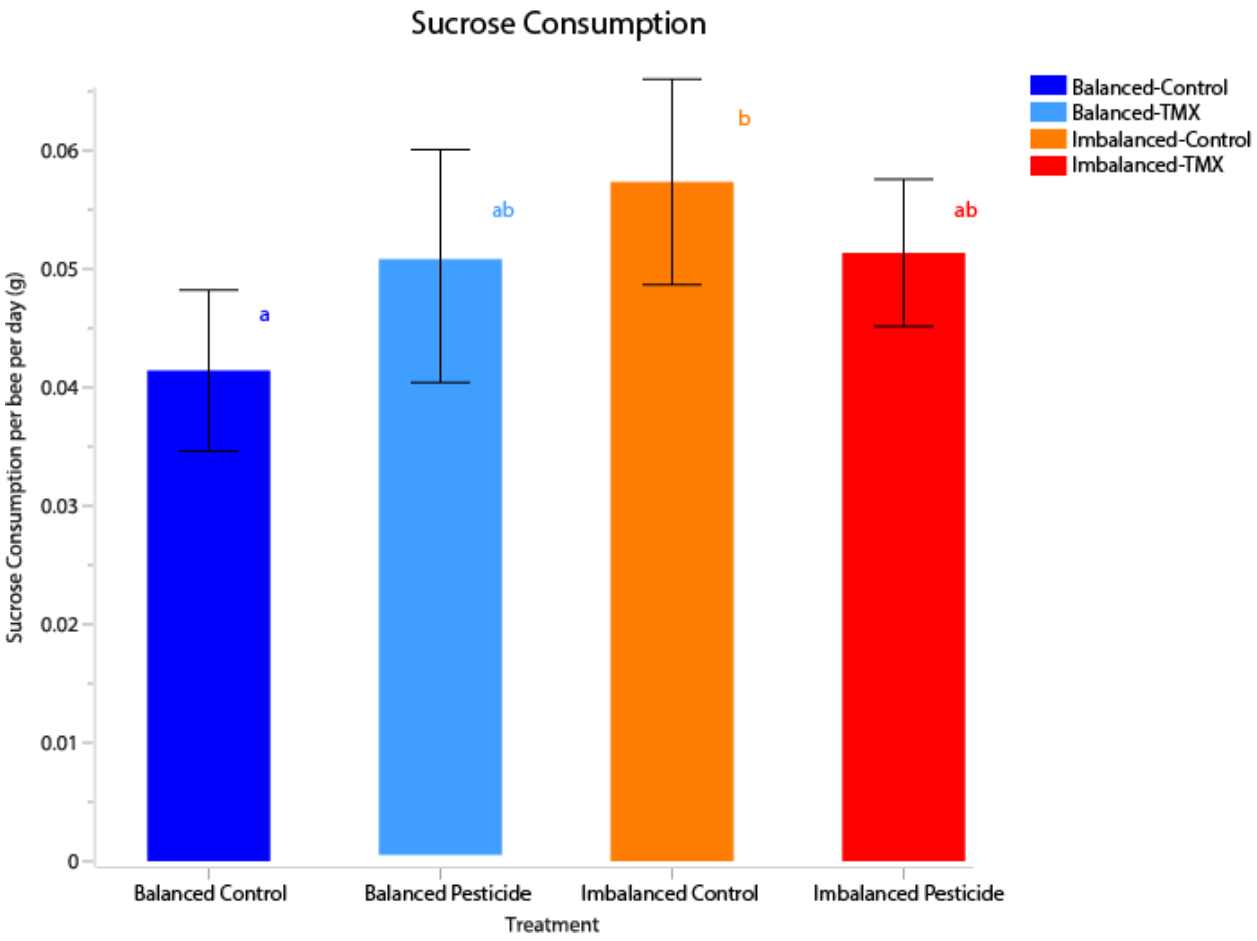

Figure 2. Bee sucrose consumption. Imbalanced-control bees consumed significantly more sucrose than balanced-control bees. Different letters show significant differences (Tukey HSD test, $p<0.05$), $N=138$ average observations per treatment group. Means and standard errors are shown.

Experiment 2: Bee learning

A total of 343 bees from 13 colonies completed our learning trials ($N_{\text{balanced-control}}$ bees=82, $N_{\text{imbalanced-control}}$ bees=93, $N_{\text{balanced-TMX}}$ bees=82, and $N_{\text{imbalanced-TMX}}$ bees=86). In this learning experiment, pesticides did not affect the number of bees that completed all six learning trials: balanced-control (94.3%), imbalanced-control (92.1%) (Fischer's exact test 2x2, $p>0.05$); or across pesticide: balanced-TMX (94.3%), imbalanced-TMX (91.5%) (Fischer's exact test 2x2, $p>0.05$).

Bees showed learning: there was a significant effect of trial ($F_{5,1694}$=73.17, p<0.0001). However, there was no significant effect of pesticide treatment ($F_{1,1015}$=0.0655, p=0.80), diet treatment ($F_{1,1021}$=0.04, p=0.83), or their interaction ($F_{1,1015}$=0.0189, p=.8906). Colony accounted for <4% of model variance. Bee identity accounted for 42.5% of model variance.

In the 6th trial, we divided the data by CS+. For CS+ decanol: diet treatment was significant ($F_{1,196}$=10.47, p=0.0014), pesticide treatment was significant ($F_{1,195}$=4.20, p=0.042) and diet x pesticide was not significant ($F_{1,195}$, p=0.16). For hexanol: diet treatment was significant ($F_{1,132}$=5.52, p=0.020), pesticide treatment was not significant ($F_{1,130}$=0.0037, p=0.95) and diet x pesticide was not significant ($F_{1,130}$=0.22, p=0.64).

In this 6th trial, considering all CS+, 45.1% of balanced-control bees, 31.2% of imbalanced-control bees, and 43.9% of balanced-TMX bees showed learning (not significantly different, Tukey HSD test, p<0.05). In contrast, fewer bees (16.3% of imbalanced-TMX bees) showed learning, a significant difference (Tukey HSD test, p<0.05). *This suggests that balanced-TMX bees were resistant to the effects of TMX* since the learning of this treatment group was significantly higher than the imbalanced-TMX group and equal to the unbalanced-control group.

Across all punished trials, bees showed no learning, as expected: no significant effect of trial ($F_{5,1695}$=2.18, p=0.054). The punished trials had no significant differences for pesticide ($F_{1,334}$=0.077, p=0.36), diet ($F_{1,339}$=0.86, p=0.36), and no other interactions were significant (F≤1.92, p≥0.087). Colony accounted for <1% of variance.

Experiment 2: Bee memory

In the rewarded 1 h memory test, 45.1% of balanced-control bees, 37.6% of imbalanced-control bees, 43.9% of balanced-TMX bees and 14.0% of imbalanced-TMX bees showed

memory. These bees all exhibited some learning in last three learning trials, and these comparisons were therefore made only with bees that had learned and could therefore exhibit memory.

Both diet ($F_{1,130}$=7.40, p=0.074) and pesticide ($F_{1,128}$=6.84, p=0.0099) had significant effects on honey bee learning in the 1 h memory test. There was a significant interaction of pesticide x diet treatments ($F_{1,118}$=14.57, p=0.0002). As in the learning results, no groups showed significantly different memory than any other, except the unbalanced-TMX group (Tukey's HSD, p<0.05), which had significantly lower memory. Thus, the balanced diet likely increased the resistant of bees to TMX given that their memory was not significantly different from the balanced-control and unbalanced-control groups (**Fig. 3A**)

For punished "memory" trials U1 and U2 in the punished trials, there were no significant effects of diet ($F_{1,339}$=0.28, p=0.60), pesticide ($F_{1,336}$=0.35, p=0.55), diet x pesticide was also not significant ($F_{1,335}$=0.39, p=.53). Colony accounted for <1% of variance.

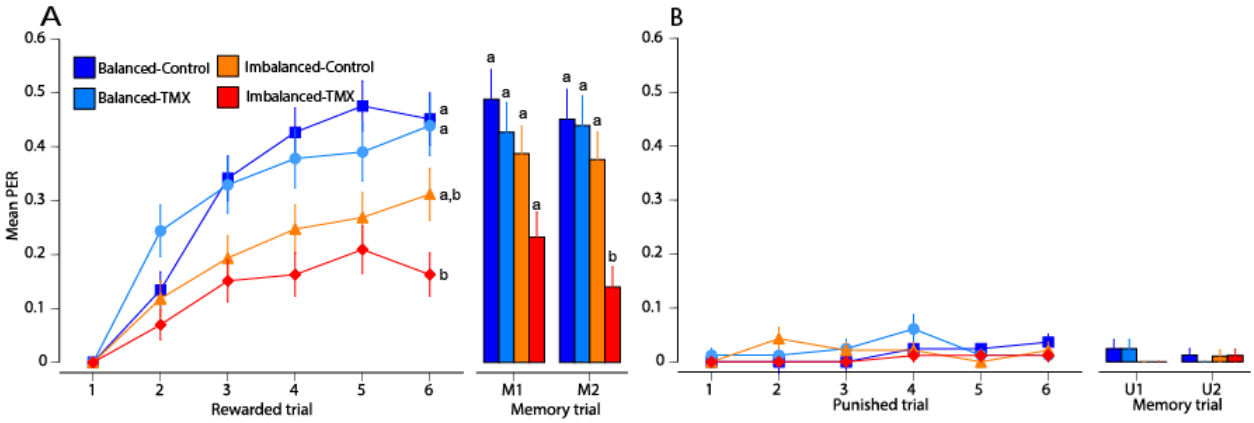

Figure 3. Bees fed a balanced omega-3 diet were resistant to the harmful effects of TMX. Means and standard errors are shown. In any given trial, bees that shown learning have a PER score=1. Data is shown from 343 bees, $N_{\text{balanced-control}}$ bees=82, $N_{\text{imbalanced-control}}$ bees=93, $N_{\text{balanced-TMX}}$ bees=82, and $N_{\text{imbalanced-TMX}}$ bees=86. (A) Rewarded trials show learning and memory. Different letters indicate significant differences (Tukey HSD, p<0.05). (B) Punished trials show no learning or memory, as expected (p>0.05).

Discussion

We found evidence that a balanced omega-3/6 fatty acid diet, in comparison with an imbalanced fatty acid diet, could improve the survival of bees chronically fed with field-realistic levels of a common neonicotinoid pesticide, thiamethoxam (TMX). In addition, this balanced diet increased cognitive resistance to TMX in honey bee olfactory learning and memory. In the 6th learning trial, the balanced-TMX bees showed higher learning than the unbalanced-TMX bees. The same was true of 1 h memory. For learning, there was no significant pesticide x diet interaction in the 6th trial, whereas this interaction was significant for the memory test. This result may reflect the higher variation in PER in the 6th learning trial as compared to the memory trial. As expected, there was no significant difference between any of the treatment groups in the punished trials because bees did not extend their proboscises when given quinine solution.

Surprisingly, the balanced-TMX group had higher survival than the balanced-control group. Studies on multiple organisms suggest that a low level of short-term stress can be beneficial (Dhabhar, 2018). For example, mitochondrial stress can restore the heat shock response and increase vitality in *C. elegans* (Labbadia et al., 2017). In honey bees (*A. mellifera*), heat shock proteins can increase worker bee survival (Li et al., 2020). Researchers have also demonstrated that a relatively brief 24 h exposure to a neonicotinoid pesticide, imidacloprid, increased a heat shock protein in honey bee cell nuclei and cytoplasm (Skerl and Gregoc 2010). We also exposed our bees for 24 h to the neonicotinoid, thiamethoxam. Thus, the increase in survival of the balanced-TMX bees may reflect the beneficial effect of a short-term stressor—potentially, heat shock proteins induced by TMX exposure. Multiple insect studies have also found evidence for this general phenomenon of hormesis in which a low dose of insecticide can be beneficial and increase insect fecundity and longevity (reviewed in Cutler et al 2013).

Moreover, the balanced-control group had lower survival than the imbalanced-control group. Arien et al. (2020) found the opposite, with the balanced diet showing significantly better survival than the imbalanced diet. We used the same diet formulations and fatty acid ratios, and are unable to account for these differences. Haddad et al. (2007) showed worker honey bees have a higher ratio of omega-6 relative to omega-3 fatty acids in their bodies. In Arien et al. (2020), feeding bees such an unbalanced ratio resulted in greater worker mortality, whereas our study showed feeding bees an imbalanced omega 3:6 ratio of significantly increased longevity. Our results may therefore correspond better with Haddad et al. (2007), but how consumption translates into the contents of worker bodies is somewhat unclear. We are currently completing new experiments testing the effects of these same diets on bees fed a field-realistic formulation of TMX and a common organosilicone adjuvant. These experiments also include direct comparisons between balanced-control and imbalanced-control treatments and should shed light upon the results reported here.

In terms of sucrose consumption, the only significant difference was between the balanced-control and imbalanced-control group. It is possible the imbalanced-control group consumed more sucrose as a foraging reflex to supplement their dietary deficiencies. Honey bees are known to increase foraging to balance their pollen diet when given pollen deficient in some essential amino acids (Hendriksma et al., 2016). Although, this applies to pollen, not nectar, foraging, we speculate that a drive to compensate for poor protein diet could increase bees' tendency to "forage" by drinking artificial nectar from the cage syringe. We note that the cages only contained artificial nectar after day 14. If this hypothesis is correct, then perhaps the TMX treatment affected the "foraging" drive since neither balanced-TMX nor unbalanced-TMX increased sucrose consumption (**Fig. 2**).

The results of our learning and memory experiments accord with prior research (Mustard et al. 2020). Bees learned to correctly distinguish between odors associated with reward and punishment, even when fed TMX. There was no inherent difference in bee responses to the CS+ odors. Between trials, which used different sets of bees, we changed the CS+ odor and the type of CS+ odor did not significantly alter our results ($p>0.05$). Thus, the significantly elevated learning of balanced-TMX bees as compared to unbalanced-TMX bees likely reflects increased cognitive resistance to TMX. The similar elevated 1 h memory of balanced-TMX bees as compared to unbalanced-TMX bees supports this result. It is possible that memory simply reflects learning, but multiple studies show that this is not necessarily the case. Bees can have increased or decreased (Tan et al., 2017) memory relative to the final learning trial when exposed to neonicotinoid pesticides.

Unlike Mustard et al. (2020), we provided TMX in the rewarded sucrose solution. Kessler et al. (2015) showed that honey bees did not avoid consuming sucrose solution laced with TMX and, in fact, had a slight preference for it. Thus, our rewarding sucrose solution was potentially slightly more rewarding because of the TMX in it and could have biased bees to have greater than if they were rewarded with pure sucrose. We choose to provide TMX bees with TMX in their rewarded sucrose solution because this more closely matches the natural situation of bees foraging on floral nectar contaminated with TMX. These doses were sublethal since there was no difference the proportion of bees that completed learning trials with or without TMX.

Our results also demonstrate a broader phenomenon, that the effects of diet and pesticide can interact. Prior studies showed that pesticide exposure alone (include TMX exposure) can decreased olfactory learning (Mustard et al. 2020). Separately, we know that bees fed an imbalanced diet have reduced olfactory learning (Arien et al., 2016). Here, we show a clear

interaction of diet and pesticide on memory and a somewhat weaker result for learning. The exact mechanisms for how TMX affected honey bee learning and memory are unclear, but these are known to be related phenomenon in which learning must first occur, followed by additional processing in different brain areas to create memory (Galizia, 2011).

It is unclear exactly why omega-3 fatty acids are important for honey bee cognition. Research on the effects of polyunsaturated fatty acid (PUFA) imbalances shows that a deficiency in alpha-linolenic acid (ALA, an omega-3 essential fatty acid) is strongly linked with cognitive decline in mammals (Arien et al. 2015 and Arien et al. 2018). Bees also require sufficient dietary ALA balanced with linoleic acid (LA, an omega-6 essential fatty acid) to maximize learning (Arien et al., 2018; Arien et al., 2015). Recently, researchers found an intriguing result: bees incorporate ALA and LA into their membrane phospholipids when they are 2-4 day old adults and, thereafter, these levels remain constant (Martin et al., 2021). This result reflects our understanding that such dietary effects are important early on in life and supports data, including ours, that bees subsist largely on sugars, consuming very little protein or fatty acids, later in life (Winston, 1991). It is unclear if bees store ALA and LA to regenerate key cellular membranes later in life or if the membranes are sufficiently robust, based upon early nutrition, throughout the remainder of their life. However, the action of neonicotinoids on neuron membranes, which may be connected to their action on nicotinic acetylcholine receptors, and the potential for a balanced fatty acid diet to help these membranes resist disruption is worthy of study.

Finally, actual pesticides used on crops contain multiple chemicals. Some tank mix additives such as organosilicone adjuvants can harm bees (Ciarlo et al. 2012), and thus studies on how these real-world mixes affect honey bee health are needed. Perhaps unsurprisingly, the diets bees are raised on and their ability to learn given pesticide exposure are closely linked and

researcher may find that addressing these issues together, rather than independently, are a fruitful way to improve the health of agricultural pollinators.

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