DISSERTATION

ANALYSIS AND MODELING OF WHAT HONEY BEES (*Apis mellifera* L.) BRING BACK TO THE HIVE AND HOW THAT AFFECTS THE HEALTH OF THE HIVE AND HUMANS

Submitted by

Mai Mousa Awad

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Doctoral Committee:

Advisor: Randall Boone
Co-advisor: Kato Takamitsu

Thomas Borch
Paul Ode

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ABSTRACT

ANALYSIS AND MODELING OF WHAT HONEY BEES (*Apis mellifera* L.) BRING BACK TO THE HIVE AND HOW THAT AFFECTS THE HEALTH OF THE HIVE AND HUMANS

Apis mellifera L. populations are decreasing at an alarming rate. Over the past 20 years, the number of managed honey bee colonies has declined, and this decline has become a global concern. This study focuses on chemical stressors that are found to affect the bee population. We used direct sampling to examine the variation of pesticides and heavy metals concentrations in two different landscape contexts. Subsequently, we extrapolated the risk of these toxins' residues on *Apis* sp. based on current literature. We found no spatial variation in metal concentrations in pollen and honey samples collected from urban and agricultural areas. Likewise, we observed no spatial variation in pesticide concentrations in pollen and honey samples collected from urban versus agricultural areas.

In addition to chemical factors, we studied the nutritional factor by investigating the effect of spatial variability on the amount of stored pollen and the floral diversity of in-hive pollen. Furthermore, we estimated the most abundant botanical families that will identify honey bees' protein-source preferences. The results indicated a spatial variation in Shannon-Weaver diversity, demonstrating a higher diversity index with a wider variety of pollen taxa collected from urban sites compared to the agricultural ones with lower diversity index with less pollen taxonomic variety.

The alarming decrease in honey bees' population urges researchers to investigate the factors that enhance the deterioration of bees' population. A few models explained these factors individually. We designed a NetLogo model to assess multiple factors that would intensify the

impact of the Colony Collapse Disorder phenomenon, by investigating the spatial variation of bees' exposure to a distinctive environmental toxin and the quantities of these toxins in hive products. The model indicated that there were significant spatial variation effects on the pesticides and heavy metal concentrations in the accumulated pollen and nectar inside the beehive.

Pesticides and heavy metal accumulation in bees' products are mainly caused by human activities, which can affect human health by their consuming contaminated honey. Based on the results of honey analysis for pesticides and heavy metals we performed in the first study, we decided to select one pesticide and a pesticide synergist along with the most two abundant heavy metals to investigate the synergistic effect of cytotoxicity and genotoxicity that would result from the interaction of one major pesticide in honey: Imidacloprid and a pesticide synergist: Piperonyl butoxide, and two major heavy metals in honey: Lead and Selenium, at the cellular level in mammalian cells, where we found different interactional effects of these compounds on cell survival, cell apoptosis, oxidative stress and sister chromatid induction.

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DEDICATION

To my family, who encouraged and prayed for me to follow my passion and always believed in my success. To my father and brother who passed away before seeing this moment.

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CHAPTER ONE: ANALYSIS AND MODELING OF WHAT HONEY BEES (*Apis mellifera* L.) BRING BACK TO THE HIVE AND HOW THAT AFFECTS HUMAN HEALTH

Apis mellifera Linnaeus (honey bees) are an essential ecosystem component; they are among the most significant pollinators, visiting more than 90% of the top 107 agricultural plants worldwide (Potts et al., 2016). Unfortunately, over the past 20 years, the number of managed honey bee colonies has declined, and this decline has become a global concern. According to FAO (2009), honey bee populations declined in both North America (49.5%) and Europe (26%) between 1961 and 2007.

Colony Collapse Disorder is a phenomenon associated with the bee population decline. Among the factors that cause this phenomenon are chemical stressors like pesticides (Mitchell et al., 2017, Tison et al., 2016, Pisa et al., 2015, Henry et al., 2012), and these have been widely investigated as the main stressors that cause the loss of bee populations, mainly in agricultural landscapes because of pesticide applications (Sabatino et al., 2013, Pohorecka et al., 2013, Castilhos et al., 2019, Zawislak et al., 2019), with less focus on the presence of pesticides in urban settings. Heavy metals are another chemical stressor that has been less studied than pesticide stressors. However, some studies have reported a relationship between the increase of heavy metal concentration in soil and plants and the decline of bee species richness, health and foraging behavior (Moron et al., 2012, Hladun et al., 2016, Sivakoff & Gardiner et al. 2017).

In addition to chemical factors, inadequate nutrition plays a significant role in a hive's deterioration. Bees' immune systems can weaken due to malnutrition, making them more vulnerable to environmental contaminants like pesticides (Wheeler and Robinson 2014). Therefore, understanding the floral resources used in different environments and their preferred foraging strategies at the colony level is essential to promoting bee health and aiding bee

conservation initiatives (Bloom et al., 2022). One method used to study bee foraging behavior and the floral selection process was tracking pollen botanical origins (Sommeijer 1983).

The impact of landscape variation on toxins deposited in bees' products has been poorly studied (Waiker et al., 2022). The importance of assessing the impact of spatial variation on bees came from the fact that the bees' exposure to toxins is not discrete and depends on the intersection of the spatiotemporal presence of toxins with bee foraging activity, which will later affect the amount and type of contaminants that bees might be exposed to and that might end up in the hive and hive products (Van Der Steen et al. 2012).

Few models have tried to explain the mechanism behind honey bees' exposure to contaminants, mainly pesticides. Instead, computer models were developed to simulate a honey bee colony and bees' foraging behavior; some models represent the multiple stressors that impact honey bee colonies, like pathogens, pesticides, and forage availability, to understand better colony development and survival, like the model in Henry et al. (2017) and the BEEHAVE model (Becher et al. 2014). Another model focused on individual bees' exposure to pesticides by considering the effect of pesticide movement in the hive and the behavior of different age stages of individual bees (Rumkee et al., 2017). However, no model addresses the heterogeneity of contaminant exposure in different landscape settings.

Considerations mentioned above about pesticides and heavy metal accumulation in bee products are mainly caused by human activities, which return to humans through consuming contaminated honey. Although there are allowable limits for the presence of these contaminants in honey, honey with different contaminants like pesticides and heavy metals has been reported in many countries worldwide (Mitchell et al., 2017; Smith et al., 2019; Ru et al., 2013; Ruschioni et al. 2013; Silici et al. 2015). Levels of pesticides and heavy metals in honey can be under the level

of concern for human health; however, little is known about the potential synergistic effects of pesticides and heavy metals mixtures and how they can interact to enhance toxicity levels.

Chapter two assesses the spatial variation of pesticide and heavy metal residues in pollen and honey samples. Furthermore, I performed a risk assessment to determine the potential risk to honey bees when exposed to these contaminants through pollen and nectar consumption by comparing our data with previous research that tested the effect of these toxins.

Chapter three investigated the influence of landscape heterogeneity on pollen diversity by inspecting the botanical and geographical origins of bee pollen collected from different land use areas in Colorado. Moreover, the most abundant botanical families were estimated to identify honey bees' protein-source preferences.

In chapter four, I used agent-based modeling to simulate bees' exposure to toxins, considering landscape variations, pesticide breakdown, and the ability of honey bees to detoxify contaminants. I used the NetLogo software platform in scenario analyses.

Based on the results of the honey analysis for pesticides and heavy metals I performed in chapter two, I selected one most abundant pesticide and a pesticide synergist and two major heavy metals to investigate the synergistic effect of cytotoxicity and genotoxicity that would result from the interaction pesticides in honey. I determined synergistic effects on cell attributes and survival of Imidacloprid and Piperonyl butoxide, and two major heavy metals in honey: Lead and Selenium, at the cellular level in mammalian cells.

In Appendix V, I included a published commentary paper (Awad and Brown 2021) discussing the lack of graduate students of color in STEM fields and the significance of involving underrepresented undergraduate students in ecology. The commentary discusses the need for a

mentoring and research program to encourage minority students to become more engaged in science in general and ecology in particular, which will have positive socioeconomic effects in the future. This paper also discusses the role of the Rocky Mountain Science and Sustainability Program (RMSSN), which offers a framework for adopting mentored research as a high-impact practice in undergraduate ecology teaching in engaging students of color in ecology-focused experiential learning. Although this paper is not directly related to the work I mentioned above, it does reflect the difficulties and roadblocks I encountered as an underrepresented student during my research journey and how it would have been simpler, less complicated, and time-consuming if I had been adequately engaged and guided early in my program.

LITERATURE CITED

Awad, M. M., & Brown, M. A. (2021). Undergraduate research as a high-impact practice for engaging students of color in ecology.

Becher, M. A., Grimm, V., Knapp, J., Horn, J., Twiston-Davies, G., & Osborne, J. L. (2016). BEESCOUT: A model of bee scouting behaviour and a software tool for characterizing nectar/pollen landscapes for BEEHAVE. Ecological modelling, 340, 126-133.

Bloom, E. H., Graham, K. K., Haan, N. L., Heck, A. R., Gut, L. J., Landis, D. A., ... & Isaacs, R. (2022). Responding to the US national pollinator plan: a case study in Michigan. Frontiers in Ecology and the Environment, 20(2), 84-92.

Castilhos D., Dombroski J.L.D., Bergamo G.C., Gramacho K., Goncalves L.S. (2019). Neonicotinoids and fipronil concentrations in honeybees associated with pesticide use in Brazilian agricultural areas. Apidologie 50: 657–668. Retrieved from https://doi.org/10.1007/s13592-019-00676-x

Food and Agriculture Organization of the United Nations (FAO), 2009. FAOSTAT. <http://faostat.fao.org>.

Henry M., Beguin M., Requier F., Rollin O., Odoux J.F., Aupinel P., Aptel J., Tchamitchian S., Decourtye A. (2012). A common pesticide decreases foraging success and survival in honey bees. Science. 336 (6079): 348-350.

Henry, M., Becher, M. A., Osborne, J. L., Kennedy, P. J., Aupinel, P., Bretagnolle, V., ... & Requier, F. (2017). Predictive systems models can help elucidate bee declines driven by multiple combined stressors. Apidologie, 48(3), 328-339.

Hladun, K. R., Di, N., Liu, T. X., & Trumble, J. T. (2016). Metal contaminant accumulation in the hive: Consequences for whole-colony health and brood production in the honey bee (Apis mellifera L.). Environmental toxicology and chemistry, 35(2), 322-329.

Mitchell E., Mulhauser B., Mulot M., Mutabzi A., Glauser G., Abei A. (2017). A world survey of neonicotinoids in honey. Science. 358: 109-11.

Moron D., Grzes I., Skroka P., Szentgyorgyl H., Laskowski R., Potts S., Woyciechowski M. (2012). Abundance and diversity of wild bees along gradients of heavy metals pollution. Journal of applied ecology. 49: 118-125.

Pisa, L.W., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Downs, C.A., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., McField, M., Morrissey, C.A.; Noome, D.A.; Settele, J., Simon-Delso, N., Stark, J.D., Van der Sluijs, J.P., Van Dyck, H., Wiemers, M. (2015). Effects of neonicotinoids and fipronil on non-target invertebrates. Environmental science and pollution research international, volume 22, issue 1, pp. 68 – 102.

Pohorecka, K., Skubida, P., Semkiw, P., Miszczak, A., Teper, D., Sikorski, P., Zagibajło, K., Skubida, M., Zdańska, D., & Bober, A. (2013). Effects of exposure of honey bee colonies to neonicotinoid seed–treated maize crops, Journal of Apicultural Science, 57(2): 199-208. doi: https://doi.org/10.2478/jas-2013-0029.

Potts S., Fonseca V., Ngo H., Aizen M., Biesmeijer J., Breeze T., Dicks L., Garibaldi L., Hill R., Settele J., Vanbergen A. (2016). Safeguarding pollinators and their values to human well-being. Nature 540: 220-229.

Ru, Q. M., Feng, Q., & He, J. Z. (2013). Risk assessment of heavy metals in honey consumed in Zhejiang province, southeastern China. Food and chemical toxicology, 53, 256-262.

Rumkee, J. C., Becher, M. A., Thorbek, P., & Osborne, J. L. (2017). Modeling effects of honeybee behaviors on the distribution of pesticide in nectar within a hive and resultant in-hive exposure. Environmental Science & Technology, 51(12), 6908-6917.

Ruschioni, S., Riolo, P., Minuz, R. L., Stefano, M., Cannella, M., Porrini, C., & Isidoro, N. (2013). Biomonitoring with honeybees of heavy metals and pesticides in nature reserves of the Marche Region (Italy). Biological trace element research, 154(2), 226-233.

Sabatino L., Scordino M., Pantò V., Chiappara E., Traulo P., & Gagliano G. (2013) Survey of neonicotinoids and fipronil in corn seeds for agriculture. Food Additives & Contaminants: Part B, 6:1, 11-16, DOI: 10.1080/19393210.2012.717969.

Silici, S., Uluozlu, O. D., Tuzen, M., & Soylak, M. (2016). Honeybees and honey as monitors for heavy metal contamination near thermal power plants in Mugla, Turkey. Toxicology and industrial health, 32(3), 507-516.

Sivakoff F., Gardiner M. (2017). Soil lead contamination decreases bee visit duration at sunflowers. Urban ecosystems. 20 (6): 1221-1228.

Smith, K. E., Weis, D., Amini, M., Shiel, A. E., Lai, V. W. M., & Gordon, K. (2019). Honey as a biomonitor for a changing world. Nature Sustainability, 2(3), 223-232.

Sommeijer, M. J., De Rooy, G. A., Punt, W., & De Bruijn, L. L. M. (1983). A comparative study of foraging behavior and pollen resources of various stingless bees (Hym., Meliponinae) and honeybees (Hym., Apinae) in Trinidad, West-Indies. *Apidologie*, *14*(3), 205-224.

Tison L., Hahn M., Holtz S., Rößner A., Greggers U., Bischoff G., Menzel R. (2016). Honey bees' behavior is impaired by chronic exposure to neonicotinoid thiacloprid in the field. environmental science and technology. 50 (13): 7218-7227.

Van Der Steen, J. J., de Kraker, J., & Grotenhuis, T. (2012). Spatial and temporal variation of metal concentrations in adult honeybees (Apis mellifera L.). Environmental monitoring and assessment, 184(7), 4119-4126.

Waiker, P., Ulus, Y., Tsui, M. T. K., & Rueppell, O. (2022). Mercury accumulation in honey bees trends upward with urbanization in the USA. Agricultural & Environmental Letters, 7(2), e20083.

Wheeler, M. M., & Robinson, G. E. (2014). Diet-dependent gene expression in honey bees: honey vs. sucrose or high fructose corn syrup. Scientific reports, 4(1), 1-5.

Zawislak J., Adamczyk J., Johnson D.R., Lorenz G., Black J., Hornsby Q., Stewart S.D., Joshi N. (2019). Comprehensive survey of area-wide agricultural pesticide use in Southern United States row crops and potential impact on honey bee colonies. Insects. 10: 280; doi:10.3390/insects10090280.

CHAPTER TWO: ASSESSMENT OF SPATIAL VARIATION OF PESTICIDES AND HEAVY METAL RESIDUES IN HONEY BEE (*APIS MELLIFERA* L.) PRODUCTS

SUMMARY

Apis mellifera L. is considered one of the most important pollinators in nature. Unfortunately, along with other insect species, honey bee populations are decreasing at an alarming rate, urging researchers to investigate the causes and stressors that precipitated this decline.

This study focuses on chemical stressors that are found to affect bee populations. We used pollen and honey samples to examine the variation of pesticides and heavy metals in two different landscapes. Subsequently, we extrapolated the risk of these toxins' residues on *Apis* spp. based on current literature. We found no spatial variation in metal concentrations in pollen and honey samples collected from urban and agricultural areas. Likewise, we observed no spatial variation in pesticide concentrations in pollen and honey samples. Based on previous literature and comparing the residues of heavy metals and pesticides in our pollen and honey samples, we found that heavy metal residues in some honey and pollen likely pose a severe health risk to honey bees. Although levels of pesticide residues were below documented thresholds of risk, we consider the possibility of synergistic chemical impacts. Our findings support future efforts to investigate the health risks associated with multiple factor combinations.

INTRODUCTION

As pollinators, *Apis mellifera* Linnaeus (honey bees) are a vital part of the ecosystem, visiting more than 90% of the 107 leading global crop plants (Potts et al. 2016). However, the number of managed honey bee hives has decreased, and this reduction has become an international issue over the past two decades. Managed hives have reduced by 25% over the last 20 years in

Europe and 59% over the previous 58 years in North America (Struttmann, 2016). This is supported by further evidence documenting the decline of honey bee colonies in Europe since at least 1972 (Potts et al. 2016). In addition, the Food and Agriculture Organization of the United Nations (FAO) documents a broader frame of reference, including the years 1961-2007, during which honey bee colonies decreased in both Europe and North America (-49.5%) (FAO, 2009). Growing concern about the declining number of bees worldwide has prompted scientists and researchers to investigate the factors contributing to their demise.

One phenomenon associated with bee population decline is called Colony Collapse Disorder. This phenomenon is defined as a dead colony where most worker bees inexplicably disappear from the colony, leaving behind the queen, a few immature bees, and plentiful food (USDA 2020). Many stressors were found to escalate this phenomenon and have been classified into different categories based on their nature and origin: 1) biological stressors, including pathogens and parasites, such as deformed wing viruses and Varroa mites (Grozinger and Flenniken 2019, Goulson et al. 2015); 2) physical stressors including habitat fragmentation and the decline of foraging resources (Aizen and Feinsinger 1994) as well as climate change (Potts et al. 2010); 3) chemical stressors including pesticides (Mitchell et al. 2017, Tison et al. 2016, Pisa et al. 2015, Henry et al. 2012), fertilizers (Wernecke et al. 2019) and heavy metals (Polykretis et al. 2016, Monchanin et al. 2021); and 4) nutritional stressors including poor diet and inadequate beekeeping practices (Alaux et al. 2010, Goulson et al. 2015).

Pesticides have been extensively investigated and documented as the primary stressor affecting honey bees. Chief among them include neonicotinoids which, as neurotoxins, have a wide range of effects on pollinators, including 1) impairment of foraging behavior (Tison et al.

2016); 2) impairment of colony reproduction (Woodcock et al. 2017); 3) lethal damage to the nervous system (Fairbrother et al. 2014); and 4) inhibition of immunity (Brandt et al. 2016).

Another stressor apart from pesticides is heavy metals, although less is known about their effects on bee species relative to those of pesticides. However, an increasing number of studies have reported the relationship between the increase of heavy metal concentration in soil and plants and the decline of bee species diversity richness, health and foraging behavior (Moron et al. 2012, Hladun et al. 2016, Sivakoff & Gardiner, 2017).

Heavy metals are ubiquitous in the environment and are often amplified in the environment as a result of either natural events such as forest fires, volcanic emissions, and sea spray (Zhou et al. 2018) or through human activities such as industrial emissions, hydraulic fracturing, and coal-burning power plants (Aghamirlou et al. 2015).

In general, bees can encounter toxins by consuming contaminated nectar or pollen and/or through exposure to contaminated dust from direct spray or contacting contaminated surfaces (Pisa et al. 2015). Most research has focused on honey bee exposure to pesticides in agricultural settings because of the associated pesticide applications (Sabatino et al. 2013, Pohorecka et al. 2013, Castilhos et al. 2019, Zawislak et al. 2019). However, recent studies also document the exposure of honey bees to pesticides in urban areas (Sheldon et al. 2019, Sadowska et al. 2019). A few studies have considered the significance of the combinatorial implications of both pesticides and heavy metal residues in honey bees and their products (Naccari et al. 2014).

This study will assess the spatial variation of pesticide and heavy metal residues in pollen and honey. It will ascertain if there is a significant difference in the pesticides and heavy metal content in honey bee products collected from agricultural versus urban areas. It will also address the combinatorial risk to honey bees when exposed to these contaminants through pollen and

nectar consumption by relating our findings with previously documented toxicological evidence related to the isolated effects of pesticides and heavy metals.

MATERIALS AND METHODS

Study Sites and Site Selection

We surveyed 24 hives distributed in 7 counties in Northern Colorado. Pollen and honey samples were collected from 10 hives during the summer of 2019 and 14 hives during the summer of 2020. A total of 13 hives were in urban areas, while 11 hives were located in agricultural areas, as shown in Figure 1. Dates of sample collection and weight of each sample were reported in Appendix I, Table A1.

Sampling sites were selected based on how urban and agricultural areas are classified. According to the United States Census Bureau, urban areas are defined as continuously built-up areas with a population of 2,500-50,000 or more with an average density of at least 1,000 inhabitants per square mile (Census.gov, 2022), while agricultural land is defined by the Colorado General Assembly as “A parcel of land, whether located in an incorporated or unincorporated area and was used the previous two years and presently is used as a farm or ranch” (Leg.colorado.gov). We had to identify the landscape type using Google Earth, taking into consideration the observation that honey bees can fly for more than 3 km searching for food (Utaipanon et al. 2019). The difference between agricultural and urban sampling sites is demonstrated in Figure 2 and 3, which represent actual sampling sites.

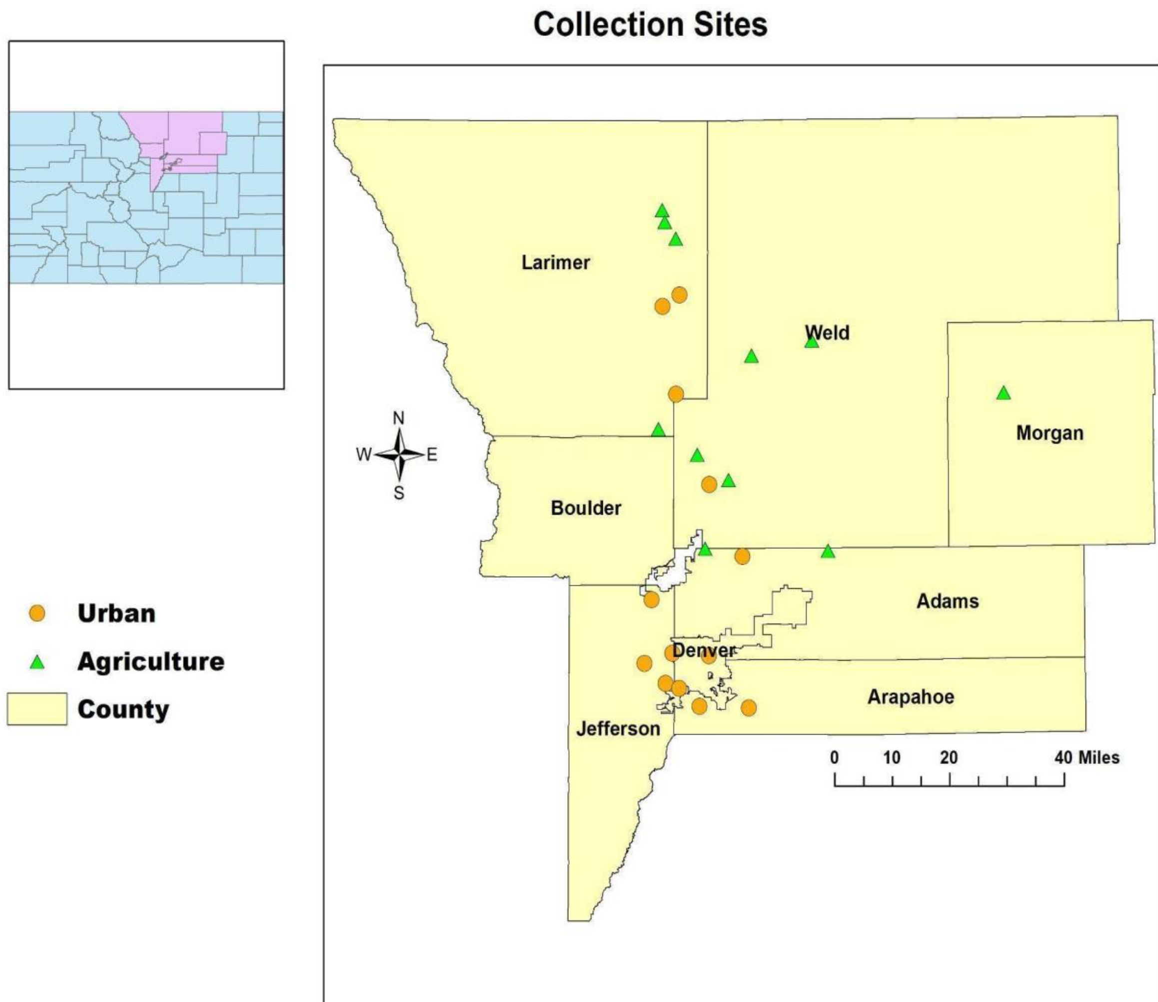


Figure 1: Hive sampling sites for pollen and honey in Northern Colorado, USA, 2019-2020.

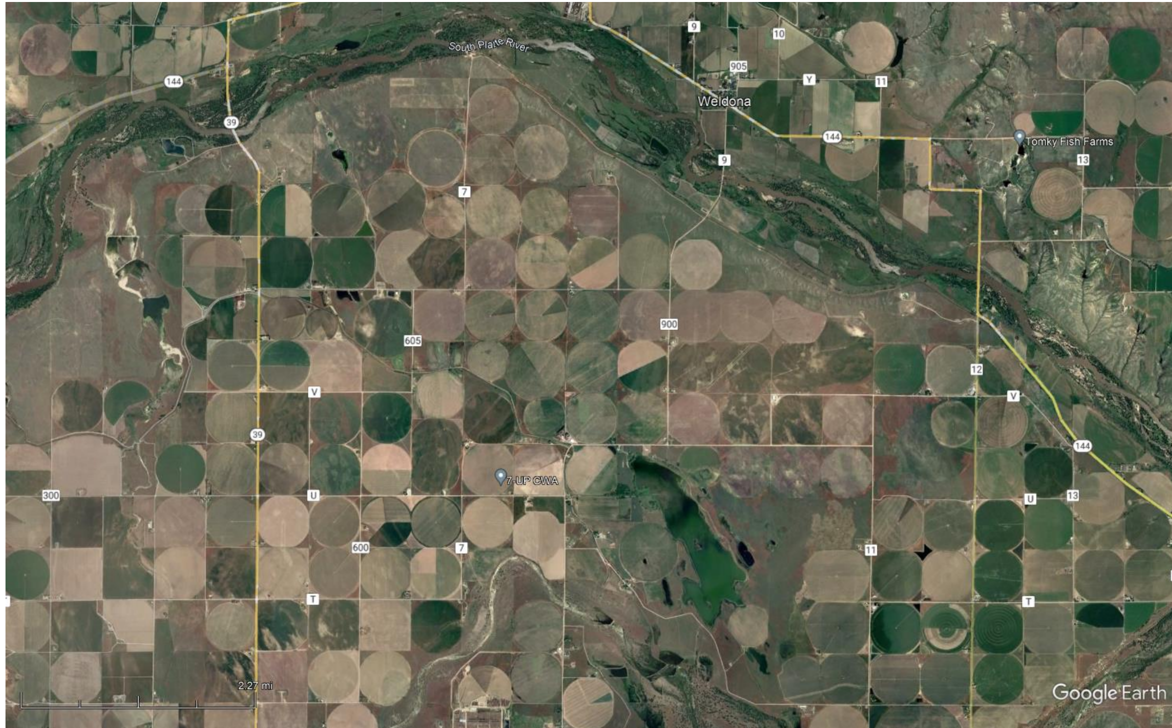


Figure 2: Example of an agricultural sampling site.

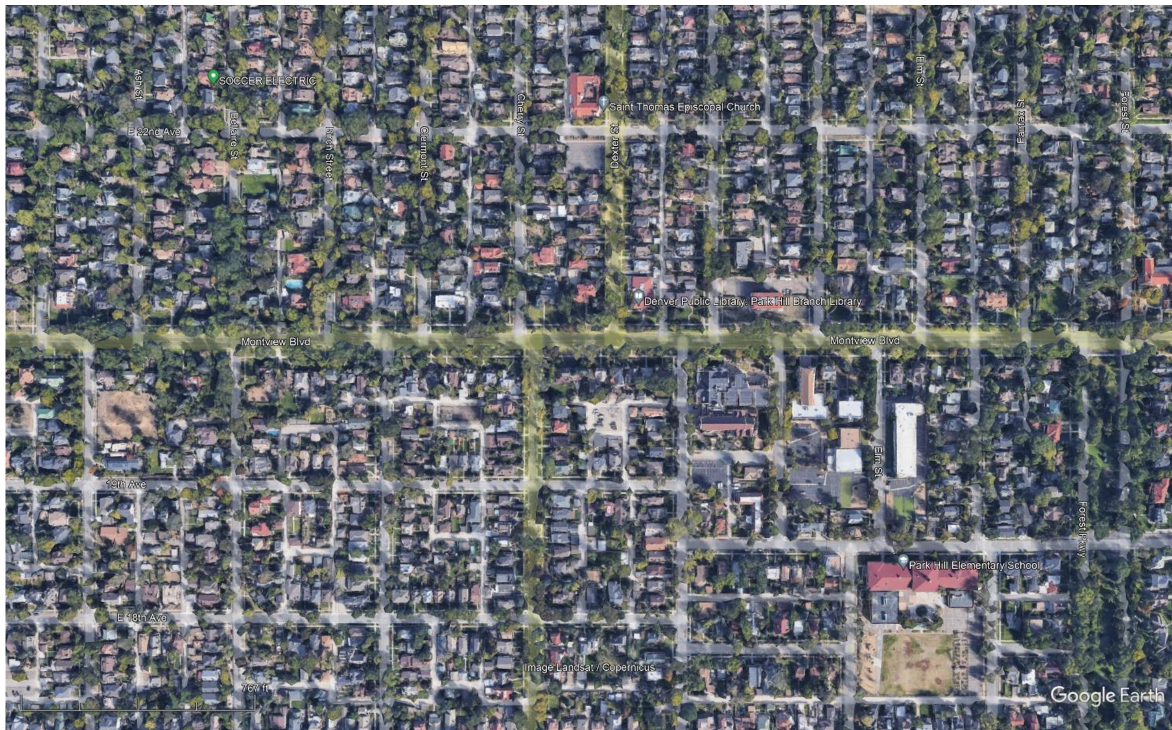


Figure 3: Example of an urban sampling site.

Sample Collection, Preparation, and Analysis

Pollen samples were collected using pollen traps (Bee Flower, Gyengbuk High-tech village, Korea) at the entrance of each hive, as shown in Figure 4. These traps force forager bees to enter the hive through a screen where they drop their pollen loads, which fall into the trap box. Pollen samples were collected from the trap boxes and stored in the laboratory at -20°C until analysis. Two to three samples were collected from each hive between the months of June and September in the years 2019 and 2020. Honey samples were collected from each site between September and October (during the harvest season) and stored in the laboratory at -20°C until analysis (Ruiz-Toledo et al. 2018).

Figure 4: Pollen trap fixed at the hive entrance.

Heavy Metals Analysis

Fifty-nine pollen samples and 21 honey samples were weighed (five grams of pollen and ten grams of honey for each sample), arranged in a box at room temperature, then submitted to the Soil, Water, and Plant Testing Laboratory, Colorado State University for analysis (Kilic Altun et al. 2017). Heavy metals analysis was performed to detect the residues of Arsenic (As), Cadmium (Cd), Lead (Pb), and Selenium (Se) heavy metals using the Nitric and Perchloric Acids method (Soltanpour et al. 1982).

Pesticide Analysis

We gathered a total of 61 pollen samples and 21 honey samples. Five grams of pollen and ten grams of honey were taken from each sample, then samples were arranged in a cooler box at ~ 4° C (Ruiz-Toledo et al. 2018) and shipped overnight to the Chemical Ecology Core Facility at Cornell University (Ithaca, New York). Pesticide analysis was performed to detect the residues of 92 types of pesticides, including some metabolites and breakdown products using the EN 15662 QuEChERS procedure (European Committee for Standardization 2018) by Liquid Chromatography-Mass Spectrometry (LC-MS/MS), as shown Appendix II, Table A2.

STATISTICAL ANALYSIS

Spatial differences in pesticides and heavy metal concentrations were assessed by comparing the mean values of the entire study period between locations. Descriptive statistics (means, standard deviations, and median) were calculated from all analyzed samples. When a compound was below the limit of detection (< LOD), the concentration of half LOD was used for statistical analysis (EPA 2000). Multiple t-tests analysis was performed to compare between samples from

agricultural settings versus urban ones using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered to be statistically significant.

RESULTS

Heavy Metals

Differences were observed in the concentrations of heavy metals (As, Pb, Cd, and Se) detected in pollen samples collected from agricultural versus urban areas. The mean concentration of Se and As detected in pollen samples was 1.16 and 1.08 times higher in agricultural sites than it was in urban sites. The mean concentration of Cd and Pb detected in pollen samples was 1.05 and 1.62 times higher in urban sites than it was in agricultural sites. Table 1 summarizes the statistical data of heavy metal concentrations in pollen samples collected from hives in agricultural and urban areas.

Figure 5 represents boxplots with Tukey whiskers showing concentrations (ppb) of As, Cd, Pb, and Se detected in pollen samples collected in urban or agricultural locations. Overall, no significant variation was observed for these metals in pollen samples in all sites (all with $p > 0.05$).

Table 1. Statistical data summary of heavy metals detected in pollen samples.

Heavy Metal	Agriculture	Urban	Multiple t-test			
	Mean (ppb)	Mean (ppb)	± SEM*	P-value	t	df
As	377	347	159.5	0.99	0.009	20

Cd	174	182	21.91	0.99	0.012	20
Pb	333	540	144.8	0.16	1.432	21
Se	1307	1124	378.4	0.63	0.483	21

* SEM: Standard Error of the Mean

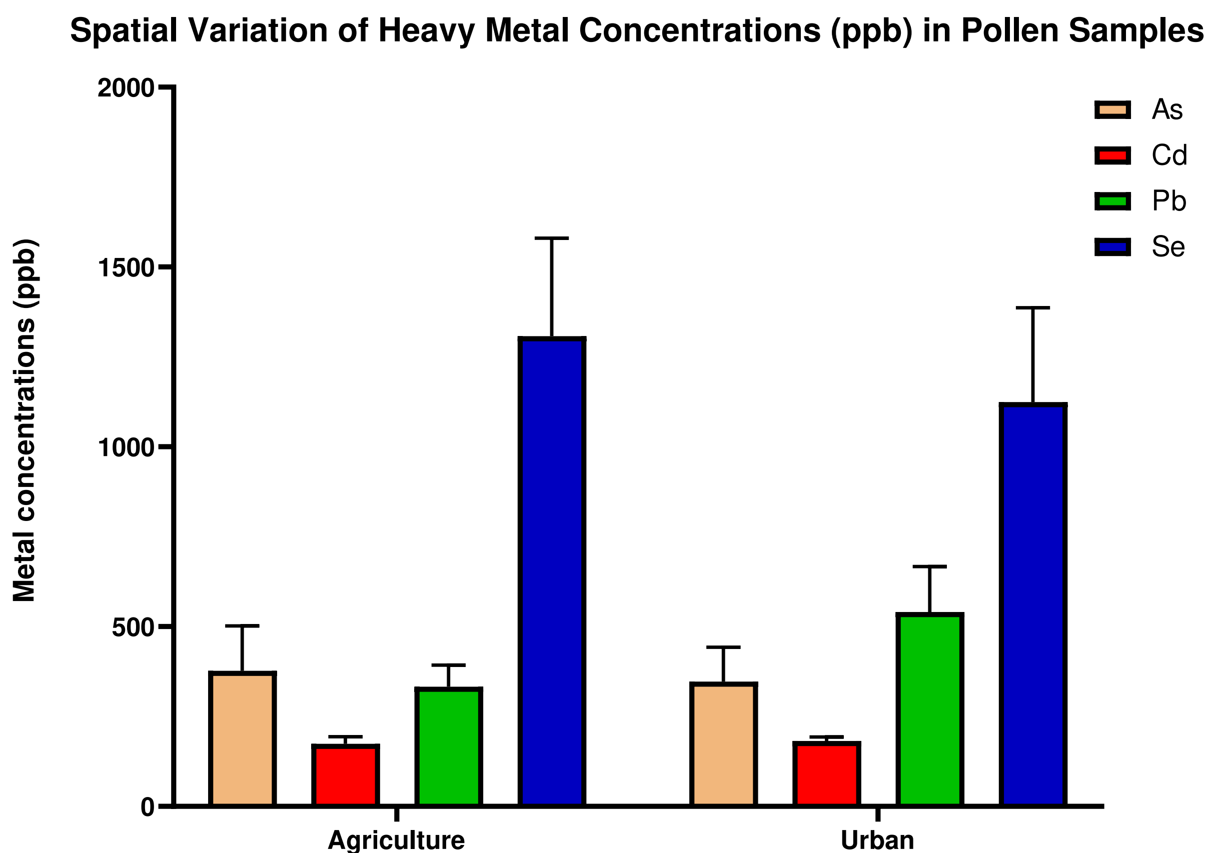


Figure 5. Mean and SEM of heavy metal (As, Cd, Pb, and Se) concentrations (ppb) in pollen samples. Pollen samples are identified as originating in either urban or agricultural locations. Significant differences were determined using multiple t-tests.

Variation was observed in the concentrations of heavy metals (As, Pb, Cd, and Se) detected in honey samples collected from agricultural versus urban areas. The mean concentration of As, Cd and Pb detected in honey samples was 1.12, 1.15 and 1.31 times higher in agricultural sites

than it was in urban sites, respectively. The mean concentration of Se detected in honey samples was 1.04 and 1.62 times higher in urban sites than it was in agricultural sites. Table 2 summarizes the statistical data of heavy metals detected in honey samples collected from hives located in agricultural and urban areas. Figure 5 represents boxplots with Tukey whiskers showing concentrations (ppb) of As, Cd, Pb, and Se detected in honey samples identified as originating in urban versus agricultural locations. Overall, no significant variation was observed between urban and agricultural locations for these metals in honey samples (all with $p > 0.05$).

Table 2. Statistical data summary of heavy metals detected in honey samples.

Heavy Metal	Agriculture	Urban	Multiple t-test			
	Mean (ppb)	Mean (ppb)	± SEM	P-value	t	df
As	271	188	148.9	0.46	0.7423	18
Cd	53	36	30.67	0.60	0.5296	19
Pb	539	409	174.0	0.64	0.4628	19
Se	1061	1006	± 500.4	0.91	0.1086	19

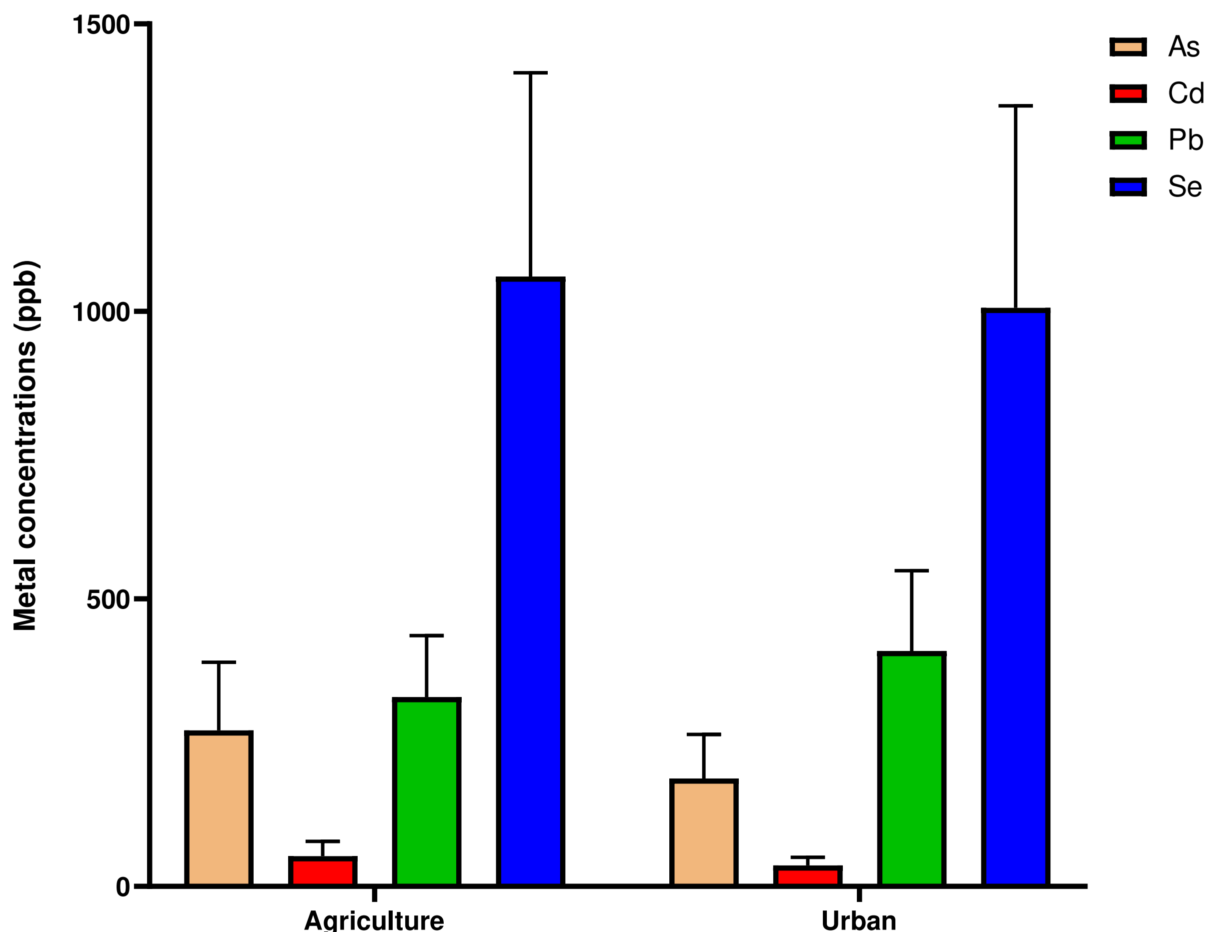


Figure 6. Mean and SEM of heavy metal (As, Cd, Pb, and Se) concentrations (ppb) were detected in honey samples. Honey samples are identified as originating from either urban or agricultural locations. Significant differences were determined using multiple t-tests.

Pesticides

A total of 61 pollen samples were collected and analyzed for the presence of 92 different pesticides. Sixty-four pesticide types were not detected in pollen samples or were less than the limit of quantitation. Of the 38 chemicals that were detected, 15 were fungicides, 13 were insecticides, 7 were herbicides, 2 were acaricides, and 1 was a pesticide synergist. Chlorpyrifos, Atrazine, Diuron, and Metconazole were observed at the highest levels among pollen samples collected from agricultural areas with mean concentrations of 17.3, 3.44, 3.21, and 1.74 ppb,

respectively. Among samples collected from urban locations, Triphenylmethyl, Chlorpyrifos, Carbaryl, and Chlorantraniliprole were observed at the highest levels with mean concentrations of 105.24, 26.01, 16.28, and 11.06 ppb, respectively. Table A3, Appendix III shows the summary of statistical data for pesticide residues detected in pollen samples. Figure 7 provides the mean and standard error of mean of pesticide concentrations observed in pollen samples collected from urban and agricultural areas. Overall, no significant variation was observed for these pesticides in pollen samples in all areas (all with $p > 0.05$).

Twenty-one honey samples were collected from hives located in urban versus agricultural landscapes and analyzed for 92 different pesticide residues. Seventy-six pesticide types were not detected in honey samples or were less than the limit of quantitation. However, 16 chemicals were detected in honey samples, of which nine were insecticides, three were herbicides, two were fungicides, one was an acaricide, and one was a pesticide synergist. Coumaphos, Piperonyl butoxide, and Tebuconazole were observed at the highest concentrations in honey samples collected from agricultural areas with mean concentrations of 0.22, 0.11, and 0.11 ppb respectively. Coumaphos, Acephate, and 2,4-DMPF were observed at the highest concentrations in honey samples collected from urban areas with mean concentrations of 1.09, 0.93, and 0.64, respectively. Table 4, Appendix IV shows the summary of statistical data for pesticide residues detected in honey samples. Figure 8 shows the mean and standard error of mean of different pesticide concentrations (ppb) found in honey samples collected from urban and agricultural areas. Overall, significant variation in pesticides concentrations was observed in honey samples collected from urban and agricultural areas (all with $p < 0.05$).

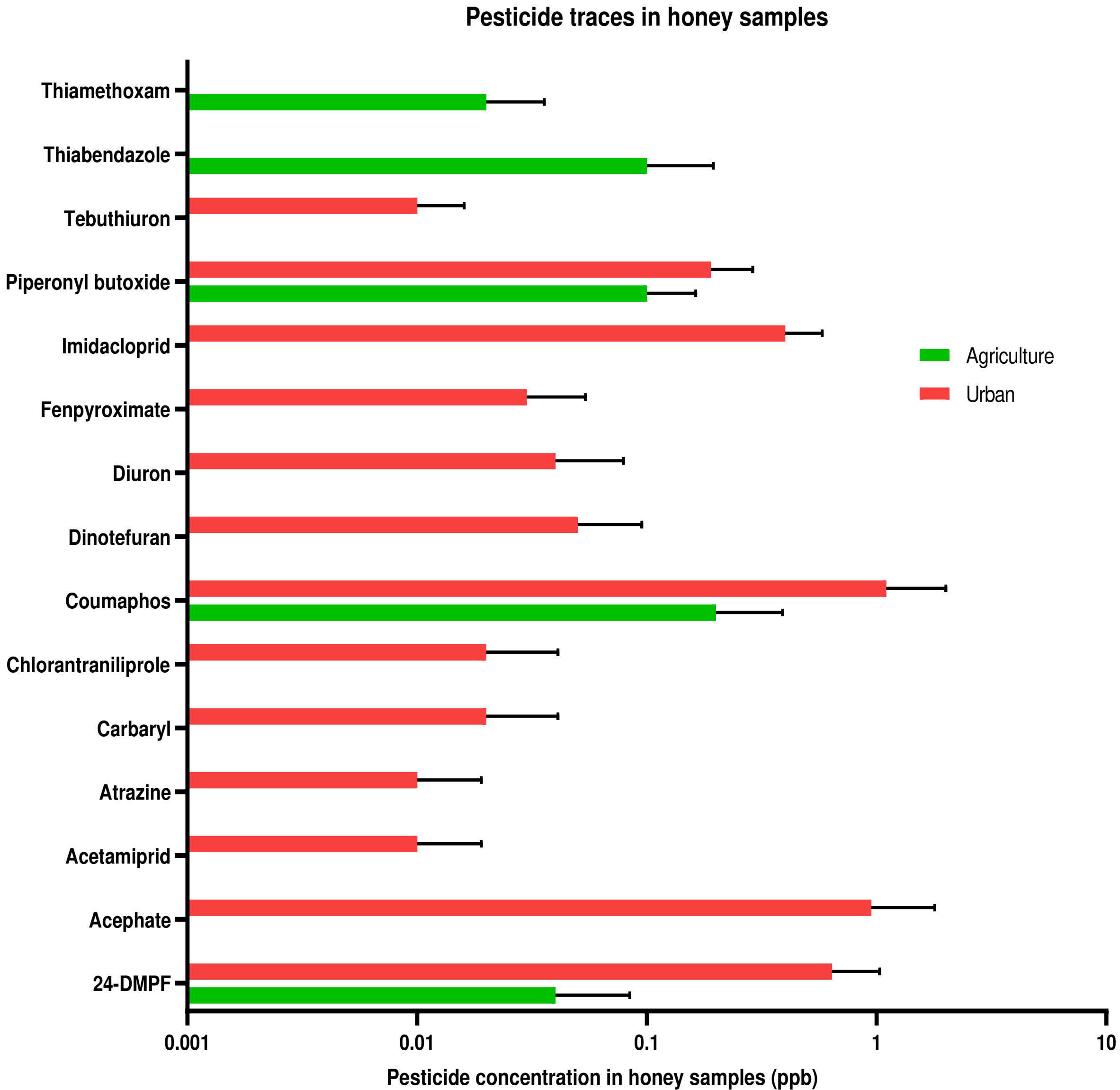


Figure 7. Mean and SEM concentrations (ppb) of different pesticide residues were detected in honey samples collected from urban and agricultural areas. Significant differences were determined using multiple t-tests.

RISK ASSESSMENT

To determine the risk level that honey bee populations may be exposed to, we compared the data for concentrations of heavy metals and pesticides detected in pollen and honey samples in this study to previously reported concentrations for lethal and chronic health impacts of pesticides and heavy metals on honey bees.

Heavy metals

In Table 3, we compare the results of heavy metal concentrations in pollen and honey samples with previously reported concentrations associated with lethal or chronic health impacts of heavy metals.

Table 3: Effect of different concentrations of heavy metals on honey bees, compared with the concentrations we detected in pollen and honey samples.

Metal	Matrix Conc. range (ppb)		Metal conc./ range in other research (ppb)	Effect	Reference
	Pollen	Honey			
As	1-1243	1-1280	10-50	Slow down learning and reduce long-term memory	Monchanin et al. (2020)
			3000	Lethal	Knowlton et al. (1950)
Pb	5-624	1-1168	60	Slow down learning and reduce long-term memory	Monchanin et al. (2020)
			1120- larvae 345,000- foragers	Lethal	Di et al. (2016)
Cd	79-258	1-298	275- larvae 78,000- foragers	Lethal	Di et al. (2016)
			100-1000	Immuno-competence reduction	Polykretis et al. (2016)
Se	20-7460	1-3491	500-700	Disrupt foraging behavior	Hladun et al. (2012)

Pesticides

In Table 4, we compare the results of pesticide concentrations in pollen and honey samples with previously reported concentrations associated with lethal or chronic health impacts of pesticides.

Table 4: Effect of different concentrations of pesticides on honey bees, compared with the concentrations we detected in honey and pollen samples.

Pesticide	Matrix Avg. of conc. (ppb)		Pesticide conc./ range in other research (ppb)	Effect	Reference
	Pollen	Honey			
Coumaphos	0.2	1.1	$1 X10^{6}$	Failure of queen development	Pettis et al (2004)
Acephate	ND	0.93	$15X10^{5}$	Lethal	Nakar et al. (2017)
			6970	Inhibit detoxification enzyme	Yao et al. (2018)
Chlorpyrifos	17.30-26.01	ND	$25 X10^{5}$	Lethal	Nakar et al. (2017)
Imidacloprid	0.14-1.34	0.37	50 25	Lethal Negatively affect development and behavior	Abbo et al. (2017)
			2-3	Negatively affect the development of the hypopharyngeal glands (HPGs) and respiratory rhythm	Hatjina et al. (2013)
Atrazine	2.03-3.44	0.01	46,700-	Lethal	Sonnet et al.

			65,300		(1978)
Tebuconazole	0.38-3.77	0.11-11.83	51	Lethal	Wang et al. (2022)

DISCUSSION

Honey bees and their products (pollen, honey, and wax) have been widely used as a bioindicator for environmental pollution, either through the accumulation of different toxins in their products or through the high mortality rates caused by these toxins (Celli and Maccagnani 2003). Most research has focused on pesticides as major chemical stressors that increase risk for the phenomenon of Colony Collapse Disorder (VanEngelsdrop et al. 2009, Frazier et al. 2011). Studies by Fairbrother et al. (2014), Zhu et al. (2019) and Demares et al. (2022) investigated a variety of risks associated with honey bees' exposure to pesticides, either inside hives or during foraging. Although there were confirming results of the effect of heavy metals on honey bees' survival (Di et al. 2016), memory, and foraging behavior (Monchanin et al. 2020), few studies examined the contribution of heavy metals in bringing about the Colony Collapse phenomenon. The presence of pesticides and heavy metals in honey bees and their products, simultaneously, can alert investigators to the possibility of combinatorial impacts of these toxins and that may exacerbate the risks of harm to bee colonies. The primary objective of this study was to determine the spatial variation of pesticides and heavy metals within pollen and honey samples.

A wide range of heavy metal residues (As, Pb, Cd, and Se) was observed in pollen and honey samples collected from urban and agricultural landscapes. Based on our data, the lowest and highest (As) concentrations in honey samples collected from different landscapes were 1 ppb and 1280 ppb, respectively. For all metals, there were no significant differences in mean concentrations detected in honey and pollen samples collected from different locations. Thus, there

was no spatial variation in metal concentrations in pollen and honey samples collected from urban and agricultural areas.

Based on our data, there were no significant differences in mean pesticide concentrations detected in pollen and honey samples collected from urban and agricultural locations. Thus, there was no spatial variation in pesticide concentrations in pollen and honey samples collected from urban and agricultural areas.

Most bee-health research has focused on the effect of insecticides, such as neonicotinoids, by reporting their immediate toxicity and close connection between these insecticides and the corresponding impacts on bee populations (Frazier et al. 2011, Hatjina et al. 2013, Fairbrother et al. 2014, Woodcock et al. 2017). Fewer studies have reported the effects of other pesticides like herbicides, acaricides, and fungicides (Johnson et al. 2013), or different types of adjuvant chemicals that are used along with pesticides like piperonyl butoxide (PBO). Piperonyl butoxide is a pesticide synergist used in combination with insecticides to enhance their active properties by inhibiting insect detoxification activity. Although the effect of PBO on different organisms has been reported [for example, reduction of developmental and behavioral orientation in mice (Tanaka 1992) and lethality to cotton whitefly (Devine and Denholm,1998)], PBO effects on honey bees are typically examined for its combinatorial impacts when added to other compounds (Zhu et al. 2019). For example, the application of PBO with methyl benzoate has been shown to decrease the orientation and flight ability of bees (Zhu et al. 2019).

Synergistic effects of some pesticide mixtures on bee health were reported, where the toxicity of some pesticides was enhanced by the presence of another one, such as in Johnson et al. (2009), where tau-ßuvalinate toxicity increased with coumaphos application. Sometimes,

protective beekeeping practices to save bee colonies expose bees to interactive toxins. Applying different types of acaricide and fungicide at the same time to control Varroa mite and bacterial infection can interact and produce higher toxicity to bee populations (Johnson et al. 2013). Pesticide synergism can also reduce bees' detoxification ability, which in turn increases their sensitivities to environmental toxins (Berenbaum & Johnson 2015). There has been less focus on the synergistic effect of pesticide- heavy metal combination or the synergistic effects of multiple stressors.

Based on the findings of this study, and by comparison with previously published findings, heavy metal levels observed in some pollen and honey samples pose severe risks to honey bees, whereas pesticide levels were observed to be below the established levels of risk for honey bee health. However, future risk-based studies will be necessary to consider the potential for combinatorial effects resulting from the interaction of pesticides with heavy metals. The inclusion of other factors that exacerbate health risks such as the presence of pathogens, variables of climate change, loss of foraging habitat, etc. may, likewise, reveal lower thresholds relative to established concentrations for risk levels.

Most of the pesticide residues detected were below established levels of risk. However, the detected levels of heavy metals in some honey and pollen samples posed a lethal or acute risk to honey bees. This investigation sets the stage for future studies to explore the effects of exacerbating combinatorial variables that can impact the health or loss of honey bee populations.

LITERATURE CITED

Abbo, P. M., Kawasaki, J. K., Hamilton, M., Cook, S. C., DeGrandi-Hoffman, G., Li, W. F., ... & Chen, Y. P. (2017). Effects of Imidacloprid and Varroa destructor on survival and health of European honey bees, Apis mellifera. Insect science, 24(3), 467-477.

Aghamirlou H., Khadem M., Rahmani A., Sadeghian., Mahvi A., Akbarzadeh A., Nazmara S. (2015). Heavy metals determination in honey samples using inductively coupled.

Aizen MA, Feinsinger P (1994). Habitat fragmentation, native insect pollinators, and feral honey bees in Argentine Chaco Serrano. Ecological Applications. 4(2): 378-392

Alaux C., Ducloz F., Crauser D., Le Conte Y. (2010). Diet effects on honeybee immunocomptence. Biology letters. 6 (4): 562-565

Berenbaum, May R., and Reed M. Johnson. "Xenobiotic detoxification pathways in honey bees." Current opinion in insect science 10 (2015): 51-58.

Brandt, A., Gorenflo, A., Siede, R., Meixner, M., & Büchler, R. (2016). The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). Journal of insect physiology, 86, 40-47.

Castilhos D., Dombroski J.L.D., Bergamo G.C., Gramacho K., Goncalves L.S. (2019). Neonicotinoids and fipronil concentrations in honeybees associated with pesticide use in Brazilian agricultural areas. Apidologie 50: 657–668. Retrieved from https://doi.org/10.1007/s13592-019-00676-x

Celli, G., & Maccagnani, B. (2003). Honey bees as bioindicators of environmental pollution. Bulletin of Insectology, 56(1), 137-139.

Colorado General Assembly (2022, September 14). Colorado revised statutes 2016. Retrieved from: https://leg.colorado.gov/sites/default/files/images/olls/crs2016-title-39.pdf

Devine, G. J., & Denholm, I. (1998). An unconventional use of piperonyl butoxide for managing the cotton whitefly, Bemisia tabaci (Hemiptera: Aleyrodidae). Bulletin of Entomological Research, 88(6), 601-610.

Di, N., Hladun, K. R., Zhang, K., Liu, T. X., & Trumble, J. T. (2016). Laboratory bioassays on the impact of cadmium, copper and lead on the development and survival of honeybee (*Apis mellifera* L.) larvae and foragers. Chemosphere, 152, 530-538.

European Committee for Standardization. (2018). Foods of Plant Origin–Multimethod for the Determination of Pesticide Residues using GC–and LC–Based Analysis following Acetonitrile Extraction/Partitioning and Clean-up by Dispersive SPE–Modular QuEChERS–Method.

Fairbrother A., Purdy J., Anderson T., Fell R. (2014). Risks of neonicotinoid insecticides to honeybees. Environmental toxicology and chemistry. 33 (4). Retrieved from: https://doi.org/10.1002/etc.2527

Food and Agriculture Organization of the United Nations (FAO), 2009. FAOSTAT. <http://faostat.fao.org>.

Frazier, J., Mullin, C., Frazier, M., & Ashcraft, S. (2011). Pesticides and their involvement in colony collapse disorder. Am Bee J, 151, 779-781.

Goulson D., Nicholls E., Botias C., Rotheray E. (2015). Bee declines driven by combined stress from parasites, pesticides and lack of flowers. Science. 347: 1255957

Grozinger CM, Flenniken ML. (2019). Bee viruses: ecology, pathogenicity and impacts. Annual review of entomology. 64:205–226.

Hatjina, F., Papaefthimiou, C., Charistos, L., Dogaroglu, T., Bouga, M., Emmanouil, C., & Arnold, G. (2013). Sublethal doses of imidacloprid decreased size of hypopharyngeal glands and respiratory rhythm of honeybees in vivo. Apidologie, 44(4), 467-480.

Henry M., Beguin M., Requier F., Rollin O., Odoux J.F., Aupinel P., Aptel J., Tchamitchian S., Decourtye A. (2012). A common pesticide decreases foraging success and survival in honey bees. Science. 336 (6079): 348-350.

Hladun, K. R., Di, N., Liu, T. X., & Trumble, J. T. (2016). Metal contaminant accumulation in the hive: Consequences for whole-colony health and brood production in the honey bee (*Apis mellifera* L.). Environmental toxicology and chemistry, 35(2), 322-329.

Hladun, K. R., Smith, B. H., Mustard, J. A., Morton, R. R., & Trumble, J. T. (2012). Selenium toxicity to honey bee (*Apis mellifera* L.) pollinators: effects on behaviors and survival. PloS one, 7(4), e34137.

Johnson, R. M., Dahlgren, L., Siegfried, B. D., & Ellis, M. D. (2013). Acaricide, fungicide and drug interactions in honey bees (*Apis mellifera*). PloS one, 8(1), e54092.

Johnson, R. M., Pollock, H. S., & Berenbaum, M. R. (2009). Synergistic interactions between in-hive miticides in Apis mellifera. Journal of economic entomology, 102(2), 474-479.

Kessler S., Tiedeken E., Simcock K., Derveau S., Mitchell J., Softley S., Radcliffe A, Stout J., Wright G.A. (2015). Bees prefer foods containing neonicotinoid pesticides. Nature. 521: 74–76.

Kılıç Altun, S., Dinç, H., Paksoy, N., Temamoğulları, F. K., & Savrunlu, M. (2017). Analyses of mineral content and heavy metal of honey samples from south and east region of Turkey by using ICP-MS. International Journal of Analytical Chemistry, 2017.

Knowlton, G. F., Sturtevant, A. P., & Sorenson, C. J. (1950). Adult honey bee losses in Utah as related to arsenic poisoning. Utah State Univ., Agric. Exp. Stn., Bull. ;(United States), 340.

Mitchell E., Mulhauser B., Mulot M., Mutabzi A., Glauser G., Abei A. (2017). A world survey of neonicotinoids in honey. Science. 358: 109-11.

Monchanin, C., Drujont, E., Devaud, J. M., Lihoreau, M., & Barron, A. B. (2021). Metal pollutants have additive negative effects on honey bee cognition. Journal of Experimental Biology, 224(12), jeb241869.

Moron D., Grzes I., Skroka P., Szentgyorgyl H., Laskowski R., Potts S., Woyciechowski M. (2012). Abundance and diversity of wild bees along gradients of heavy metals pollution. Journal of applied ecology. 49: 118-125.

Naccari, C., Macaluso, A., Giangrosso, G., Naccari, F., & Ferrantelli, V. (2014). Risk assessment of heavy metals and pesticides in honey from Sicily (Italy). Journal of Food Research, 3(2), 107.

Pettis, J. S., Collins, A. M., Wilbanks, R., & Feldlaufer, M. F. (2004). Effects of coumaphos on queen rearing in the honey bee, *Apis mellifera*. Apidologie, 35(6), 605-610.

Pisa, L.W., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Downs, C.A., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., McField, M., Morrissey, C.A.; Noome, D.A.; Settele, J., Simon-Delso, N., Stark, J.D., Van der Sluijs, J.P., Van Dyck, H., Wiemers, M. (2015). Effects of neonicotinoids and fipronil on non-target invertebrates. Environmental science and pollution research international, volume 22, issue 1, pp. 68 – 102.

Pohorecka, K., Skubida, P., Semkiw, P., Miszczak, A., Teper, D., Sikorski, P., Zagibajło, K., Skubida, M., Zdańska, D., & Bober, A. (2013). Effects of exposure of honey bee colonies

to neonicotinoid seed–treated maize crops, Journal of Apicultural Science, 57(2): 199-208. doi: https://doi.org/10.2478/jas-2013-0029.

Polykretis, P., Delfino, G., Petrocelli, I., Cervo, R., Tanteri, G., Montori, G., ... & Gulisano, M. (2016). Evidence of immunocompetence reduction induced by cadmium exposure in honey bees (*Apis mellifera*). Environmental Pollution, 218, 826-834.

Potts S., Biesmeijer J., Kremen C., Neumann P., Schweiger O., Kunin W. (2010). Global pollinator declines: Trends, impacts and drivers. Trends in ecology and evolution. 25 (6): 345-53.

Potts S., Fonseca V., Ngo H., Aizen M., Biesmeijer J., Breeze T., Dicks L., Garibaldi L., Hill R., Settele J., Vanbergen A. (2016). Safeguarding pollinators and their values to human well-being. Nature 540: 220-229.

Ratnakar, V., Koteswara, R., Sridevi, D., & Vidyasagar, B. (2017). Contact toxicity of certain conventional insecticides to European honeybee, *Apis mellifera* Linnaeus. International Journal of Current Microbiology and Applied Sciences, 6(8), 3359-3365.

Ruiz-Toledo, J., Vandame, R., Castro-Chan, R. A., Penilla-Navarro, R. P., Gómez, J., & Sánchez, D. (2018). Organochlorine pesticides in honey and pollen samples from managed colonies of the honey bee *Apis mellifera* Linnaeus and the stingless bee Scaptotrigona mexicana Guérin from Southern, Mexico. *Insects*, *9*(2), 54.

Sabatino L., Scordino M., Pantò V., Chiappara E., Traulo P., & Gagliano G. (2013) Survey of neonicotinoids and fipronil in corn seeds for agriculture. Food Additives & Contaminants: Part B, 6:1, 11-16, DOI: 10.1080/19393210.2012.717969.

Sadowska, M., Gogolewska, H., Pawelec, N., Sentkowska, A., & Krasnodębska-Ostręga, B. (2019). Comparison of the contents of selected elements and pesticides in honey bees with regard to their habitat. Environmental Science and Pollution Research, 26(1), 371-380.

Sheldon, M., Pinion Jr, C., Klyza, J., & Zimeri, A. M. (2019). Pesticide Contamination in Central Kentucky Urban Honey: A Pilot Study. Journal of Environmental Health, 82(1).

Sivakoff F., Gardiner M. (2017). Soil lead contamination decreases bee visit duration at sunflowers. Urban ecosystems. 20 (6): 1221-1228.

Soltanpour, P.N., J. Benton Jones, Jr., and S.M Workman. Optical Emission Spectroscopy. in Methods of Soil Analysis. Part 2. A.L. Page, et al., ed., pp29-65, 1982.

Sonnet, P. E., Lye, T. L., & Sackett, R. R. (1978). Effects of selected herbicides on the toxicity of several insecticides to honey bees. Environmental Entomology, 7(2), 254-256.

Struttmann N. (2016). The complex causes of worldwide bee declines. Public library of science. Retrieved from https://phys.org/news/2016-01-complex-worldwide-bee-declines.html.

Tison L., Hahn M., Holtz S., Rößner A., Greggers U., Bischoff G., Menzel R. (2016). Honey bees' behavior is impaired by chronic exposure to neonicotinoid thiacloprid in the field. environmental science and technology. 50 (13): 7218-7227.

Toyohito, T. (1992). Effects of piperonyl butoxide on F1 generation mice. Toxicology letters, 60(1), 83-90.

United States Census Bureau. (2022, September 14). The Urban and Rural Classifications. Retrieved from: https://www2.census.gov/geo/pdfs/reference/GARM/Ch12GARM.pdf

United States. Environmental Protection Agency. Office of Pesticide Programs. (2000). Assigning values to non-detected/non-quantified pesticide residues in human health food exposure assessments. Office of Pesticide Programs, US Environmental Protection Agency.

USDA ARS. (2020, June 5). ARS Honey Bee health. Retrieved from: https://www.ars.usda.gov/oc/br/ccd/index/

Utaipanon, P., Holmes, M. J., Chapman, N. C., & Oldroyd, B. P. (2019). Estimating the density of honey bee (*Apis mellifera*) colonies using trapped drones: area sampled and drone mating flight distance. Apidologie, 50(4), 578-592.

VanEngelsdorp, Dennis, Jay D. Evans, Claude Saegerman, Chris Mullin, Eric Haubruge, Bach Kim Nguyen, Maryann Frazier et al. "Colony collapse disorder: a descriptive study." PloS one 4, no. 8 (2009): e6481.

Wang, F., Wang, Y., Li, Y., Zhang, S., Shi, P., Li-Byarlay, H., & Luo, S. (2022). Pesticide residues in beebread and honey in *Apis cerana* cerana and their hazards to honey bees and human. Ecotoxicology and Environmental Safety, 238, 113574.

Wernecke, A., Frommberger, M., Forster, R., & Pistorius, J. (2019). Lethal effects of various tank mixtures including insecticides, fungicides and fertilizers on honey bees under laboratory, semi-field and field conditions. Journal of Consumer Protection and Food Safety, 14(3), 239-249.

Woodcock B.A., Bullock J.M., Shore R.F., Heard M.S., Pereira M.G., Redhead J., Ridding L., Dean H., Sleep D., Henrys P., Peyton J., Hulmes S., Hulmes L., Sarospataki M., Saure C., Edwards M., Generrsch E., Knabe S., Pywell R.F. (2017). Country-specific effects of neonicotinoid pesticides of honey bees and wild bees. Science. 365(6345): 1393-1395.

Woodcock, B. A., Bullock, J. M., Shore, R. F., Heard, M. S., Pereira, M. G., Redhead, J., Redding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Sarospataki, M., Saure, C., Edwards, M., Genersch, E., Knab, S., & Pywell, R. F. (2017). Country-specific effects of neonicotinoid pesticides on honey bees and wild bees. Science, 356(6345), 1393-1395.

Yao, J., Zhu, Y. C., Adamczyk, J., & Luttrell, R. (2018). Influences of acephate and mixtures with other commonly used pesticides on honey bee (*Apis mellifera*) survival and detoxification enzyme activities. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 209, 9-17.

Zawislak J., Adamczyk J., Johnson D.R., Lorenz G., Black J., Hornsby Q., Stewart S.D., Joshi N. (2019). Comprehensive survey of area-wide agricultural pesticide uses in Southern United States row crops and potential impact on honey bee colonies. Insects. 10: 280; doi:10.3390/insects10090280.

Zhou X., Taylor M., Davies P., Prasad S. (2018). Identifying sources of environmental contamination in European honey bees (*Apis mellifera*) using trace elements and lead isotopic compositions. Environmental science and technology. 52:991-1001.

Zhu, Y. C., Wang, Y., Portilla, M., Parys, K., & Li, W. (2019). Risk and toxicity assessment of a potential natural insecticide, methyl benzoate, in honey bees (*Apis mellifera* L.). Insects, 10(11), 382.

CHAPTER THREE: IMPACTS OF SPATIAL VARIATION ON POLLEN TAXONOMIC PREFERENCES OF HONEY BEES

SUMMARY

Malnutrition of honey bees plays a significant role in a hive's deterioration. Therefore, understanding the floral resources that bees use in different environments, and their preferred foraging strategies at the colony level are essential to promoting bee health and aiding bee colony survival. In this study, we assessed the influence of landscape heterogeneity on pollen diversity by investigating the botanical and geographical origins of bee pollen collected from different land use areas in Colorado. We also estimated the most abundant botanical families that will identify honey bees' protein-source preferences. The results indicated a spatial variation in Shannon-Weaver diversity, demonstrating a higher diversity index with a wide variety of pollen taxa collected from urban sites compared to the agricultural ones with a lower diversity index with less pollen taxonomic variety.

INTRODUCTION

Honey bees (*Apis mellifera* L.) are one of the major groups of pollinators that aid plants in reproduction by collecting and transferring pollen from one flower to another. However, bees also collect pollen for its high nutritive value, as it is composed of a rich profile of amino acids, lipids, fatty acids, carbohydrates, phenolic compounds, minerals, and vitamins (Campos et al. 2008) and is considered an essential protein source for colony growth (Haydak 1970).

Besides chemical stressors that trigger the decline of bee populations, poor nutrition is another contributing factor to hive’s decline. Malnutrition compromises the viability of bees' immune systems, thereby reducing their resilience to exposure to environmental toxins such as pesticides (Wheeler and Robinson 2014). Although nectar is the primary nutritional source of bees,

extreme conditions of food shortage can dramatically change bees' behavior, including cannibalistic patterns of feeding upon their larvae (Brodschneider and Crailsheim 2010).

To promote bee health and support bee conservation efforts, it is critical to understand the floral resources that bees use in various landscapes and their colony-level foraging preferences (Bloom et al., 2022). Tracking pollen botanical sources is a technique that has been employed to determine bees' foraging behavior and floral selection process (Sommeijer 1983). Using techniques of palynology (identification of pollen grains), the morphology of pollen grains is used to determine the family, genus, and, often, the species of the originating plant (Weber 1998, Erdtman 1969). To date, few studies have investigated the identification and quantity of in-hive stored pollen (Wood et al. 2018) to identify bees' floral preferences and foraging behavior.

Urban areas represent only 2% of the total land use in the U.S., while agricultural areas spread over 17% of the total land base (Ghosh 2020), yet little is known about their unique impacts on honey bees' population and foraging behavior. There have been conflicting reports on the impact of landscape variation on the quantities of stored pollen, hive health, and productivity. While some findings indicate that agricultural land usage is associated with lower amounts of stored pollen in hives relative to urban or forested locations (Donkersley et al. 2014), others have observed that agricultural landscapes are associated with higher levels of stored pollen when compared to urban or forest areas (Sponsler and Johnson 2015). Yet another study linked the variation in the amount of stored pollen to seasonal change with little or no influence associated with the heterogeneity of land use (Danner et al. 2017).

Quality of pollen, in addition to quantity, has been increasingly considered among key factors for hive health. For example, the diversity of pollen botanical origin and its corresponding profile of key biomolecules and essential nutrients play an important role in the growth of the

hypopharyngeal gland and the longevity of honey bees (Di Pasquale et al. 2016). These factors also impact bees' foraging behavior (Cook et al. 2003). Alaux et al. (2010) observed that polyfloral diets from diverse pollen boosted several immunological activities compared to monofloral diets. In particular, glucose oxidase activity was higher, suggesting that the variety of floral resources provided greater in-hive antiseptic protection.

This study aims to assess the influence of landscape heterogeneity on pollen diversity by investigating the botanical and geographical origins of bee pollen collected from different land use areas in Colorado. This study also contributes to a growing body of data for estimating honey bee preference among botanical families.

MATERIAL AND METHODS

Study Sites and Pollen Collection

We surveyed 16 hives distributed in 7 counties in Northern Colorado. Pollen samples were collected during the summer of 2019 and 2020. A total of eight hives were located in urban areas, while eight hives were located in agricultural areas, as shown in Figure 8.

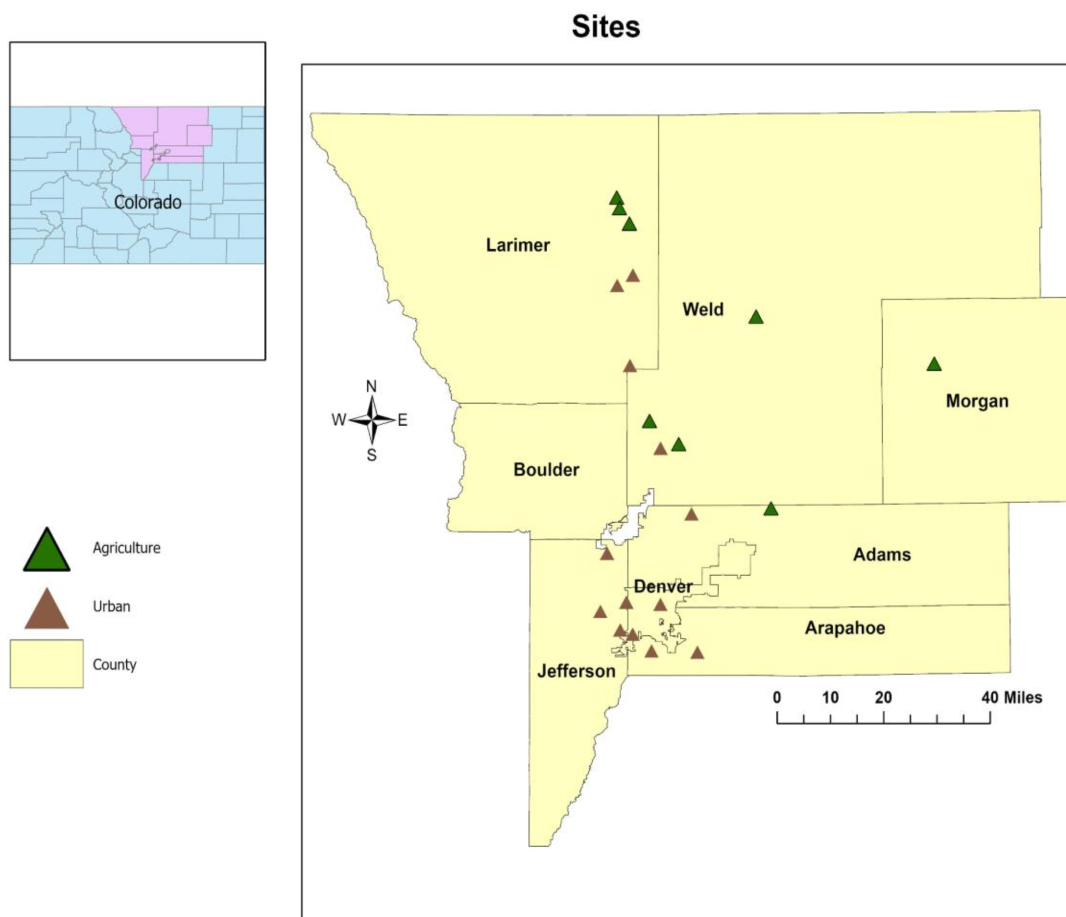


Figure 8: Map of Northern Colorado showing hive sampling sites for pollen from different land use categories, 2019-2020.

Sample collection, preparation, and analysis:

Pollen samples were collected using pollen traps (Bee Flower, Gyengbuk High-tech village, South Korea) at the entrance of each hive. These traps force forager bees to enter the hive through a screen where they drop their pollen loads, which fall into the trap box. Pollen samples were collected from the trap boxes and stored in the laboratory at -20 °C until analysis. A minimum of two samples were collected from each hive between June and September in the years 2019 and 2020 with a total of 61 pollen samples.

Pollen Staining and Identification

To prepare pollen for staining and identification, each pollen sample was weighed and subsampled at 10% of the original sample's total mass. Gross differences in taxa visited by bees in different areas can lead to pollen balls of different color, and so the subsample was sorted by color to help with botanical source identification (Brodschneider et al. 2021; Sponsler et al. 2017). To prepare a microscopic slide for each subsample, the subsample was homogenized according to Burden (2012) then mixed with a few drops of water and shaken to yield a homogeneous mixture, then approximately one drop of the mixture was mounted on a glass slide and fused with glycerin jelly and basic fuchsin suspension according to Kearns and Inouye (1993). Pollen was examined using a light microscope at x100-400 magnification and identified to family using the Palynological database ("PalDat," 2022), pollen wiki ("Pollen. wiki", 2022), and the Global Pollen Project ("The global pollen project," 2022).

Relative Abundance

The relative abundance of each pollen taxonomic family was estimated by identifying and quantifying all pollen pellets under a microscope and sorting them according to families. The total pollen pellets counted under each family were divided by the total pollen pellets counted for each landscape type and multiplied by 100 (Sponsler et al. 2017).

Diversity Index

To describe floral taxonomic diversity for urban versus agricultural landscapes, the Shannon-Weaver Diversity Index was employed (Shannon and Weaver, 1949). The Shannon-Weaver diversity index (H’) was calculated using the equation:

$$H' = -\Sigma\,[(pi) * (ln\,pi)]$$

Where *pi* is the proportion of each pollen type (*i*) in the sample and ln is the natural logarithm. A greater H’ value indicates greater taxonomic diversity (Shannon and Weaver, 1949). The Shannon-

Weaver diversity index was employed to compare taxonomic diversity between different land uses. Species relative abundance percentages were also calculated to show the differences in plant biodiversity in urban versus agricultural landscapes.

STATISTICAL ANALYSIS

Differences in pollen taxonomy and richness between agricultural and urban sites were assessed by analyzing diversity values through mixed model analysis. Spatial variation of pollen relative abundance was analyzed using Welch's t-test. Both analyses were performed with GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). A p-value of less than 0.05 was considered statistically significant for all analyses.

RESULTS

A total of 55 pollen taxonomic families were identified in samples collected from urban sites between June-September, and 41 pollen families were identified in samples collected from agricultural sites during the same period. In the subset samples, the mean count and standard deviation of each pollen family is reported by landscape designation in Table 5. Representative pollen family images are provided in Table 6.

Table 5: Mean and standard error of mean of counted pollen taxonomic families that were identified in 10% of samples collected from agricultural and urban sites.

Plant family	Agriculture	Urban
	Mean ± SEM	Mean ± SEM
Asteraceae	8518.10 ± 1637.39	9095.00 ± 1430.00
Fabaceae	7016.00 ± 1512.24	5065.00 ± 892.24
Brassicaceae	3504.40 ± 860.01	3096.00 ± 592.53
Lamiaceae	445.00 ± 305.12	1269.00 ± 1005.34
Amaranthaceae	292.15 ± 109.51	3273.00 ± 844.588

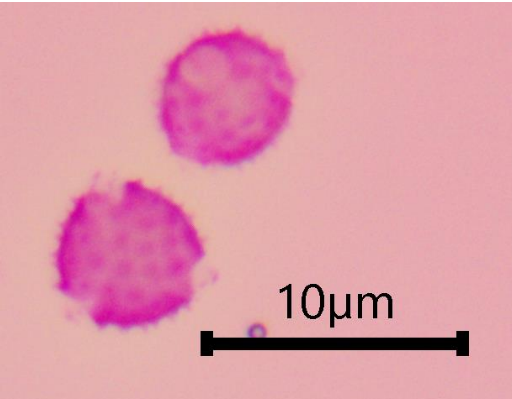
Caprifoliaceae	2993.30 ± 1876.75	206.30 ± 99.99
Amaryllidaceae	ND	1878.00 ± 812.67
Malvaceae	86.3 ± 58.40	264.50 ± 170.88
Polemoniaceae	1415.6 ± 391.06	2045.00 ± 718.06
Oxalidaceae	1500 ± NA	4878.00 ±1645.33
Dipsacaceae	553.33 ± 337.90	112.00 ± 73.10
Poaceae	666.43 ± 171.95	44.00 ± 15.36
Aceraceae	2028.00 ± 807.70	5310.00 ± 2334.53
Primulaceae	3650.00 ± 2775.34	2723.00 ± 1101.23
Plantaginaceae	560.00 ± 520.00	2045.00 ± 969.13
Pinaceae	20.00 ± 10.00	30.00 ± 18.01
Papaveraceae	ND	660.00 ± 640
Tiliaceae	ND	12740.00 ± 4099.04
Scrophulariaceae	10.00 ± NA	1155.00 ± 1065.00
Rosaceae	3991.10 ± 1906.02	6969.00 ± 2071.84
Polygonaceae	ND	1078.00 ± 751.22
Liliaceae	10.00 ± NA	192.20 ± 437.80
Campanulaceae	217.50 ± 178.76	4231.00 ± 1392.26
Cannabaceae	10.00 ± NA	5050.00 ± 3950.00
Gymnospermae	ND	130.00 ± 45.00
Iridaceae	10.00 ± NA	70.00 ± 94.16
Solanaceae	106.00 ± 62.18	4383 ± 4020.62
Geraniaceae	ND	966.00 ± 403.77
Lythraceae	ND	7593.00 ± 4181.31
Ranunculaceae	1740.00 ± 899.64	206.00 ± 103.01
Violaceae	20.00 ± NA	100 ± NA
Hyacinthaceae	ND	70.00 ± 60.00
Convolvulaceae	108.57 ± 178.18	234.00 ± 61.77
Elaeagnaceae	30.00 ± NA	70.00 ± 21.91
Onagraceae	145.00 ± 55.00	183.30 ± 144.40
Cyperaceae	ND	1700.00 ± 784.48

Magnoliaceae	539.00 ± NA	396.00 ± 98.01
Portulacaceae	1430.00 ± NA	322.50 ± 178.90
Anacardiaceae	970.00 ± 549.75	5025.00 ± 2465.27
Cactaceae	20.00 ± 10.00	15.00 ± 5.00
Caryophyllaceae	ND	130.00 ± 61.10
Rubiaceae	200.00 ± NA	1495.00 ±1475.00
Alliaceae	ND	80.00 ± NA
Apiaceae	ND	2948.00 ± 2937.50
adoxaceae	2017.50 ± 365.23	6548.00 ± 3505.76
Cucurbitaceae	ND	70.00 ± 50.33
Boraginaceae	400.00 ± NA	126.70 ± 111.70
Passifloraceae	ND	1730.00 ± NA
Zygophyllaceae	ND	10.00 ± NA
Grossularaceae	ND	4390.00 ± NA
Ephedraceae	ND	1250.00 ± NA
Salicaeae	3600.00 ± NA	9770.00 ± NA
Polypodiaceae	ND	4240.00 ± NA
Leguminosae	ND	700.00 ± NA
Asparagaceae	20.00 ± NA	ND
Oleaceae	4150.00 ± NA	ND
Acanthaceae	1485.00 ± 485.00	ND
Rhamnaceae	380.00 ± NA	ND

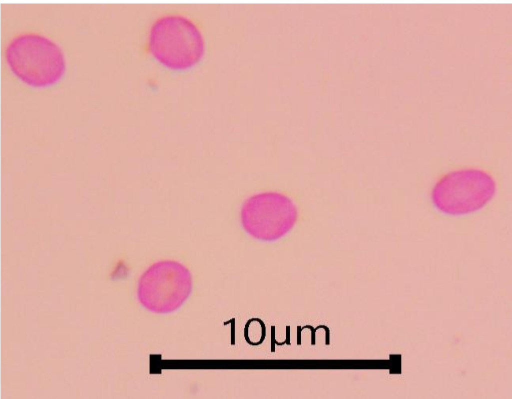
Table 6: Sample images of the pollen taxa analyzed in our study.

Family	Microscopic Image (x100)

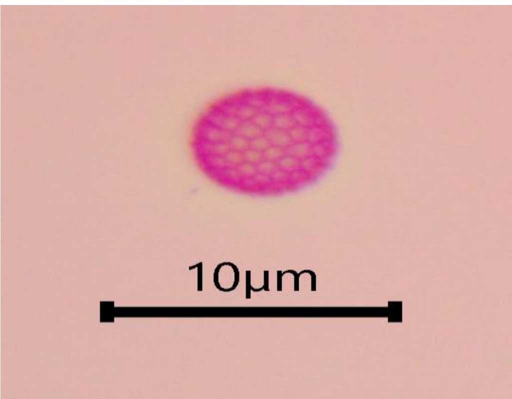
Asteraceae



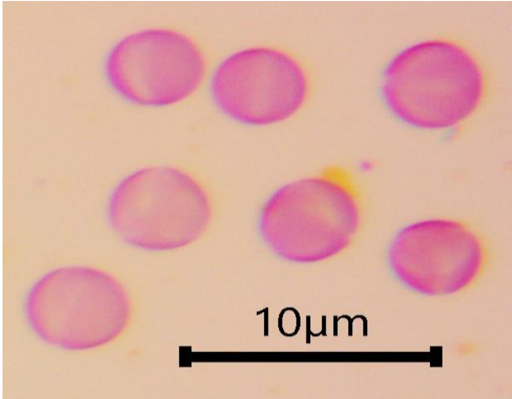
Fabaceae



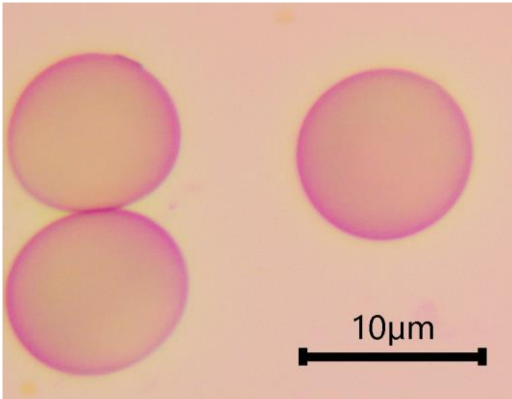
Polemoniaceae



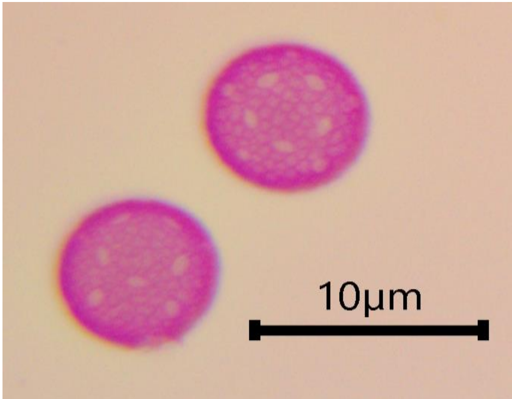
Brassicaceae



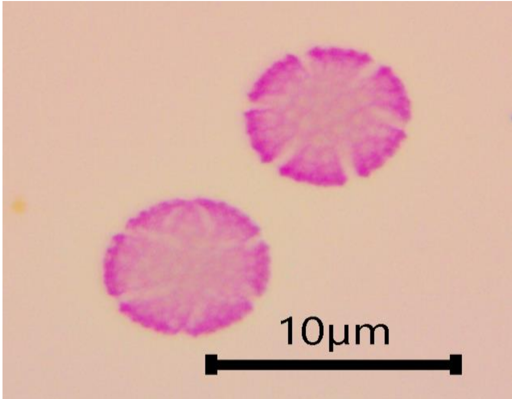
Poaceae



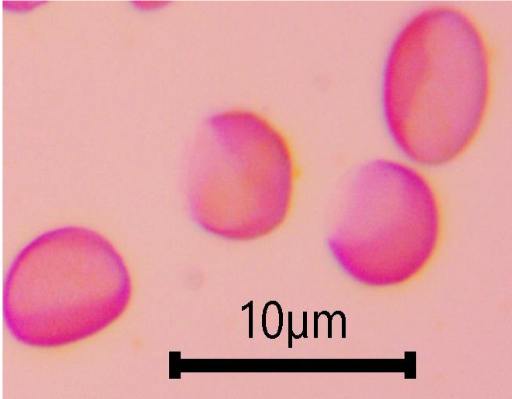
Portulacaceae



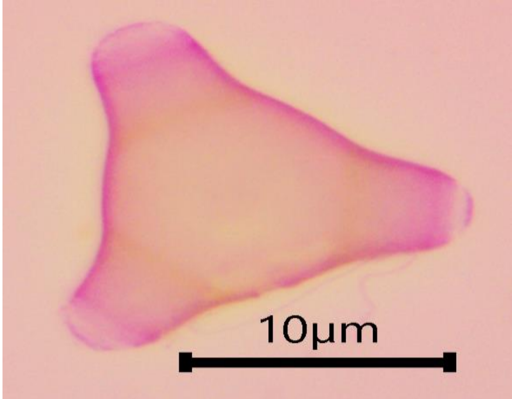
Lamiaceae



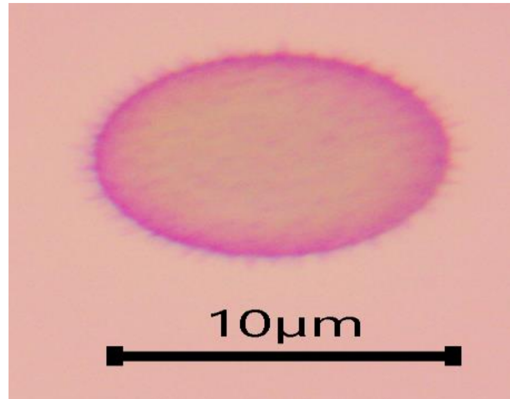
Amaryllidaceae



Onagraceae



Malvaceae



Diversity Index

There was a significant difference between urban and agricultural landscapes regarding pollen taxonomic diversity. Predictably, the pollen botanical diversity index was higher in urban landscapes (Mean ±SEM, 1.69 ± 0.12) than in agricultural sites (Mean ±SEM, 1.16 ± 0.18). The summary of Shannon-Weaver Diversity Index for each site is in Figure 9.

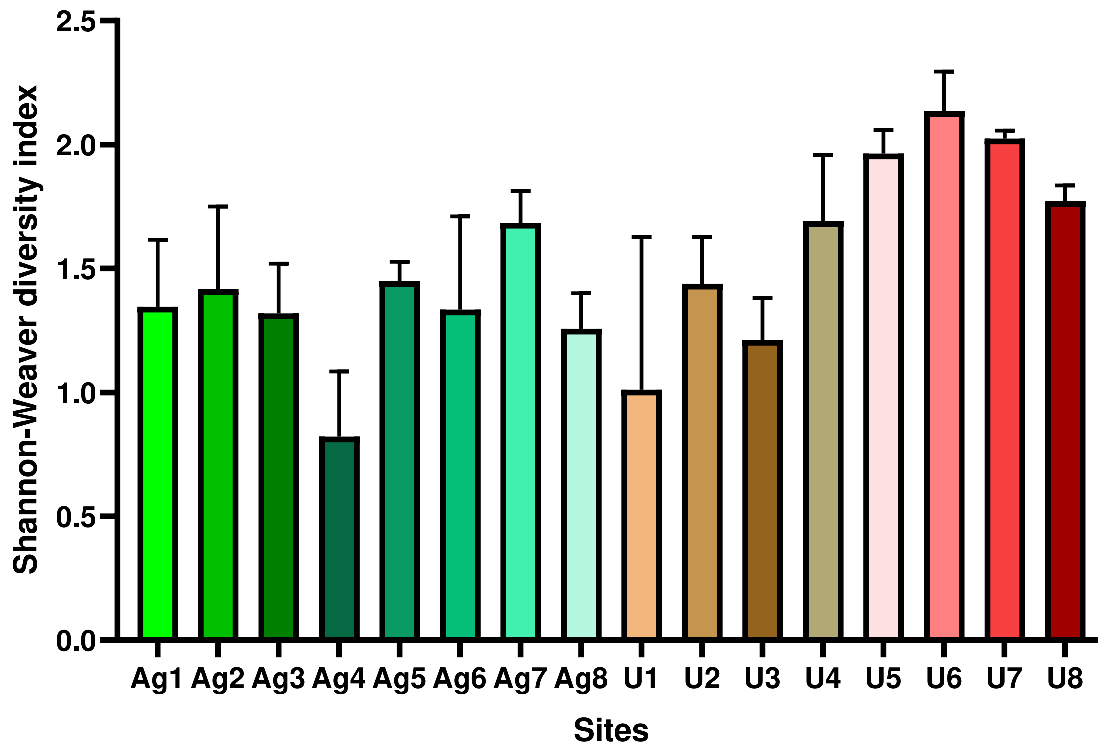


Figure 9: Summary of Shannon-Weaver diversity index values (mean ± SEM) for all sites classified by landscape type. Mixed model analysis was performed to determine the diversity difference between the two landscapes, which were significant (p-value = 0.0069), F (DFn, DFd) = 8.22.

Relative Abundance

Welch's t-test was performed to indicate the spatial variation of pollen relative abundance. There was no significant difference in pollen relative abundance between agriculture and urban, with p-value = 0.435, t = 0.784 and df = 121.9. However, and as shown in figures 3 and 4, there were more identified families located in samples collected from urban areas. The most abundant pollen families in both landscape sites are summarized in Figures 10 and 11.

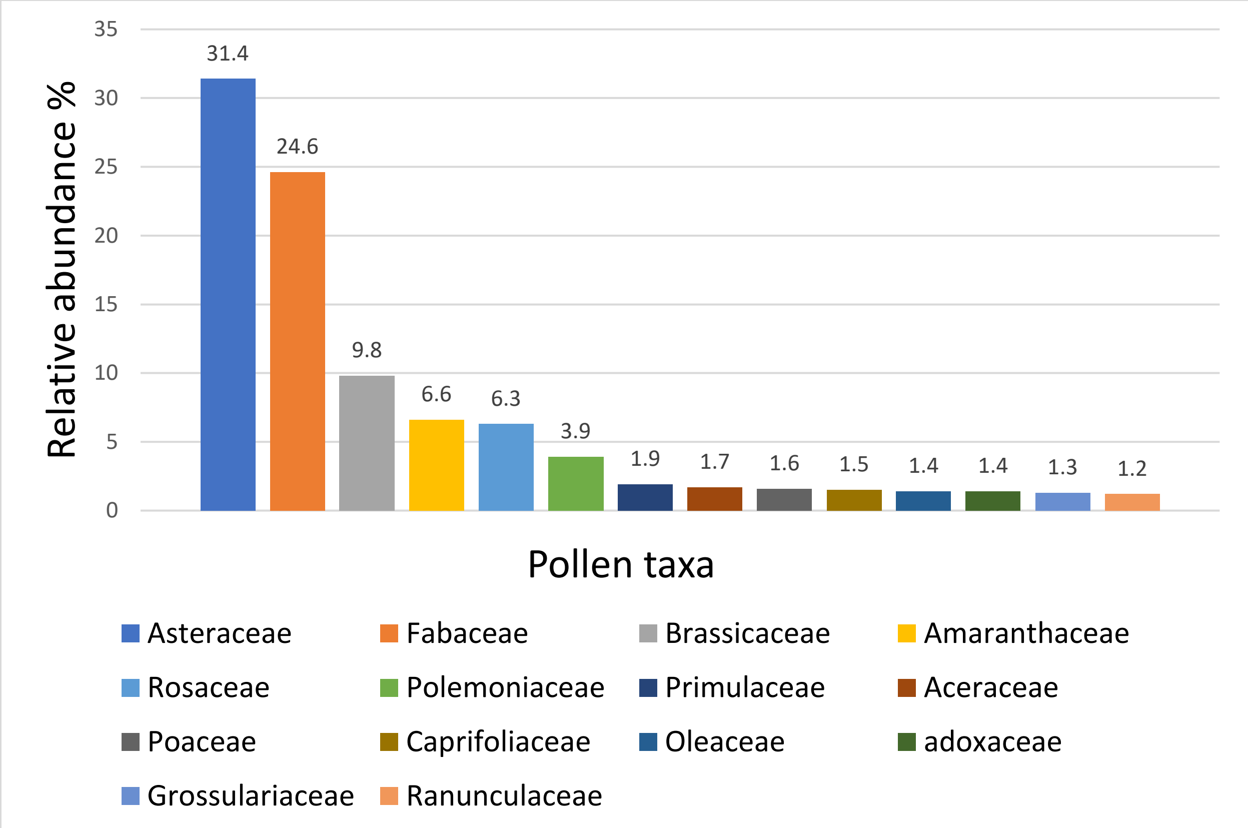


Figure 10: Relative abundance (%) of major pollen taxonomic families found in pollen samples collected from agricultural sites.

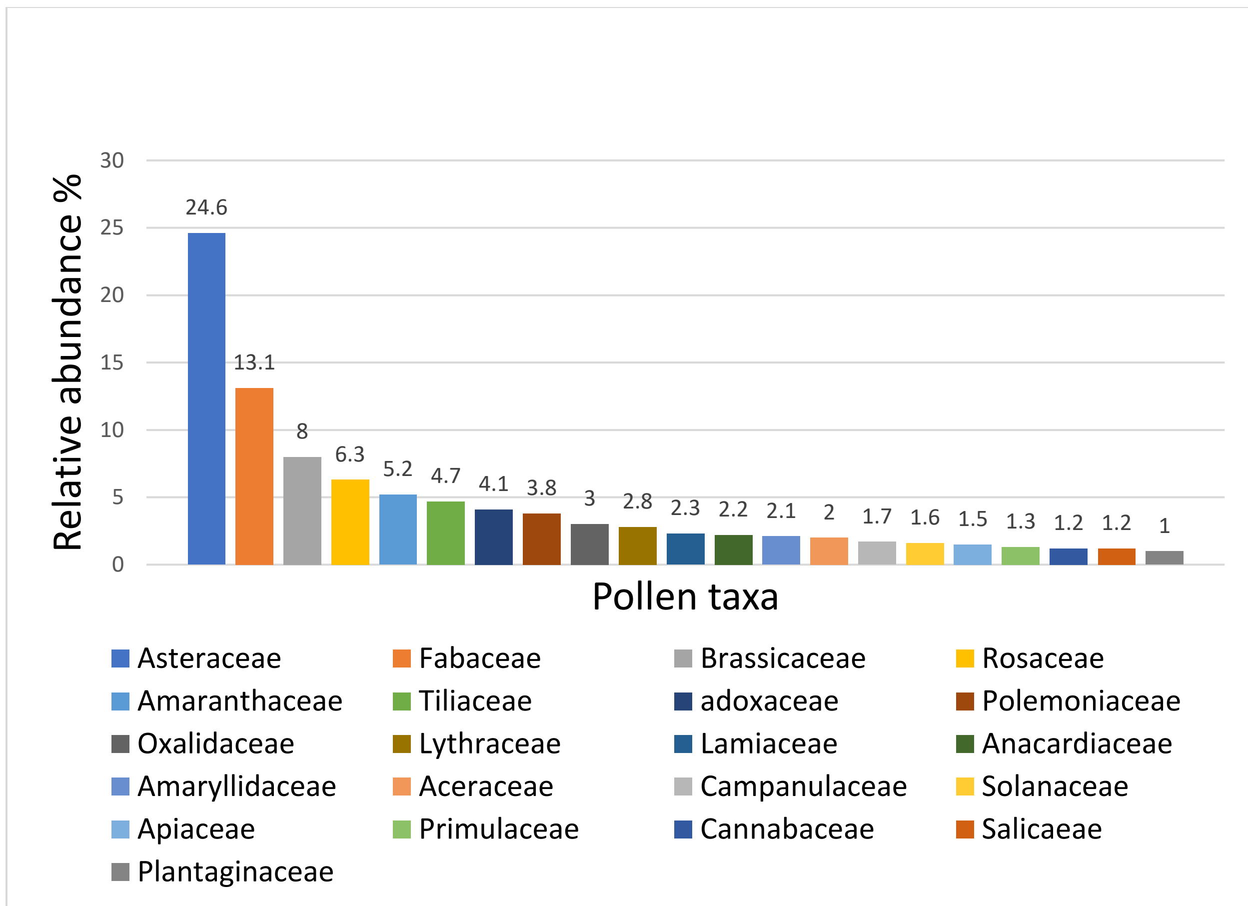


Figure 11: Relative abundance (%) of major pollen taxonomic families found in pollen samples collected from urban sites.

DISCUSSION

We conducted a two-year survey of the floral sources of pollen collected by honey bees in urban and agricultural settings in 16 locations between June and September of 2019 and 2020. Pollen pellets were examined under a light microscope and identified to the family taxonomic level. The results indicated spatial variation in Shannon-Weaver diversity, demonstrating a higher diversity index and a wider variety of pollen taxa collected from urban sites compared to agricultural sites. However, the relative abundance of plant families in both landscape settings showed the same two dominant families each time, namely the Asteraceae and Fabaceae plant families. This supports existing studies about bees' predisposition to forage on a relatively few

selected species (Baum et al. 2004), although here we cannot say if the dominance was due to selection, availability, or aspects of both.

Pollen source and quality are of great importance as an essential source of protein, lipids, vitamins, and minerals in the diet of the honey bee (Campos et al., 2008). Worker bees ingest pollen to produce jelly fluid through their hypopharyngeal glands while nursing bees feed this nutritive-rich jelly to the newly emerged adults and queen (Brouwers, 1984). Stress or poor pollen quality adversely impacts the development of the hypopharyngeal gland in nurser bees, which, in turn, impairs the growth of the queen and larvae (Zaytoon et al. 1988; Schmidt et al. 1995). It would be worthwhile to focus future studies on the impact of changing pollen diets and the variation in protein and amino acid contents on bees' survival and development and immunocompetence (Omar et al. 2016; Alaux et al. 2010)

Few studies have attempted to account for the taxonomic diversity of the foraged pollen as it relates to the prosperity of a specific plant group. Lower diversity of pollen families and species may not necessarily correlate with the poor nutrition of bees. However, it is well documented that honey bees prefer to consume a variety of pollen species that tend to give them a higher nutritional value and to use these varieties to dilute the effect of toxic pollen species (Schmidt 1984). Besides nutrition, honey bees tend to look for specific pollen families with toxic compounds to defend the colony against pathogens like *Nosema ceranae* (Giacomini et al. 2018).

Asteraceae was the most abundant pollen family in all sites. However, the protein content of Asteraceae is low compared with other pollen taxa and has been reported to impair the ability of honey bees to rear broods if Asteraceae species were their only source for protein (Roulston et al., 2000). Despite that, Asteraceae pollen is consumed by honey bees due to the large number of species that belong to this family that supply pollen and nectar (Santos et al., 2019). This is also

true for the second most dominant pollen present from the Fabaceae family (Bilisik et al., 2008) and explains the high relative abundance of Asteraceae and Fabaceae in collected samples from all study sites. This supports the classification of honey bees as generalists with regard to floral resources (Goulson, 2003).

Our observations indicating the higher diversity index and relative abundance of floral species in urban sites compared to agricultural support are similar to findings by Donkersley et al. (2014). They contradict the findings of Sponsler et al. (2017) on this point, who found higher variation in pollen taxa in samples collected from agricultural sites. Future studies on the impact of spatial variation on bees' food collection process should take into consideration the influence of seasonal variation of floral availability and honey bees' nutritional demands.

LITERATURE CITED

Alaux, C., Ducloz, F., Crauser, D., & Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. Biology letters, 6(4), 562-565.

Baum, K. A., Rubink, W. L., Coulson, R. N., & Bryant Jr, V. M. (2004). Pollen selection by feral honey bee (Hymenoptera: Apidae) colonies in a coastal prairie landscape. Environmental Entomology, 33(3), 727-739.

Bilisik, A., Cakmak, I., Bicakci, A., & Malyer, H. (2008). Seasonal variation of collected pollen loads of honeybees (*Apis mellifera* L. anatoliaca). Grana, 47(1), 70-77.

Bloom, E. H., Graham, K. K., Haan, N. L., Heck, A. R., Gut, L. J., Landis, D. A., ... & Isaacs, R. (2022). Responding to the US national pollinator plan: a case study in Michigan. Frontiers in Ecology and the Environment, 20(2), 84-92.

Brodschneider, R., & Crailsheim, K. (2010). Nutrition and health in honey bees. Apidologie, 41(3), 278-294.

Brodschneider, R., Kalcher-Sommersguter, E., Kuchling, S., Dietemann, V., Gray, A., Božič, J., ... & van der Steen, J. J. (2021). CSI pollen: diversity of honey bee collected pollen studied by citizen scientists. Insects, 12(11), 987.

Brouwers, E. V. M. (1984). Glucose/fructose ratio in the food of honeybee larvae during caste differentiation. Journal of Apicultural Research, 23(2), 94-101.

Burden, D.W. (2012). Guide to the Disruption of Biological Samples. Random Primers, 12(1) 1-25.

Campos, M. G., Bogdanov, S., de Almeida-Muradian, L. B., Szczesna, T., Mancebo, Y., Frigerio, C., & Ferreira, F. (2008). Pollen composition and standardization of analytical methods. Journal of Apicultural Research, 47(2), 154-161.

Colorado General Assembly (2022, September 14). Colorado revised statutes 2016. Retrieved from: https://leg.colorado.gov/sites/default/files/images/olls/crs2016-title-39.pdf

Cook, S. M., Awmack, C. S., Murray, D. A., & Williams, I. H. (2003). Are honey bees' foraging preferences affected by pollen amino acid composition? Ecological Entomology, 28(5), 622-627.

Danner, N., Keller, A., Härtel, S., & Steffan-Dewenter, I. (2017). Honey bee foraging ecology: Season but not landscape diversity shapes the amount and diversity of collected pollen. PloS one, 12(8), e0183716.

Di Pasquale, G., Alaux, C., Le Conte, Y., Odoux, J. F., Pioz, M., Vaissière, B. E., ... & Decourtye, A. (2016). Variations in the availability of pollen resources affect honey bee health. PloS one, 11(9), e0162818.

Donkersley, P., Rhodes, G., Pickup, R. W., Jones, K. C., & Wilson, K. (2014). Honeybee nutrition is linked to landscape composition. Ecology and evolution, 4(21), 4195-4206.

Erdtman, G. (1969). Handbook of palynolgy: Morphology, taxonomy, ecology. An introduction to the study of pollen grains and spores. Hafner.

Ghosh, I. Mapped: The Anatomy of Land use in America (2022, September 15). Retrieved from https://www.visualcapitalist.com/america-land-use/

Giacomini, J. J., Leslie, J., Tarpy, D. R., Palmer-Young, E. C., Irwin, R. E., & Adler, L. S. (2018). Medicinal value of sunflower pollen against bee pathogens. Scientific reports, 8(1), 14394.

Goulson, D. (2003). Effects of introduced bees on native ecosystems. Annual Review of Ecology, Evolution, and Systematics, 1-26.

Kearns, C. A., & Inouye, D. W. (1993). Techniques for pollination biologists. University press of Colorado.

Lau, P., Bryant, V., Ellis, J. D., Huang, Z. Y., Sullivan, J., Schmehl, D. R., ... & Rangel, J. (2019). Seasonal variation of pollen collected by honey bees (*Apis mellifera*) in developed areas across four regions in the United States. PLoS One, 14(6), e0217294.

Omar, E., Abd-Ella, A. A., Khodairy, M. M., Moosbeckhofer, R., Crailsheim, K., & Brodschneider, R. (2017). Influence of different pollen diets on the development of hypopharyngeal glands and size of acid gland sacs in caged honey bees (*Apis mellifera*). Apidologie, 48(4), 425-436.

PalDat-Palynological Database. (2022, September 9). Retrieved from https://www.paldat.org/

Pollen.wiki. (2022, September 9). Retrieved from https://pollen.tstebler.ch/MediaWiki/index.php?title=Pollenatlas#gsc.tab=0

Ritchie, H., & Roser, M. (2018). Urbanization. Our world in data.

Roulston, T. A. H., Cane, J. H., & Buchmann, S. L. (2000). What governs protein content of pollen: pollinator preferences, pollen–pistil interactions, or phylogeny?. Ecological monographs, 70(4), 617-643.

Santos, A. P. D. S. M., Assis, S. L., Lourenço, A. P., & Machado, E. L. M. (2019). Bees diversity on flowers of Eremanthus spp. (Asteraceae). Floresta e Ambiente, 26.

Schmidt, J. O. (1984). Feeding preferences of *Apis mellifera* L. (Hymenoptera: Apidae): individual versus mixed pollen species. Journal of the Kansas entomological society, 323-327.

Schmidt, L. S., Schmidt, J. O., Rao, H., Wang, W., & Xu, L. (1995). Feeding preference and survival of young worker honey bees (Hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. Journal of Economic Entomology, 88(6), 1591-1595.

Shannon, C. E., and Weaver, W., 1949. The Mathematical Theory of Communication. Urbana: University of Illinois Press.

Sommeijer, M. J., De Rooy, G. A., Punt, W., & De Bruijn, L. L. M. (1983). A comparative study of foraging behavior and pollen resources of various stingless bees (Hym., Meliponinae) and honeybees (Hym., Apinae) in Trinidad, West-Indies. Apidologie, 14(3), 205-224.

Spear, D. M., Silverman, S., & Forrest, J. R. (2016). Asteraceae pollen provisions protect Osmia mason bees (Hymenoptera: Megachilidae) from brood parasitism. The American Naturalist, 187(6), 797-803.

Sponsler, D. B., & Johnson, R. M. (2015). Honey bee success predicted by landscape composition in Ohio, USA. PeerJ, 3, e838.

Sponsler, D. B., Matcham, E. G., Lin, C. H., Lanterman, J. L., & Johnson, R. M. (2017). Spatial and taxonomic patterns of honey bee foraging: A choice test between urban and agricultural landscapes. Journal of Urban Ecology, 3(1).

Standifer, L. N. (1967). A comparison of the protein quality of pollens for growth-stimulation of the hypopharyngeal glands and longevity of honey bees, Apis mellifera L. (Hymenoptera: Apidae). Insectes Sociaux, 14(4), 415-425.Alaux, C., Ducloz, F., Crauser, D., & Le Conte, Y. (2010). Diet effects on honeybee immunocompetence. Biology letters, 6(4), 562-565.

The global pollen project. (2022, September 9). Retrieved from https://globalpollenproject.org/

United States Census Bureau. (2022, September 14). The Urban and Rural Classifications. Retrieved from: https://www2.census.gov/geo/pdfs/reference/GARM/Ch12GARM.pdf

Waiker, P., Ulus, Y., Tsui, M. T. K., & Rueppell, O. (2022). Mercury accumulation in honey bees trends upward with urbanization in the USA. Agricultural & Environmental Letters, 7(2), e20083.

Weber, R. W. (1998). Pollen identification. Annals of Allergy, Asthma & Immunology, 80(2), 141-148.

Wheeler, M. M., & Robinson, G. E. (2014). Diet-dependent gene expression in honey bees: honey vs. sucrose or high fructose corn syrup. Scientific reports, 4(1), 1-5.

Wood, T. J., Kaplan, I., & Szendrei, Z. (2018). Wild bee pollen diets reveal patterns of seasonal foraging resources for honey bees. Frontiers in Ecology and Evolution, 6, 210.

Zaytoon, A. A., Matsuks, M., & Saski, M. (1988). Feeding efficiency of pollen substitutes in a honeybee colony: Effect of feeding site on royal jelly and queen production. Applied Entomology and Zoology, 23(4), 481-487.

CHAPTER 4: MODELING THE IMPACT OF LANDSCAPE VARIATION ON HONEY BEES' FORAGING BEHAVIOR AND WHAT RETURNS TO THE BEEHIVE

SUMMARY

The alarming decrease in honey bees' population is the impetus for more studies investigating contributing factors. We assessed overlapping contributing environmental factors related to the Colony Collapse Disorder phenomenon. We investigated the spatial variation of bees' exposure to a distinctive environmental toxin and the corresponding quantities of these toxins in floral products collected by bees. We used agent-based modeling to simulate bees' exposure to toxins, considering landscape variations, pesticide breakdown, occurrence percentage of pesticides or heavy metals in the environment, and the ability of honey bees to detoxify contaminants. The results of this study indicated significant spatial variation effects on the accumulated quantities of the collected floral products in the hive, which in turn significantly affected the amounts of accumulated pesticides and heavy metal inside the beehive.

INTRODUCTION

Honey bees are classified as bioindicators whose level of function, population, products, and behavior are predictive of qualitative environmental conditions (Celli and Maccagani 2003). They have been used to monitor the level of environmental pollutants like heavy metals (Conti and Botre 2001, Lambert et al. 2012, Hladun et al. 2016, Matin et al. 2016), pesticides (Balayiannis and Balayiannis 2008, Malhat et al. 2015, de Oliveira et al. 2016) and radionuclides (Mujic et al. 2011). The presence of pesticides and heavy metals in the beehive matrix has been reported worldwide (Mitchell et al. 2017, Tison et al. 2016, Hladun et al. 2016), with more focus on pesticides, which are considered the main chemical stressor that triggers Colony Collapse Disorder phenomena. In Colony Collapse Disorder, most forager bees abandon the colony,

leaving behind adequate quantities of food, a queen, and insufficient workers to take care of eggs and larvae (USDA 2020). The impact of pesticides on bee colonies has been addressed with approaches including field sampling (Zawislak et al. 2019), laboratory-based assays (Di et al. 2016), and computer models and simulations (Becher et al. 2014).

Bees' exposure to various toxins most often involves foraging in a contaminated environment. This may result in the direct exposure of the foragers themselves to the toxins through collecting and consumption of contaminated pollen and nectar (Krupke et al. 2012, Lambert et al. 2012) and/or the delivery of toxins to the rest of the colony, by bringing the contaminated pollen and nectar to the hive (Villa et al. 2000). Bees' exposure to toxins may also occur due to certain beekeeping practices (T O'Neal et al. 2018).

However, the most common source of contamination is due to the use of agrochemical products in surrounding landscapes (Krupke et al. 2012, Castilhos et al. 2019, Zawislak et al. 2019). Although many prior studies have investigated the consequences of human activities and urbanization on the distribution of contaminants in the environment (Hanafi et al. 2020, Duzgoren-Aydin et al. 2006, Wang et al. 2017), fewer studies have considered the impact of landscape variation on toxins deposited in bee's products (Waiker et al. 2022). Bees' exposure to toxins is not discrete and depends on the intersection of the spatiotemporal presence of toxins with bee foraging activity. This affects the amount and type of contaminants to which bees, hives, and their products are exposed (Van Der Steen et al. 2012).

Few models have attempted to explain the mechanism behind honey bees' exposure to contaminants, mainly pesticides. Computer models have been developed to simulate honey bee colonies and foraging behavior. Other models represent the multiple stressors that impact honey bee colonies, like pathogens, pesticides, and forage availability, to better understand and predict

colony development and survival (Henry et al. 2017, Becher et al. 2014). Some models have focused on individual bees' exposure to pesticides by considering the effect of pesticide movement in the hive and the behavior of different age stages of individual bees (Rumkee et al. 2017), bees' ability to detect food sources (Becher et al. 2016) or the adaptive behavior of bees against predators (Johnson and Nieh 2010). No prior model, however, has addressed the heterogeneity of contaminant exposure in different landscape contexts.

In this study, we used agent-based modeling to simulate bees' exposure to toxins, considering landscape variations, pesticide dissipation, the percentage of toxins occurrence or presence in the environment, and the ability of honey bees to detoxify contaminants. Agent-based models (ABM) are simulation tools were individuals (agents) are allowed to interact with each other and with their surrounding environment in a bottom-up approach to give explanations or predictions on how these agents interact in the real world and the outcomes (Boone and Galvin 2014). Since it is complex to explain honey bee behavior and dynamics in different landscape settings (Becher at al. 2013) as well as the complications of bees' exposure to different environmental toxins (Johnson et al. 2010), ABM serves as an effective tool to explain and predict bees' behaviors in two distinct systems. The objectives of this study are to simulate bee exposure to pesticides and heavy metals in different landscape contexts (agriculture vs. urban areas) using agent-based modeling to assess the spatial variation effect on what bees are exposed to and what they might bring back to the hive in terms of contaminants.

MODEL AND METHODS

To inspect the effect of landscape variation on bee's exposure to different types of toxins and how much of these toxins are accumulating in floral products brought back to the hive (pollen and nectar), we have developed an ABM implemented in Netlogo 6.1.1 (Wilensky 1999).

Model Description:

To illustrate the simulation coding, a flow chart for the model procedures is provided in Figure 12.

The model is described in detail following the ODD protocol (Overview, Design concepts, Details), which is used for describing individual-and agent-based models (Railsback and Grimm 2011).

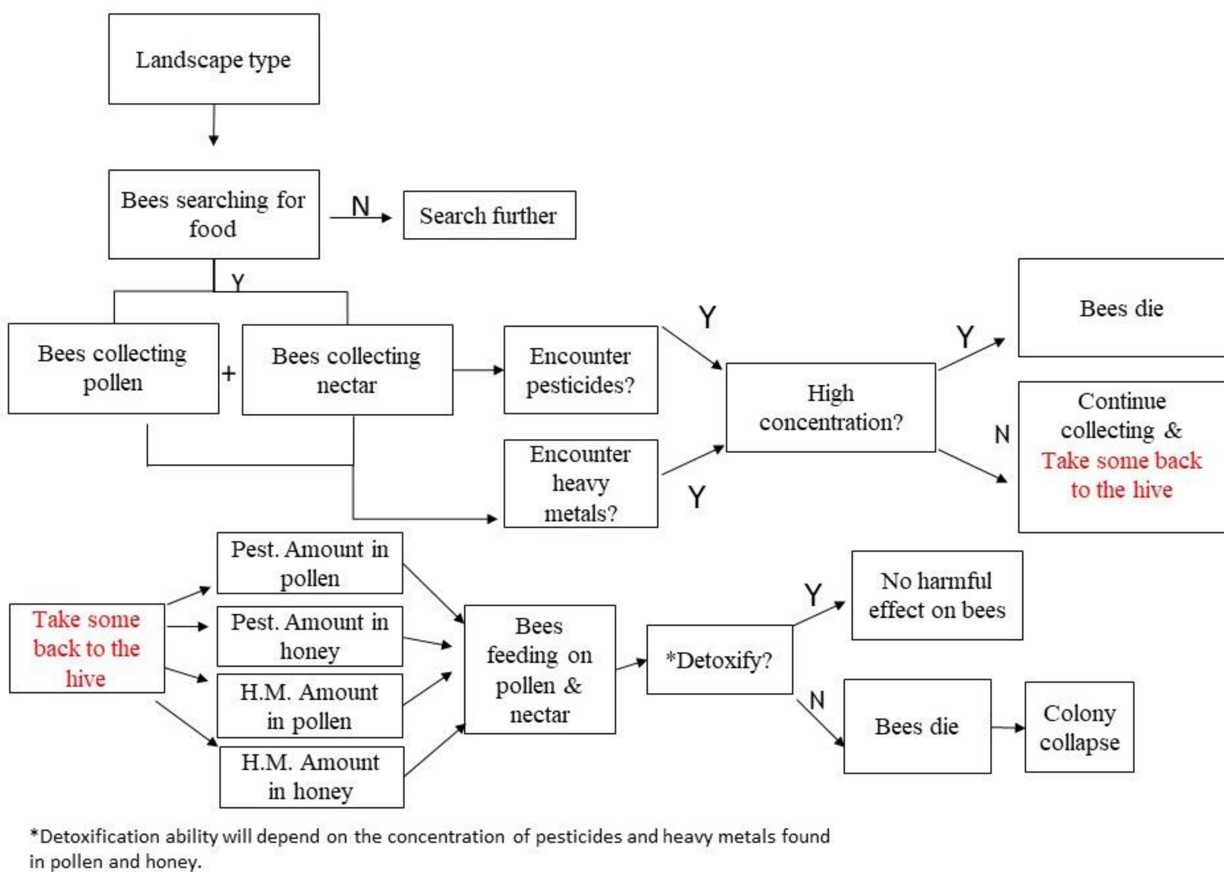


Figure 12. Flow chart of the model processes.

Overview

a. Purpose: This model is designed to assess how much pesticide and heavy metal contaminants honey bees bring back to the hive. It is also designed to establish scenarios including landscape changes and how they will impact bee foraging

behavior and the net contaminants, taking into consideration the many causative factors including foraging time, landscape setting, contaminants occurrence percentage which indicate the presence or absence of multiple contaminant sources, lethal dose of the contaminants, pesticide degradation, and bees' ability to detoxify contaminants. The model is not designed to give accurate values of bees' exposure to pesticides or heavy metals or to assess the effect of these toxins on the hive's health or capacity; too many unknowns remain for that to be within the scope of this effort. Rather, it is designed to inspect the variation of toxin accumulation in hive products influenced by the change of landscape type (urban vs. agriculture).

b. Entities, state variables, and scales:

i. Individuals: The individuals in this model will be forager honey bees, flowers with pollen and nectar, houses, and a hive.

ii. State Variables: The state variables are owned by a hive, flowers, houses, and plants. The hive is characterized by the: 1) Daily accumulation of pollen; 2) Daily accumulation of nectar; 3) Daily accumulation of pesticides; 4) Daily accumulation of heavy metals; 5) Total accumulated amount of pollen; 6) Total accumulated amount of nectar; 7) Total accumulated amount of pesticides; and 8) Total accumulated amount of heavy metals. Daily and the accumulated amounts of pesticides or heavy metals are affected by pesticides or metal occurrence percentage in the environment. The amount of these toxins in flowers and bees' foraging behaviors later affects the amount of pesticide and heavy metal residues in pollen and nectar that they are carrying back to the hive.

Flowers are characterized by the following: 1) Each flower is established to have a range between 1-5 mg of pollen (Buchmann and Shipman 1990); 2) Nectar amount in

each flower is established to be 17 µl (Eckert et al. 1994); 3) Flower colors vary based on the pollen and nectar contents; 4) The quantities of pesticides and heavy metals in pollen and nectar are based on the values that indicate the presence or absence of pesticides and metals and the occurrence percentage of these toxins; 5) Flowers have the ability to replenish nectar after a bee's visit and pollination. Houses and plants are established to represent the variation in landscape type. Houses are associated with the existence of urban landscapes which are characterized by higher floral diversity, as suggested by Martins et al. (2017). Houses, plants, flowers, and the hive are treated as agents that vary in location each time we reset the model.

The bees are characterized by the following variables: 1) A single load of pollen is set at 2.5 mg/bee; 2) The single amount of nectar that a forager bee can hold is set at 2 µl/bee (Huang and Seeley 2004); 3) The amount of pesticides or heavy metals that bees can carry is affected by the presence or absence of pesticides or heavy metals in flower products; as well as the occurrence percentage of these toxins in the environment; 4) Bees' energy is affected by the amount of energy that they gain from food consumption. The level of energy is also affected by the presence of toxins, where forager bees lose energy whenever the percent of occurrence or application of pesticides or heavy metals in the environment exceed 30 g/L; 5) Bees have the ability to detoxify nectar from pesticides and heavy metals based on their reported ability to remove up to 33% of heavy metals from nectar through metabolism and excrete these metals from their bodies through defecation which usually happen outside the hive (Borsuk et al. 2021; Evans & Schwarz 2011); 6) Bees will return to the hive by nighttime and resume pollen and nectar collection the next day; 8) The model runs

with a 3 min time-step (i.e., tick) time, where 250 ticks = one day, or a 12.5 hr daylight foraging period. The maximum age of bees is around 2500 ticks, with each tick representing 3 min, they will age 1 day as sunset starts at 200 ticks; forager bees' maximum age was reported between 15-28 days (Vance et al. 2009).

iii. Scales: The spatial scale of the model is one landscape with one hive that produces bees and changes its location in each setup. Flowers, houses, and plants are distributed around the hive. The spatial arena of the model is composed of 2601 patches, each patch is 118 X118 m (i.e., a 6,051 m x 6,051 m area)

c. Process overview and scheduling: The process of this model is represented by the movement of forager honey bees to search for pollen and nectar; as soon as their load of pollen and/or nectar is full, they return to the hive to deposit the collected floral products. Pesticides and heavy metals start to build up in the hive as soon as they exist in the environment and are deposited in pollen and nectar. Time in the model is represented by days; bees forage during the day and return back to the hive by sunset or if their capacity to store product is reached. Regardless, bees deposit their products at the hive, and if they have returned during the day, will leave the hive to continue foraging. Nectar quantities in flowers are restored overnight to default levels, but quantities of pollen are not not restored and decrease as gathered by bees.

D. Daily update: At the start of each day, daily hive contents of pollen, nectar, pesticides, and heavy metals are added to an accumulator and then reset to 0. Flowers and bees are aged, and if they age above the age threshold, they die and are removed from the model. Pesticides are managed by the daily update and decay exponentially as suggested by Karlik (2009), using: $C_t = C_0e^{-kt}$, where C_t: is pesticide concentration at a time, C_0: is the initial

concentration of pesticide, k: is a constant that represents the half-life of the pesticide, which was 0.693, t: is time. We used this equation to represent a general dissipation rate of pesticide but not the half-life equation, because we are considering metabolites as a harmful residue for bees (O'Neal et al. 2017).

e. Foraging: As the day starts, forager bees leave the hive and start looking for flowers to collect pollen and nectar; if they pass below an energy threshold, they will consume nectar to replenish their energy and resume foraging. If pesticides or heavy metals exist in the environment, bees will collect contaminated pollen and nectar. The amount of accumulated toxins in the hive varies depending on the occurrence percentage of pesticides or heavy metals in the environment and how much of these toxins were found in floral products. Also, the accumulated toxins in nectar inside the hive are affected by the detoxification process, where nectar is subject to being detoxified and metabolized. The foraging behavior of bees is affected by pesticide and heavy metal occurrence percentages, which are associated with multiple application sources. If bees encounter pesticides or heavy metals with high presence levels, bees die.

Design Concepts

a. Basic principles: The basic principles addressed by this model are that variation of toxin residues in floral products that are collected by bees end up in the hive. Of interest is the way that the change in land use will affect bees' foraging behavior, which will later impact the amount of pollen, nectar, and toxins present in the hive.

b. Emergence: The results of this model emerged from the contaminant concentrations and bees' death relationship and how much of these contaminant residues build up in the hive.

c. Adaptation: Bees change their behavior when they are engaged in the detoxification of contaminants, they lose energy in the detoxification process, and die whenever the occurrence percentage of toxins exceed 60 g/L.

d. Sensing: Bees can identify flowers with sufficient amounts of pollen or nectar for foraging. However, they are unaware of the contaminated state of pollen and nectar as it is being sought and consumed and will either lose energy or die depending on the contaminant concentrations.

e. Interaction: There is no interaction between foragers.

f. Stochasticity: The bees will start to search randomly until they find a flower with enough pollen or nectar.

g. Observation: The output that is required to be observed in this model is what type and what quantity of contaminants are accumulated in the beehive and floral products because of contaminated pollen and nectar and the role of landscape variation in affecting the level of these accumulated contaminants. Also, it is necessary to test the effect of contaminants' breakdown and detoxification on lowering the hive's concentration of contaminants.

Details

a. Initialization: The model's initial state includes a beehive located in an agricultural area where there are many flowers with different colors that indicate the amount of pollen and nectar in each. When starting the simulation, a new day starts, where 100 bees are created and leave the hive, searching for pollen and nectar. Depending on the percent of pesticides or heavy metal occurrence in the environment, bees will either die or continue to collect pollen and nectar. When forager bees lose energy due to flying and food searching, they will eat nectar but not pollen (Nicholls and de Ibarra 2017) to gain more energy to resume the collecting process.

The accumulated concentration of pesticides and heavy metals in the hive will vary according to collected quantities of pollen and nectar and on the occurrence percentage of either pesticides or heavy metals in the environment, which can be controlled through pest-occur or metal-occur sliders in the model interface. In this model, the number of pesticides and heavy metals in stored pollen or nectar can be dissipated or reduced through degradation and detoxification processes. This model focuses on quantifying contaminants brought back to the hive, not in their fates once stored in the hive.

Figure 13: Model interface, where the type of landscape can be changed by altering the number of houses, plants, and flowers. The existence and occurrence percentage of pesticides and heavy metals can be controlled by switches and sliders.

b. Output: The output variables in this model are the amount of accumulated pollen in mg and nectar in µl, pesticide or heavy metals amount are in µg. The final quantities of pesticides or heavy metals in pollen or nectar are calculated later in the analysis to get the net

concentration of these contaminants in ppm. These outputs are recorded daily.

SCENARIOS AND ANALYSIS

The effect of spatial variation in food resources on bees' foraging behavior and the accumulated quantities of pesticides and heavy metals in hive products are predicted under two main scenarios: urban and agricultural landscapes. Changing the type of landscape is achieved by switching between urban and agricultural patches in the interface; urban landscape is characterized with a larger number of houses with associated variation in floral diversity, where the patches with only one house and more plants represents the agricultural land. Under each scenario, the occurrence percentage of pesticides or heavy metals was set at either 0, 20, 40, or 60 g/L. The lower percentage represents no presence or lower frequency or occurrence of pesticides or heavy metals in the environment; 40 g/L shows the level of toxins presence in the environment where behavior of bees change due to detoxifying toxin activities, 60 g/L represents the threshold limit of toxin occurrence were beyond that bees will not be able to survive. Also in each scenario, flower number will change from 100 to 800 flowers, this change in flower numbers represent the effect of food limitation versus the wealth presence of food source, and it will affect bees foraging behavior, the amount of floral collected product then eventually the amount of accumulated toxins in hive. Each scenario was repeated five times to allow for estimates of variability between simulations.

Outputs from the model were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Two-way ANOVA analysis multiple comparison test was used to determine the spatial variation in differences of pesticide and heavy metal accumulation in pollen and honey. A p-value of less than 0.05 was considered statistically significant for all analyses.

RESULTS

Collected pollen and nectar:

Collected pollen and nectar under different scenarios of landscape settings and different occurrence percentage of pesticides or heavy metals were observed at the end of simulations. Figure 14 shows the variation in the collected amount of pollen between urban versus agricultural landscapes under different conditions of pesticide occurrence in the environment. Figure 15 shows variations in collected nectar amount under heavy metal scenarios. Results in Table 7 showed significant spatial variation in the amount of collected pollen and nectar under different pesticide presence scenarios, while Table 8 summarized the significant differences in the collected pollen and nectar quantities between urban and agricultural landscapes under heavy metal presence scenarios.

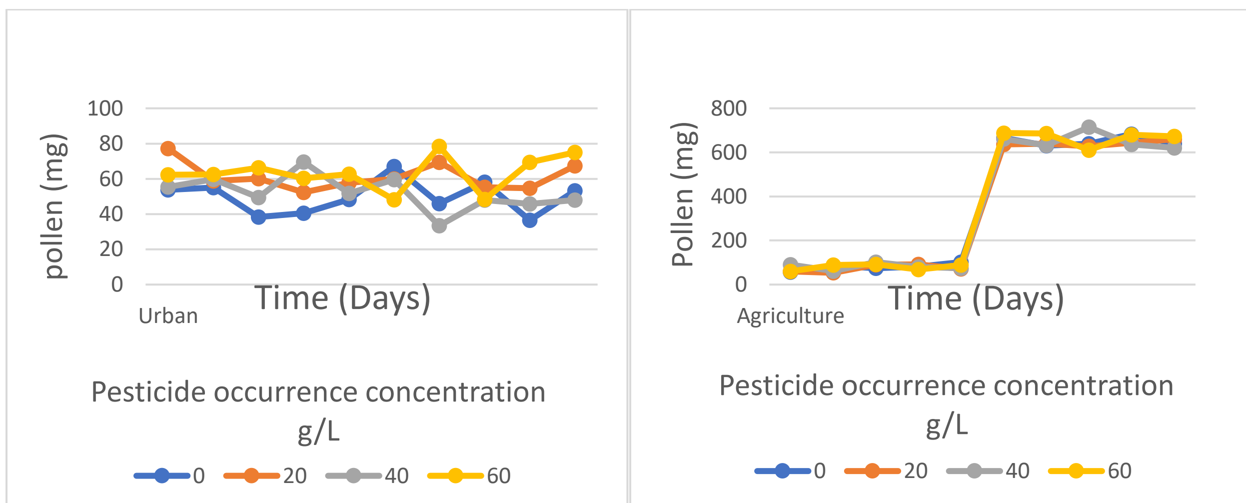


Figure 14: Variation in the amount of collected pollen (mg) between urban and agricultural settings under different pesticide occurrence scenarios over time. The sudden jump in pollen amount represents when the model switched the number of flowers from 100 to 800.

Table 7: Summary of statistical analysis for the spatial variation of collected pollen and nectar under different percentages of pesticide concentrations scenarios.

Collected floral product	Pesticide occurrence in the environment	Agriculture	Urban	Two-way ANOVA multiple comparison test		
	g/L	Mean (mg)	Mean (mg)	SE of difference between means	p-value	t	df
Pollen	0	365.70	49.67	7.32	<0.0001	43.17	27.00
	20	357.50	61.28	7.32	<0.0001	40.46	27.00
	40	367.40	52.06	7.32	<0.0001	43.07	27.00
	60	373.30	63.32	7.32	<0.0001	42.35	27.00
Nectar	0	5280	1447	95.69	<0.0001	40.05	27.00
	20	5270	1463	95.69	<0.0001	39.78	27.00
	40	5218	1423	95.69	<0.0001	39.66	27.00
	60	5374	1426	95.69	<0.0001	41.26	27.00

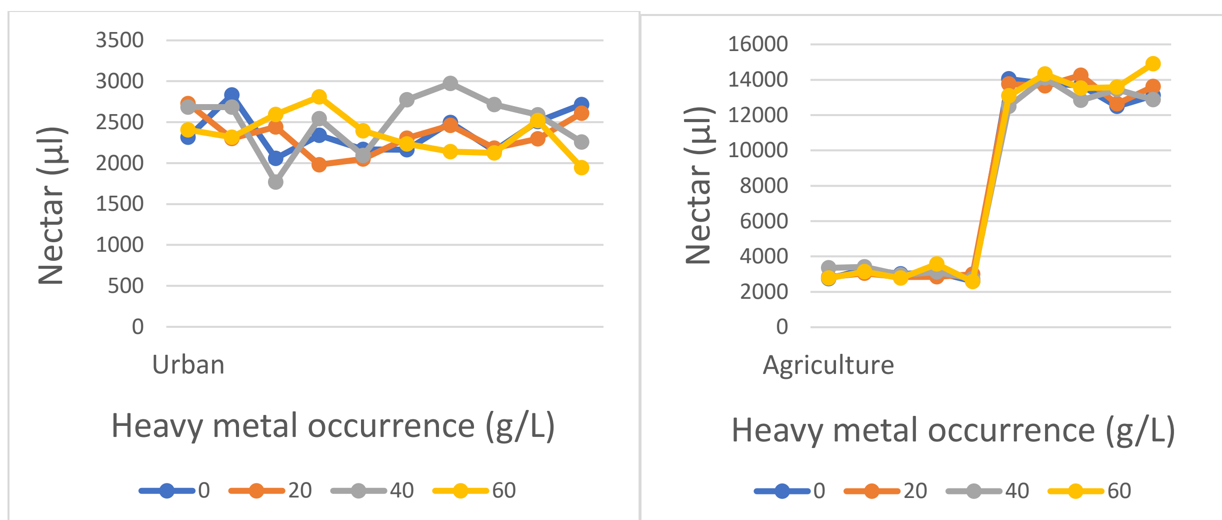


Figure 15: Variation trend in the amount of collected nectar (μl) between urban and agricultural settings under presence of heavy metal scenarios over time. The sudden jump in pollen amount represents when the model switched the number of flowers from 100 to 800.

Table 8: Summary of statistical analysis for the spatial variation of collected pollen and nectar under presence of heavy metal scenarios.

Collected floral product	HM occurrence in the environment	Agriculture	Urban	Two-way ANOVA multiple comparison test		
	g/L	Mean (mg)	Mean (mg)	SE of difference between means	p-value	t	df
Pollen	0	366.50	59.18	9.40	<0.0001	32.69	27.00
	20	366.30	55.17	9.40	<0.0001	33.10	27.00
	40	354.5	61.27	9.40	<0.0001	31.19	27.00
	60	369.40	59.20	9.40	<0.0001	33.00	27.00
	0	8184	2376	197.00	<0.0001	29.48	27.00

Nectar	20	8247	2336	197.00	<0.0001	30.00	27.00
	40	8132	2509	197.00	<0.0001	28.54	27.00
	60	8422	2349	197.00	<0.0001	30.83	27.00

Pesticide Residues in Pollen and Nectar:

After 100 days, and after calculating the concentration of pesticide residues accumulated in hive pollen and nectar, analysis showed there were significant differences in pesticide residues in pollen and nectar collected from the urban or agricultural landscape under medium to high pesticide occurrence percentages in the environment, with p-values < 0.05, as shown in Figures 16 and 17. A summary of statistical analysis results is presented in Table 9. Results also showed significant differences in heavy metal residues in pollen and nectar collected from urban or agricultural landscapes under medium to high pesticide occurrence percentages in the environment, with p-values < 0.05 (Figures 18 and 19). Table 10 presents the summary of statistical analysis for heavy metal variation in pollen and nectar between urban and agriculture.

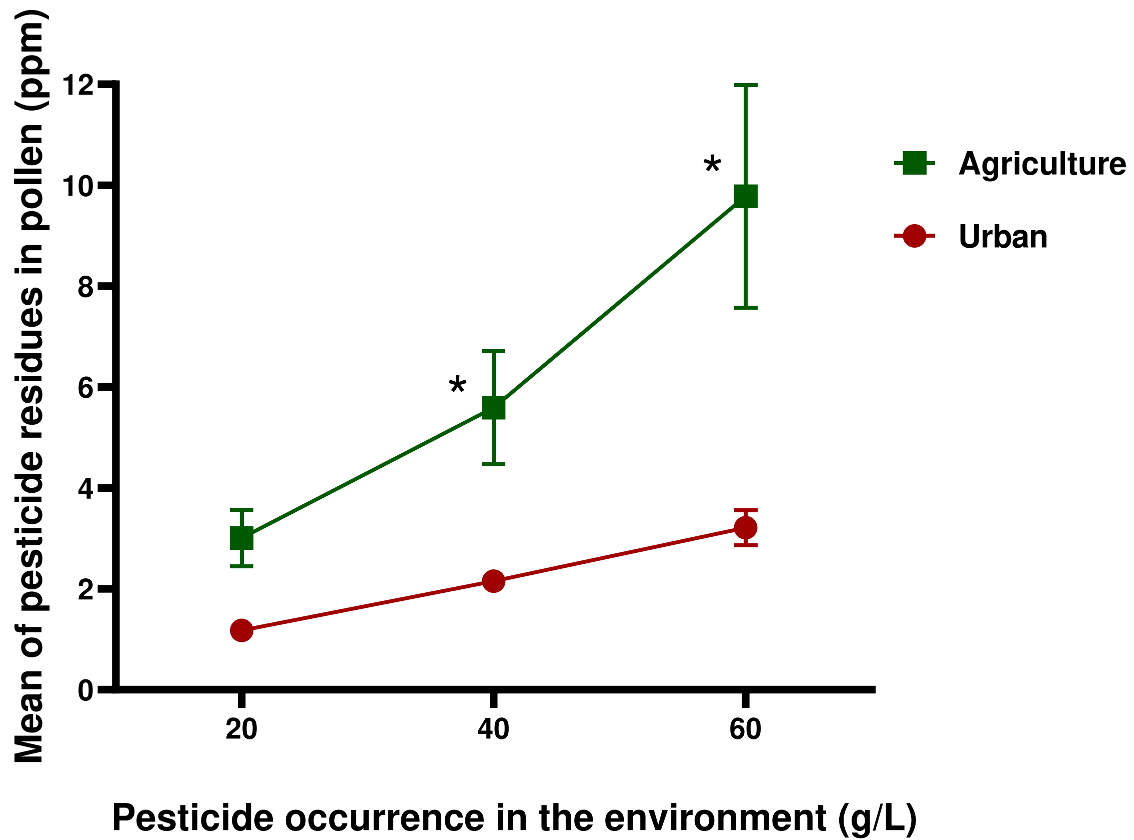


Figure 16: Spatial variation of pesticides residues in collected pollen. A two-way ANOVA multiple comparison test showed significant differences in pesticide residues in pollen collected from urban or agricultural landscapes, with * indicating p-value < 0.05.

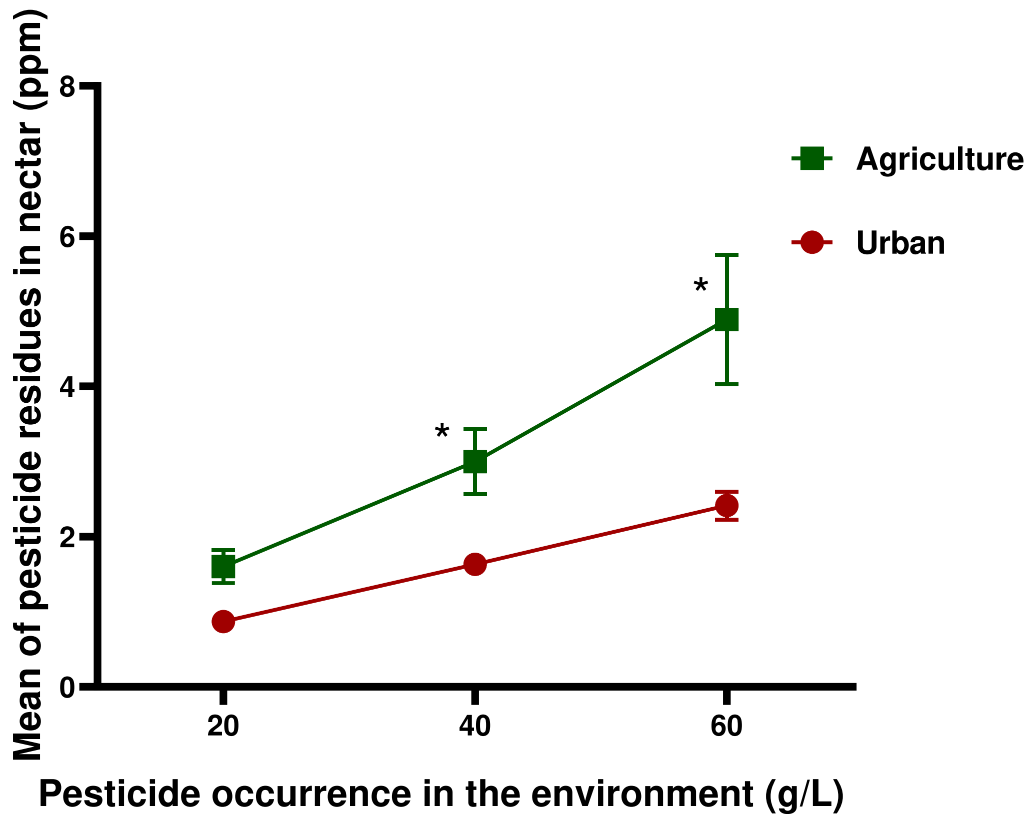


Figure 17: Spatial variation of pesticides residues in collected nectar. A two-way ANOVA multiple comparison test showed significant differences in pesticide residues in nectar collected from agricultural versus urban hives, with * indicating p-value <0.05.

Table 9: Summary of statistical analysis for the spatial variation of pesticide residues in collected pollen and nectar.

Pesticide	Pesticide occurrence in the environment	Agriculture	Urban	Two-way ANOVA multiple comparison test			
	g/L	Mean (ppb)	Mean (ppb)	SE of difference between means	p-value	t	df
	20	3.01	1.18	0.82	0.116	2.23	18

Pesticide in pollen	40	5.60	2.15	0.82	0.001	4.19	18
	60	9.78	3.22	0.82	<0.0001	7.99	18
Pesticide in nectar	20	1.60	0.87	0.34	0.31	2.13	18
	40	2.99	1.63	0.34	0.02	3.98	18
	60	4.89	2.42	0.34	<0.0001	7.21	18

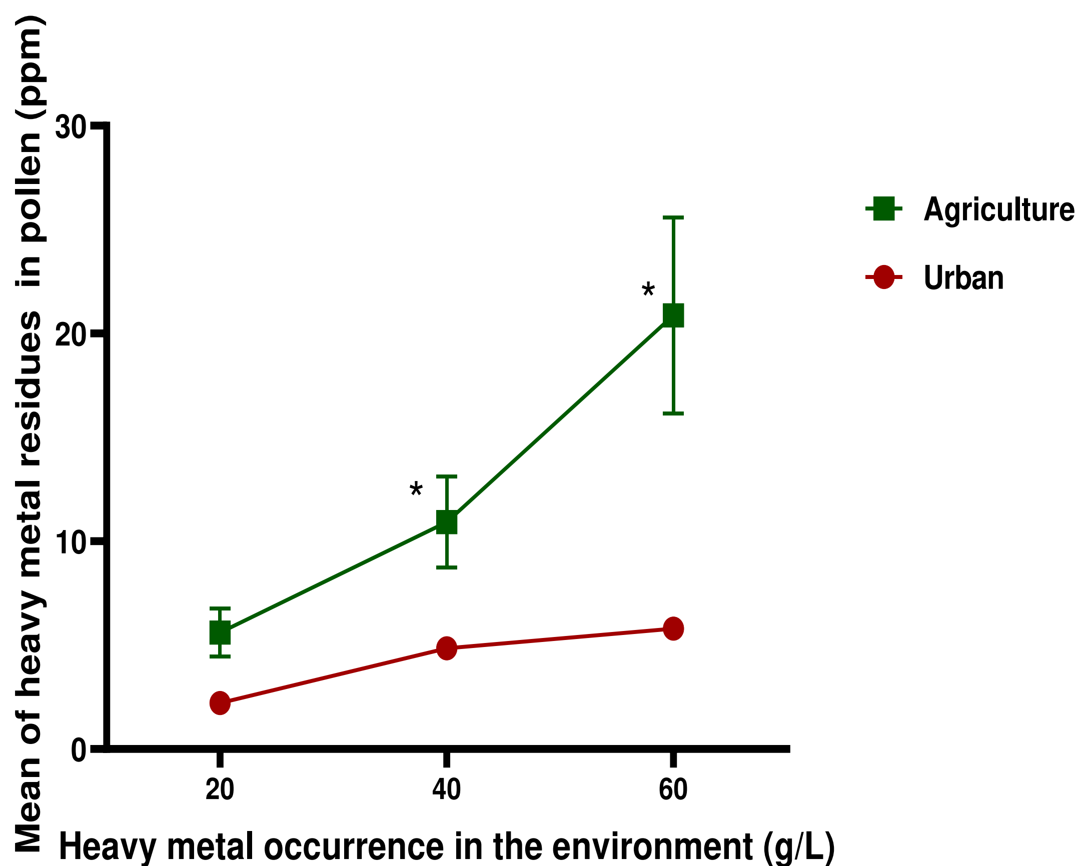


Figure 18. Spatial variation of heavy metal residues in collected pollen. The two-way ANOVA multiple comparison test showed significant differences in heavy metal residues in pollen collected from agricultural versus urban hives with * indicating p-value < 0.05.

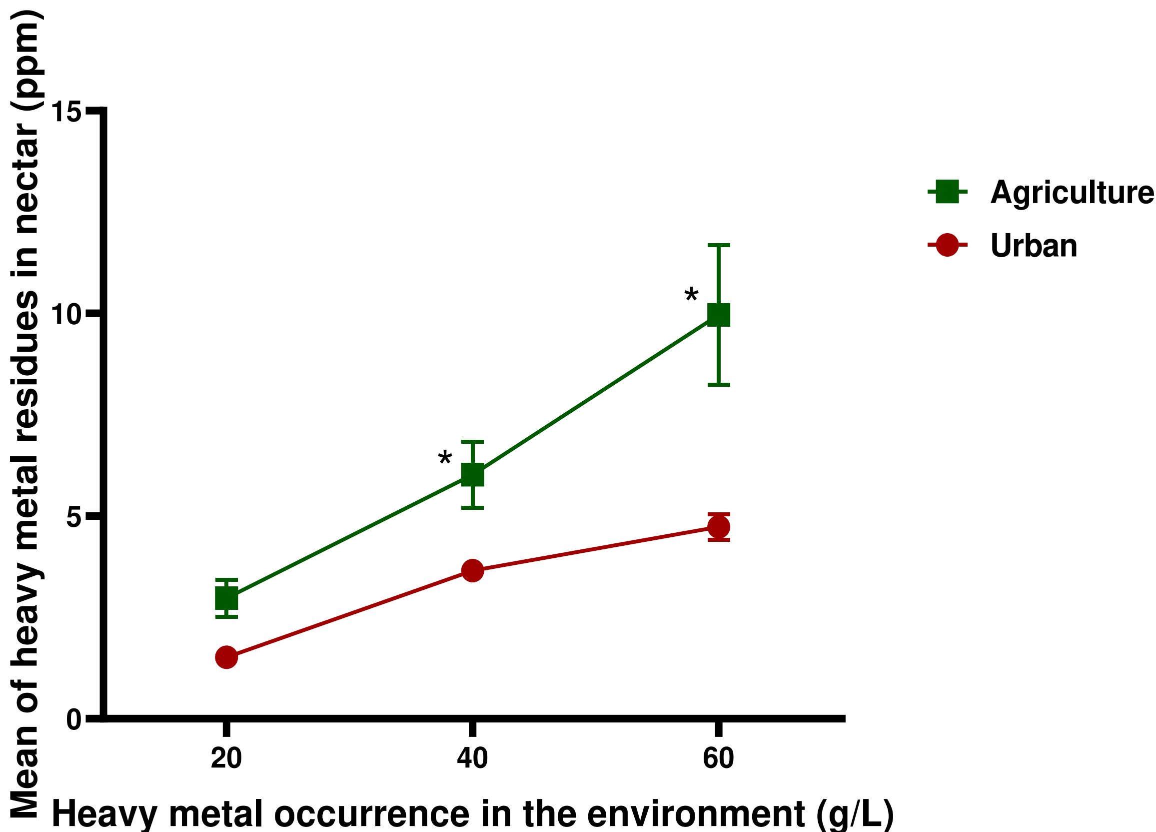


Figure 19. Spatial variation of heavy metal residues in collected pollen. The two-way ANOVA multiple comparison test showed significant differences in heavy metal residues in nectar collected from agricultural versus urban hives with * indicating p-value < 0.05.

Table 10: Summary of statistical analysis for the spatial variation of heavy metal residues in collected pollen and nectar.

Heavy metal	Heavy metal occurrence in the environment	Agriculture	Urban	Two-way ANOVA multiple comparison test			
	g/L	Mean (ppb)	Mean (ppb)	SE of difference between means	p-value	t	df
	20	5.61	2.22	2.25	0.38	1.51	18

Heavy metal in pollen	40	10.92	4.85	2.25	0.04	2.76	18
	60	20.88	5.79	2.25	<0.0001	6.71	18
Heavy metal in nectar	20	2.97	1.52	0.82	0.26	1.76	18
	40	6.02	3.66	0.82	0.03	2.85	18
	60	9.98	4.74	0.82	<0.0001	6.33	18

DISCUSSION

The main objective of this modeling exercise was to assess the spatial variation of honey bees' exposure to heterogeneous types of environmental toxins and how much of these toxins accumulate in a beehive as a result of the foraging process. The model considers multiple factors that would play a role in contaminants' accumulation inside the hive; one of these factors was the floral diversity associated with an urban setting, which means a higher chance for bees to collect pollen or nectar; however, the analysis showed that collected pollen and nectar quantities were higher in the agricultural setting (Figures 14 and 15) with statistically significant variation (Table 7 and 8), which supports earlier findings about bees' tendency to forage on a few selected species (Baum et al. 2004). This significant variation in the amount of collected floral products affected the accumulated amounts of pesticides and heavy metals in the hive; simulations and analysis indicated significant spatial variation effects on the pesticides in collected pollen and nectar (Table 9, Figures 16, 17). The results also showed significant variation in heavy metal accumulation in the collected pollen and nectar inside the beehive (Table 10, Figures 18, 19). The variation was larger at the agricultural landscape with higher toxin buildup in the collected floral products in the hive. Because of the higher toxin contents that have accumulated in the collected floral products inside the hive, it is illogical to draw the conclusion that agricultural

landscapes are detrimental for bee colonies. The honey bees, on the other hand, can be exposed to these toxins in various ways or have them build up inside the beehive, such as direct contact (Ward et al. 2022), or toxin accumulation in other hive collected materials like wax (Ravoet et al. 2015). Toxins' persistence and mobility could also affect how bees are exposed to these contaminants and how they are deposited in the hive (Krupke et al. 2012). Furthermore, beehive location and distance from a contaminated source would also affect the quantity and quality of toxins that bees are exposed to (Salman et al. 2022).

The main difference we used in our model to distinguish between urban and agricultural landscape was the number of houses, which reflects the number of residences per area. We incorporated this difference based on the definition of urban versus agriculture. While agricultural land is defined by the Colorado General Assembly as "A parcel of land, whether located in an incorporated or unincorporated area, and was used the previous two years and is currently used as a farm or ranch" (Leg.colorado.gov), urban areas are defined by the United States Census Bureau as "continuously built-up areas with a population of 2,500–50,000 or more and an average density of at least 1,000 inhabitants per square mile" (Census.gov, 2022). However, these definitions were not enough to make a distinct variation between the two landscapes, so, we used another variable to distinguish urban areas by having higher floral diversity in the urban settings (Martins et al. 2017). Yet, this factor did not show to have a large impact on bee's foraging behavior and the amount of accumulated pollen and nectar in the hive as the accumulated quantities of floral products in the hive was still higher in the agricultural setting, and that opens the door to re-define the difference between urban and agricultural landscape from an ecological perspective.

Experimental research has reported the presence of pesticides in pollen or honey (e.g., de

Olivera et al. 2016; Mitchell et al. 2017) or heavy metals (Al Naggar et al. 2017). However, a few experimental studies reported contradictory results about the effect of spatial variation of toxins in pollen and nectar. Calatayud-Vernich et al. (2018) found higher pesticides residues in pollen collected from hives located in agricultural areas, while Mahé et al. (2021) found that honey bees were exposed to higher levels of pesticides and metals in the urban areas compared with the agricultural ones. Multiple factors could serve as an explanation for these contradictions, which can be taken into consideration in designing an experiment or simulation research using scenarios, such as seasonal variation (Chauzat et al. 2011), climatic factors (Noyes et al. 2009), hive management (VanEngelsdorp et al. 2008), or types of contaminants and their mode of application as well as their persistence in the environment (Goulson et al. 2018).

Several models served as an excellent reference to elucidate the factors behind Colony Collapse Disorder. Some of them combined the dynamics of honeybee colonies along with the impact of varroa mites and the epidemiology of varroa-transmitted virus associated with pesticide treatment of this parasite like the BEEHAVE model (Becher et al. 2014). Other models narrowed in on just one stressor, such as bees' exposure to pesticides (Rumkee et al. 2017), interactions within the colony, and food management (Schmickl & Crailsheim 2007). Considering the climatic effects, Switanek et al. (2017) focused on the effects of long-term weather variabilities on honey bee winter mortality rate. Our model is intended as a tool to explain multifactor variables contributing to the collapse phenomenon. Beyond those modeled here, many other factors are known to adversely impact the honey bee population, including other environmental contaminants, habitat fragmentation, climate change, and pathogens. The model presented in this study sets the stage to address the impact of these variables collectively.

LITERATURE CITED

Al Naggar, Y. A., Naiem, E. S. A., Seif, A. I., & Mona, M. H. (2013). Honey bees and their products as a bio-indicator of environmental pollution with heavy metals. *Mellifera*, *13*, 1-20.

Balayiannis, G., & Balayiannis, P. (2008). Bee honey as an environmental bioindicator of pesticides' occurrence in six agricultural areas of Greece. Archives of Environmental Contamination and Toxicology, 55(3), 462-470.

Baum, K. A., Rubink, W. L., Coulson, R. N., & Bryant Jr, V. M. (2004). Pollen selection by feral honey bee (Hymenoptera: Apidae) colonies in a coastal prairie landscape. Environmental Entomology, 33(3), 727-739.

Becher, M. A., Grimm, V., Knapp, J., Horn, J., Twiston-Davies, G., & Osborne, J. L. (2016). BEESCOUT: A model of bee scouting behaviour and a software tool for characterizing nectar/pollen landscapes for BEEHAVE. Ecological modelling, 340, 126-133.

Becher, M. A., Grimm, V., Thorbek, P., Horn, J., Kennedy, P. J., & Osborne, J. L. (2014). BEEHAVE: a systems model of honeybee colony dynamics and foraging to explore multifactorial causes of colony failure. Journal of Applied Ecology, 51(2), 470-482.

Becher, M. A., Osborne, J. L., Thorbek, P., Kennedy, P. J., & Grimm, V. (2013). Towards a systems approach for understanding honeybee decline: a stocktaking and synthesis of existing models. Journal of Applied Ecology, 50(4), 868-880.

Boone, R. B., & Galvin, K. A. (2014). Simulation as an approach to social-ecological integration, with an emphasis on agent-based modeling. Understanding Society and Natural Resources: Forging New Strands of Integration Across the Social Sciences, 179-202.

Borsuk, G., Sulborska, A., Stawiarz, E., Olszewski, K., Wiącek, D., Ramzi, N., ... & Jędryczka, M. (2021). Capacity of honeybees to remove heavy metals from nectar and excrete the contaminants from their bodies. Apidologie, 52(6), 1098-1111.

Calatayud-Vernich, P., Calatayud, F., Simó, E., & Picó, Y. (2018). Pesticide residues in honey bees, pollen and beeswax: Assessing beehive exposure. *Environmental Pollution*, *241*, 106-114.

Castilhos, D., Bergamo, G. C., Gramacho, K. P., & Gonçalves, L. S. (2019). Bee colony losses in Brazil: a 5-year online survey. Apidologie, 50(3), 263-272.

Celli, G., & Maccagnani, B. (2003). Honey bees as bioindicators of environmental pollution. Bulletin of Insectology, 56(1), 137-139.

Chauzat, M. P., Martel, A. C., Cougoule, N., Porta, P., Lachaize, J., Zeggane, S., ... & Faucon, J. P. (2011). An assessment of honeybee colony matrices, Apis mellifera (Hymenoptera: Apidae) to monitor pesticide presence in continental France. *Environmental Toxicology and Chemistry*, *30*(1), 103-111.

Colorado General Assembly (2022, September 14). Colorado revised statutes 2016. Retrieved from: https://leg.colorado.gov/sites/default/files/images/olls/crs2016-title-39.pdf

Conti, M. E., & Botrè, F. (2001). Honeybees and their products as potential bioindicators of heavy metals contamination. Environmental monitoring and assessment, 69(3), 267-282.

de Oliveira, R. C., do Nascimento Queiroz, S. C., da Luz, C. F. P., Porto, R. S., & Rath, S. (2016). Bee pollen as a bioindicator of environmental pesticide contamination. Chemosphere, 163, 525-534.

Di, N., Hladun, K. R., Zhang, K., Liu, T. X., & Trumble, J. T. (2016). Laboratory bioassays on the impact of cadmium, copper and lead on the development and survival of honeybee (Apis mellifera L.) larvae and foragers. Chemosphere, 152, 530-538.

Duzgoren-Aydin, N. S., Wong, C., Aydin, A., Song, Z., You, M., & Li, X. D. (2006). Heavy metal contamination and distribution in the urban environment of Guangzhou, SE China. Environmental geochemistry and health, 28(4), 375-391.

Evans, J. D., & Schwarz, R. S. (2011). Bees brought to their knees: microbes affecting honey bee health. Trends in microbiology, 19(12), 614-620.

Goulson, D., Thompson, J., & Croombs, A. (2018). Rapid rise in toxic load for bees revealed by analysis of pesticide use in Great Britain. PeerJ, 6, e5255.

Hanfi, M. Y., Mostafa, M. Y., & Zhukovsky, M. V. (2020). Heavy metal contamination in urban surface sediments: sources, distribution, contamination control, and remediation. Environmental Monitoring and Assessment, 192(1), 1-21.

Henry, M., Becher, M. A., Osborne, J. L., Kennedy, P. J., Aupinel, P., Bretagnolle, V., ... & Requier, F. (2017). Predictive systems models can help elucidate bee declines driven by multiple combined stressors. Apidologie, 48(3), 328-339.

Hladun, K. R., Di, N., Liu, T. X., & Trumble, J. T. (2016). Metal contaminant accumulation in the hive: Consequences for whole-colony health and brood production in the honey bee (Apis mellifera L.). Environmental Toxicology and Chemistry, 35(2), 322-329.

Johnson, B. R., & Nieh, J. C. (2010). Modeling the adaptive role of negative signaling in honey bee intraspecific competition. Journal of Insect Behavior, 23(6), 459-471.

Johnson, R. M., Ellis, M. D., Mullin, C. A., & Frazier, M. (2010). Pesticides and honey bee toxicity–USA. Apidologie, 41(3), 312-331.

Karlik, J. F. (2009, May). Calculation of pesticide residues on roses using California's total reporting data and first order chemical kinetics. In V International Symposium on Rose Research and Cultivation 870 (pp. 121-128).

Krupke, C. H., Hunt, G. J., Eitzer, B. D., Andino, G., & Given, K. (2012). Multiple routes of pesticide exposure for honey bees living near agricultural fields. PLoS one, 7(1), e29268.

Lambert, O., Piroux, M., Puyo, S., Thorin, C., Larhantec, M., Delbac, F., & Pouliquen, H. (2012). Bees, honey and pollen as sentinels for lead environmental contamination. Environmental Pollution, 170, 254-259.

Mahé, C., Jumarie, C., & Boily, M. (2021). The countryside or the city: Which environment is better for the honeybee?. Environmental Research, 195, 110784.

Malhat, F. M., Haggag, M. N., Loutfy, N. M., Osman, M. A., & Ahmed, M. T. (2015). Residues of organochlorine and synthetic pyrethroid pesticides in honey, an indicator of ambient environment, a pilot study. Chemosphere, 120, 457-461.

Martins, K. T., Gonzalez, A., & Lechowicz, M. J. (2017). Patterns of pollinator turnover and increasing diversity associated with urban habitats. Urban Ecosystems, 20, 1359-1371.

Matin, G., Kargar, N., & Buyukisik, H. B. (2016). Bio-monitoring of cadmium, lead, arsenic and mercury in industrial districts of Izmir, Turkey by using honey bees, propolis and pine tree leaves. Ecological Engineering, 90, 331-335.

Mitchell, E. A., Mulhauser, B., Mulot, M., Mutabazi, A., Glauser, G., & Aebi, A. (2017). A worldwide survey of neonicotinoids in honey. Science, 358(6359), 109-111.

Mujić, I., Alibabić, V., Jokić, S., Galijašević, E., Jukić, D., Šekulja, D., & Bajramović, M. (2011). Determination of pesticides, heavy metals, radioactive substances, and antibiotic residues in honey. Pol J Environ Stud, 20(3), 719-724.

Nicholls, E., & Hempel de Ibarra, N. (2017). Assessment of pollen rewards by foraging bees. Functional Ecology, 31(1), 76-87.

Noyes, P. D., McElwee, M. K., Miller, H. D., Clark, B. W., Van Tiem, L. A., Walcott, K. C., ... & Levin, E. D. (2009). The toxicology of climate change: environmental contaminants in a warming world. Environment international, 35(6), 971-986.

O'Neal, S. T., Brewster, C. C., Bloomquist, J. R., & Anderson, T. D. (2017). Amitraz and its metabolite modulate honey bee cardiac function and tolerance to viral infection. Journal of invertebrate pathology, 149, 119-126.

Ravoet, J., Reybroeck, W., & de Graaf, D. C. (2015). Pesticides for apicultural and/or agricultural application found in Belgian honey bee wax combs. Bulletin of environmental contamination and toxicology, 94, 543-548.

Rumkee, J. C., Becher, M. A., Thorbek, P., & Osborne, J. L. (2017). Modeling effects of honeybee behaviors on the distribution of pesticide in nectar within a hive and resultant in-hive exposure. Environmental Science & Technology, 51(12), 6908-6917.

Salman, N. H., Mok Sam, L., Ador, K., Binjamin, B., Johny-Hasbulah, M. I. J., & Benedick, S. (2022). Linking Measure of the Tropical Stingless Bee (Apidae, Meliponini, and Heterotrigona itama) Honey Quality with Hives Distance to the Source of Heavy Metal Pollution in Urban and Industrial Areas in Sabah, Borneo. Journal of Toxicology, 2022.

Stout, J. C., & Goulson, D. (2001). The use of conspecific and interspecific scent marks by foraging bumblebees and honeybees. Animal behaviour, 62(1), 183-189.

Switanek, M., Crailsheim, K., Truhetz, H., & Brodschneider, R. (2017). Modelling seasonal effects of temperature and precipitation on honey bee winter mortality in a temperate climate. Science of the Total Environment, 579, 1581-1587.

T O’Neal, S., Anderson, T. D., & Wu-Smart, J. Y. (2018). Interactions between pesticides and pathogen susceptibility in honey bees. Current opinion in insect science, 26, 57-62.

Tison, L., Hahn, M. L., Holtz, S., Rößner, A., Greggers, U., Bischoff, G., & Menzel, R. (2016). Honey bees’ behavior is impaired by chronic exposure to the neonicotinoid thiacloprid in the field. Environmental science & technology, 50(13), 7218-7227.

United States Census Bureau. (2022, September 14). The Urban and Rural Classifications. Retrieved from: https://www2.census.gov/geo/pdfs/reference/GARM/Ch12GARM.pdf

USDA ARS. (2020, June 5). ARS Honey Bee health. https://www.ars.usda.gov/oc/br/ccd/index/

VanEngelsdorp, D., Hayes Jr, J., Underwood, R. M., & Pettis, J. (2008). A survey of honey bee colony losses in the US, fall 2007 to spring 2008. PloS one, 3(12), e4071.

Van Der Steen, J. J., de Kraker, J., & Grotenhuis, T. (2012). Spatial and temporal variation of metal concentrations in adult honeybees (Apis mellifera L.). Environmental monitoring and assessment, 184(7), 4119-4126.

Vance, J. T., Williams, J. B., Elekonich, M. M., & Roberts, S. P. (2009). The effects of age and behavioral development on honey bee (Apis mellifera) flight performance. Journal of Experimental Biology, 212(16), 2604-2611.

Villa, S., Vighi, M., Finizio, A., & Bolchi Serini, G. (2000). Risk assessment for honeybees from pesticide-exposed pollen. Ecotoxicology, 9(4), 287-297.

Waiker, P., Ulus, Y., Tsui, M. T. K., & Rueppell, O. (2022). Mercury accumulation in honey bees trends upward with urbanization in the USA. Agricultural & Environmental Letters, 7(2), e20083.

Ward, L. T., Hladik, M. L., Guzman, A., Winsemius, S., Bautista, A., Kremen, C., & Mills, N. J. (2022). Pesticide exposure of wild bees and honey bees foraging from field border flowers in intensively managed agriculture areas. Science of the Total Environment, 831, 154697.

Wang, W., Bai, J., Zhang, G., Wang, X., Jia, J., Cui, B., & Liu, X. (2017). Depth-distribution, possible sources, and toxic risk assessment of organochlorine pesticides (OCPs) in different river sediment cores affected by urbanization and reclamation in a Chinese delta. Environmental Pollution, 230, 1062-1072.

Wilensky, U. Netlogo; Center for Connected Learning and Computer-Based Modeling: Evanston, IL, 1999.

Zawislak, J., Adamczyk, J., Johnson, D. R., Lorenz, G., Black, J., Hornsby, Q., ... & Joshi, N. (2019). Comprehensive survey of area-wide agricultural pesticide use in Southern United States row crops and potential impact on honey bee colonies. Insects, 10(9), 280.

CHAPTER 5: INTRODUCTION TO SYNERGISTIC TOXICITY IN MAMMALIAN CELLS CHAPTER

The development of agricultural methods and the industrial revolution have led to a rise in the accumulation of pesticides and heavy metals in the environment. Synthetic agrochemicals like pesticides were widely used in urban and agricultural settings to protect against various diseases in plants, animals, and people (Nicolopoulou-Stamati et al. 2016). Since the late 1960s, after Rachel Carson published her well-known book (*The Silent Spring,* 1960) on the effects of DDT pesticides on the environment, the extensive and unrestrained use of pesticides on the ecosystem has attracted attention. Two chemicals we used in the following chapter that are classified as agrochemical pesticides, one is designed for insects: Imidacloprid, and the other one is Piperonal butoxide, which is a synergist chemical used to induce the activity of different pesticides.

Imidacloprid is a systemic insecticide that works on insects' central nervous systems (Bai et al. 1991) that is used to control flies, turf insects, soil insects, and fleas on pets as well as termites and sucking insects (Gervais et al. 2010). It is a member of the neonicotinoid chemical class that is designed to mimic the properties of nicotine. This insecticide affects the insect nervous system's ability to transmit stimuli, which is how it functions. It specifically blocks the nicotinergic neural pathway (Liu and Casida 1993). Imidacloprid paralyzes and eventually kills insects by inhibiting nicotinic acetylcholine receptors, which stops acetylcholine from transferring impulses between nerves (Sur and Stork 2003). Imidacloprid is more poisonous to insects than to mammals because it binds insect neuron receptors considerably more strongly than it binds mammal neuron receptors. (Tomizawa and Casida 2003).

On the other hand, **Piperonyl butoxide** is a pesticide synergist that was mainly developed to enhance the performance of insecticide pyrethrum in the early 1940s (Glynne-Jones 1998). It

mainly works by impeding the insect's defense mechanisms by inhibiting the mixed-function oxidase (MFO) system of the insect (Casida 1970). Piperonyl butoxide is considered one of the main components of 1600- 1700 registered pesticides (US EPA 2005). PBO undergoes photolysis in the environment where it is quickly degraded and digested by soil microorganisms; the estimated half-life for PBO is eight hours (Maples 2014).

Heavy metals, together with pesticides and other synthetic substances, have a dangerous effect on human health. Heavy metals are metals with high densities or atomic weights that are dangerous in low quantities. They can be found naturally in the soil or introduced through human activity (Singh et al. 2011). Due to their wide range of uses, questions have been raised concerning their potential effects on both human and environmental health. The level and frequency of exposure, as well as the pace at which these compounds are absorbed, metabolized, and eliminated, all influence the potentially harmful effects of pesticides and heavy metals on people and other species (Tchounwou et al. 2012, Nesheim et al. 1978). Two heavy metals found in the honey samples (chapter two) we used in the following chapter for toxicity testing, Lead (Pb) and Selenium (Se), defined below.

Lead (Pb), a heavy naturally occurring element, is found in the crust of the earth, usually found combined with zinc, copper, and iron (Ghazi and Millette 2004). However, human activities like mining, manufacturing and the burning of fossil fuels are what produce the majority of lead. The items that included lead were paint, cosmetics, pipes, and gasoline. The three different forms of lead (elemental, inorganic, and organic lead) are considered extremely toxic. Lead can result in neurotoxicity by replacing calcium and zinc, and it can prevent neurotransmitters from being released, which disrupts mental processes like cognition, memory, and language (Cookman et al.,

1987). The demise of the Roman Empire is thought to have a direct connection to lead toxicity (Ara and Usmani 2015).

Selenium (Se) is a trace metal that naturally exists in a variety of inorganic forms, such as selenide, selenate, and selenite. Selenium is typically found in sulfide ores of several metals as an impurity, replacing a small amount of the sulfur (Cutter 1985). Naturally, certain soils have high levels of selenium, soil's selenium content depends on the makeup of the parent material as well as any leaching or subsequent processes that may have added selenium during soil formation (Shamberger 1981). Burning coal, mining and smelting sulfide ores are major anthropogenic sources of selenium (Mehdi et al. 2013). Selenium is a crucial nutrient that is fundamentally important to human life. Low selenium levels may contribute to adverse consequences for disease susceptibility like cancer, muscular dystrophy, malaria, and cardiovascular disease (Foster and Sumar 1997). However, high selenium consumption can be poisonous and have negative health effects (Tinggi 2003).

Different scientific techniques have been developed to detect the toxicity effect of different synthetic and natural substances, among these techniques is cell culture. **Cell culture** is the process of growing human, animal, or insects' cells under controlled artificial conditions supplemented with a medium containing nutrients and growth factors (Segeritz, and Vallier 2017). Early in the 20th century, cell culture was employed to investigate tissue development, virus biology, the creation of vaccines, the function of genes, the manufacture of biopharmaceuticals and used in toxicity testing to study the effects of drugs and toxic compounds (Segeritz, and Vallier 2017). The benefit of cellular toxicity tests in human or animal cells is to simulate the response of cells to chemical exposure and serves as a model of a target tissue in the animal or human body (Ekwall et al. 1990). Different ranges of toxicological studies used cell culture to test the toxicity of a

substance and its ability to change different cell function like cytotoxicity, genotoxicity, apoptotic cell death, oxidative stress effect and others (Ferro and Doyle 2001). These tests were the main ones used in our study to measure the toxicity effects of pesticide and heavy metal combinations.

Cytotoxicity tests refer to the ability of a chemical agent or drug to destroy a cell or inhibit its ability to grow and divide after some period of incubation (Riss et. al. 2019). One of the assays we used to measure the cytotoxicity of pesticide and heavy metal combinations is called **cell growth inhibition assay**, where we measured the inhibitory effect of these chemicals and their combination using a Coulter counter. This instrument works based on the detection of variations in electrical resistance cause by cells suspended in a conductive liquid, where the voltage pulses give information on the number of cells moving through the suspension (Beckman Coulter Life Sciences 2023).

Another assay we used to measure the cytotoxicity of pesticides and heavy metals is called **clonogenic cell survival**. This assay is based on the ability of a cell to proliferate, reproduce and to form a larger colony (Munshi et al. 2005), and how this ability to proliferate and form colonies is affected by treatment with different doses and combinations of selected chemicals. This assay was performed manually by treating cells with chemicals, incubating them for a specific time, staining the cell in the plates, then counting the number of colonies formed under each treatment.

Apoptosis assay is another test we used to evaluate the cytotoxicity of pesticides and heavy metals. Apoptosis refers to the process of programmed cell death, which is characterized by nuclear shrinkage as well as the compaction and margination of chromatin, and subsequently, the presence of nuclear fragmentation and formation of apoptotic bodies (Bardales et al. 1996). Cell apoptosis can be triggered through different pathways, and we investigated two of these pathways to

understand the toxicity mechanism by which these chemicals work toward causing cell death or apoptosis. One of these mechanisms is through the generation of **reactive oxygen species (ROS)**. Increased levels of ROS in the cell upon exposure to toxins or chemicals cause disruption of redox signaling and damage various cell organelles as well as lipids, proteins, and nucleic acids. This damage can later trigger the cell death process (Redza-Dutordoir and Averill-Bates 2016). In the assay we used to measure the effect of chemicals on the amount of ROS, or what is called oxidative stress analysis. The main idea behind this analysis is that by employing an indicator, (Carboxy-H2DCFDA) in our experiments, which will oxidize in the presence of ROS and cause it to glow green (Buglewicz et al. 2020), we can determine how much ROS is present in a sample by measuring how much green fluorescence is present using a spectrophotometer.

Another pathway we used to measure the toxicity of pesticides and heavy metals is **genotoxicity**, which refers to the ability of an agent or toxins to cause damage in the genetic material of a cell (Ren et al. 2017). We used an assay called **sister chromatid exchange (SCE)** to measure the damage in the DNA caused by pesticide-heavy metal combinations. SCE refers to the exchange of DNA fragments between two sister chromatids (Tumini and Aguilera 2021). To identify sister chromatid DNA exchange, chromatids must be differentially stained in order to visually identify these processes in metaphase chromosomes (Tumini and Aguilera 2021). We can measure the genotoxic ability of agents through counting the number of exchanged DNA fragments, which proportionally increase with the genotoxic ability of an agent. We used all the aforementioned assays to determine the synergistic cytotoxicity and genotoxicity of selected pesticides and heavy metals that were detected in honey samples in the following chapter.

LITERATURE CITED

Ara, A., & Usmani, J. A. (2015). Lead toxicity: a review. Interdisciplinary toxicology, 8(2), 55-64.

Bai, D., Lummis, S. C., Leicht, W., Breer, H., & Sattelle, D. B. (1991). Actions of imidacloprid and a related nitromethylene on cholinergic receptors of an identified insect motor neurone. Pesticide science, 33(2), 197-204.

Bardales, R. H., Hailey, L. S., Xie, S. S., Schaefer, R. F., & Hsu, S. M. (1996). In situ apoptosis assay for the detection of early acute myocardial infarction. The American journal of pathology, 149(3), 821.

Buglewicz, D. J., Mussallem, J. T., Haskins, A. H., Su, C., Maeda, J., & Kato, T. A. (2020). Cytotoxicity and mutagenicity of narrowband UVB to mammalian cells. Genes, 11(6), 646.

Carson, R. (2009). Silent spring. 1962.

Casida, J. E. (1970). MFO involvement in the biochemistry of insecticide synergists. J. Agric. Food Chem. 18, 753–772.

Cellular analysis using the Coulter Principle. Beckman Coulter Life Sciences. (n.d.). Retrieved February 18, 2023, from https://www.beckman.com/resources/reading-material/application-notes/cellular-analysis-using-the-coulter-principle#:~:text=The%20Coulter%20Principle%20is%20based,they%20function%20as%20discrete%20insulators.

Cookman, G. R., King, W., & Regan, C. M. (1987). Chronic low-level lead exposure impairs embryonic to adult conversion of the neural cell adhesion molecule. Journal of neurochemistry, 49(2), 399-403.

Cutter, G. A. (1985). Determination of selenium speciation in biogenic particles and sediments. Analytical Chemistry, 57(14), 2951-2955.

Ekwall, B., Silano, V., Paganuzzi-Stammati, A., & Zucco, F. (1990). Toxicity Tests with Mammalian Cell Cultures (eds Bourdeau, P. et al.) Chapter 7.

Ferro, M., & Doyle, A. (2001). Standardisation for in vitro toxicity tests. Cell culture methods for in vitro toxicology, 1-8.

Foster, L. H., & Sumar, S. (1997). Selenium in health and disease: a review. Critical Reviews in Food Science & Nutrition, 37(3), 211-228.

Gervais, J. A., Luukinen, B., Buhl, K., & Stone, D. (2010). Imidacloprid technical fact sheet. National Pesticide Information Center, Oregon State University Extension Services, 5, 1-7.

Ghazi, A. M., & Millette, J. R. (2004). Environmental forensic application of lead isotope ratio determination: a case study using laser ablation sector ICP-MS. Environmental Forensics, 5(2), 97-108.

Glynne-Jones, D. (1998). History of PBO In "PBO—The Insecticide Synergist" (D. Glynne Jones, ed.). Academic Press, San Diego.

Liu, M. Y., & Casida, J. E. (1993). High affinity binding of [3H] imidacloprid in the insect acetylcholine receptor. Pesticide biochemistry and physiology, 46(1), 40-46.

Maples, D. (2014). Piperonyl Butoxide. Encyclopedia of Toxicology, 958-959.

Mehdi, Y., Hornick, J. L., Istasse, L., & Dufrasne, I. (2013). Selenium in the environment, metabolism and involvement in body functions. Molecules, 18(3), 3292-3311.

Munshi, A., Hobbs, M., & Meyn, R. E. (2005). Clonogenic cell survival assay. Chemosensitivity: Volume 1 In Vitro Assays, 21-28.

Nesheim, O. N., & Criswell, J. T. (1978). Toxicity of pesticides. Oklahoma State University, Cooperative Extension Service.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: the urgent need for a new concept in agriculture. Frontiers in public health, 4, 148.

Redza-Dutordoir, M., & Averill-Bates, D. A. (2016). Activation of apoptosis signalling pathways by reactive oxygen species. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1863(12), 2977-2992.

Ren, N., Atyah, M., Chen, W. Y., & Zhou, C. H. (2017). The various aspects of genetic and epigenetic toxicology: testing methods and clinical applications. Journal of Translational Medicine, 15, 1-13.

Riss, T., Niles, A., Moravec, R., Karassina, N., & Vidugiriene, J. (2019). Cytotoxicity assays: in vitro methods to measure dead cells. Assay guidance manual [internet].

Segeritz, C. P., & Vallier, L. (2017). Cell culture: Growing cells as model systems in vitro. In Basic science methods for clinical researchers (pp. 151-172). Academic Press.

Shamberger, R. J. (1981). Selenium in the environment. Science of the total environment, 17(1), 59-74.

Singh, R., Gautam, N., Mishra, A., & Gupta, R. (2011). Heavy metals and living systems: An overview. Indian journal of pharmacology, 43(3), 246.

Sur, R., & Stork, A. (2003). Uptake, translocation and metabolism of imidacloprid in plants. Bulletin of insectology, 56, 35-40.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metal toxicity and the environment. Molecular, clinical and environmental toxicology, 133-164.

Tinggi, U. (2003). Essentiality and toxicity of selenium and its status in Australia: a review. Toxicology letters, 137(1-2), 103-110.

Tomizawa, M., & Casida, J. E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annual review of entomology, 48, 339.

Tumini, E., & Aguilera, A. (2021). The Sister-chromatid exchange assay in human cells. Homologous Recombination: Methods and Protocols, 383-393.

US EPA. 2005. “Overview of the Piperonyl Butoxide Risk Assessments.” Docket ID EPA-HQ-OPP-2005-0042 p.2 (accessed Jan 2006) http://www.regulations.gov

CHAPTER 6: COMBINATORIAL IMPACTS OF PESTICIDES AND HEAVY METALS ON HUMAN CELLS

SUMMARY

As a result of the widespread use of chemicals and the presence of chemical and metal residues in a variety of foods, beverages, and other consumables, concerns have been raised about the possibility of amplified toxicity. To test the potential synergistic effects of the pesticides or their synergists or heavy metal combinatorial treatments, samples of local honey were collected and tested for pesticides and heavy metals. Based on the results, the pesticide Imidacloprid, a pesticide synergist called Piperonyl butoxide, and the heavy metals Lead and Selenium were chosen for biological treatments. Piperonyl butoxide showed a synergistic toxic impact when combined with Imidacloprid, lead chloride, or sodium selenite. The value of a more thorough investigation of the interactions between pesticides and heavy metals is highlighted by the present study's results and earlier research to determine the lower exposure thresholds more precisely. In addition, toxicological mechanisms underlying exposure to diverse combinations at varied concentrations, including concentrations determined under the permitted limit of intake or the degree of concern, as well as the changing effect of heavy metals, require further study.

INTRODUCTION

The extensive usage of chemicals and the presence of chemical and metal residues in a variety of foods, beverages, other consumables have raised concerns about the potential for amplified toxicity, in which two or more chemicals have a more detrimental effect together than they would individually (Cedergreen 2014). To understand the combined effects of pesticides, researchers have looked at the synergistic effects of compounds from the same class of toxins,

such as pesticide combinations. Combinatorial exposure to herbicides by humans is linked, in part, to an increase in chronic degenerative illnesses and cancer (Bassil et al. 2007). Additionally, it has been demonstrated that paraquat and cypermethrin pesticides can speed up DNA oxidation and damage in human liver HepG2 cells (Barron Cuenca et al., 2022). However, little is known about the potential combinatorial effects of chemical mixtures from different toxic chemical groups, such as pesticide-heavy metal mixtures, or how they can interact to exacerbate toxicity. Few studies have investigated the synergistic effect of pesticide-heavy metal mixtures; in prior studies, the exposure of rats to a mixture of dimethoate pesticide and lead acetate or cadmium chloride resulted in immunotoxic effects (Institoris et al. 1999) as well as the co-interaction of organophosphate pesticides and arsenic had shown to induce the rate of oxidative stress in blood and brains and increase the risk for hepatotoxicity in rats (Dwivedi and Flora 2011).

Among other food or drink sources, we selected honey because it has been considered a safe food compared to other animal products and processed food (Grigoryan 2016); besides that, the quality of honey has been widely employed as a bioindicator of environmental pollution that can harm honey bee populations and diminish the value of honey as a food source (Irungu et al. 2016). As reported in some studies, a range of xenobiotic contaminants like pesticides, heavy metals, and antibiotics in honey have been recorded in many countries worldwide (Al-Waili et al. 2012; Mitchell et al. 2017). A worldwide survey to test for the presence of neonicotinoids found these types of pesticides, including Acetamiprid, Clothianidin, Imidacloprid, Thiacloprid, and Thiamethoxam in 75% of samples collected from six continents (Mitchell et al. 2017). Heavy metals present in honey samples, such as Pb, As, Cd, and Cu, have also been widely documented throughout the U.S. and in other countries like Canada, China, Italy, and Turkey (Smith et al. 2019, Ru et al. 2013, Ruschioni et al. 2013, Silici et al. 2015). Levels of pesticides and heavy metals in

honey can be under a level of concern for human health; however, little is known about the potential synergistic effects of pesticides and heavy metals mixtures and how they can interact to enhance toxicity levels. (Naccari et al 2014; Yaqub et al. 2020).

Piperonyl butoxide is a pesticide synergist that was mainly developed to enhance the performance of insecticide pyrethrum in the early 1940s (Glynne-Jones 1998), it mainly works by impeding the insect's defense mechanisms by inhibiting the mixed-function oxidase (MFO) system of the insect (Casida 1970). The efficiency of Piperonyl butoxide to intensify the toxicity of pesticides against insects and lower insect's resistance to pesticides was reported widely (Wang et al. 2013; Romero et al. 2009; Young et al. 2005). On the other hand, the toxicity of Piperonyl butoxide as a single agent was tested in some mammalian cells, as it has been found to be an apoptosis inducer in Murine Splenocytes (Battaglia et al. 2010) and can induce sister chromatid exchange at Chinese hamster ovary K1 (CHO-K1) cells (Tayama 1996). However, little is known about the co-interaction of Piperonyl butoxide with pesticides or heavy metals in mammalian cells.

In this study, we collected local honey samples and confirmed the presence of Piperonyl butoxide and Imidacloprid, besides heavy metals, in honey. The most abundant metals: Lead and Selenium, were selected, as well as Imidacloprid insecticide, to test further synergistic toxic effects that might be enhanced by Piperonyl butoxide in mammalian cell models. Imidacloprid is known to induce cytotoxicity and DNA damage in HepG2 and SH-SY5Y cells (Şenyıldız et al. 2018). Lead has been shown to induce cytotoxicity and DNA damage in human lymphocyte cells (Alimba et al. 2016). Selenium is known to induce sister chromatid exchange (SCE) in Chinese hamster V79 cells, mainly when applied as sodium selenite (Sirianni and Huang 1983). Despite its protective role, there is a controversial debate on the power and risks of Se. It has been shown that it can enhance and suppress the toxicity of arsenic in human cells depending on the concentration,

so it acts in synergistic and antagonistic ways, as shown by Sun et al. (2014). It has been reported that sodium selenite can reduce the toxicity of some heavy metals like cadmium by alleviating oxidative damage (Alharithi et al. 2020), however, Se has been shown to have an antagonistic effect on Pb cytotoxicity in Leydig cells in sheep (Shi et al. 2022). On the other hand, little is known about the potential combinatorial effects of these pesticides and heavy metals or how they can interact to exacerbate toxicity, thus, it is crucial to know the combined effects of these xenobiotics for several reasons, first because of the different routes by which these chemicals can enter the human body. Second, combining exposures can change the toxicity of a single agent, which later can impose unexpected negative health effects (Singh et al. 2017).

We hypothesized that co-treatment of Piperonyl butoxide with either Imidacloprid or Lead or Selenium enhances the cytotoxicity and genotoxicity of mammalian cells. These effects were measured through cell growth inhibition assay, clonogenic cell survival assay, sister chromatid exchange (SCE) assay, and oxidative stress analysis.

MATERIALS AND METHODS

Study sites, sample collection, and analysis

Twenty-four hives distributed in 7 counties in Northern Colorado were surveyed (Figure 20). Honey samples were collected from 10 hives during the summer of 2019 and 11 hives during the summer of 2020. A total of twenty-one honey samples were collected from study sites between September and October of 2019 and 2020 and stored in the laboratory at -20°C until analysis.

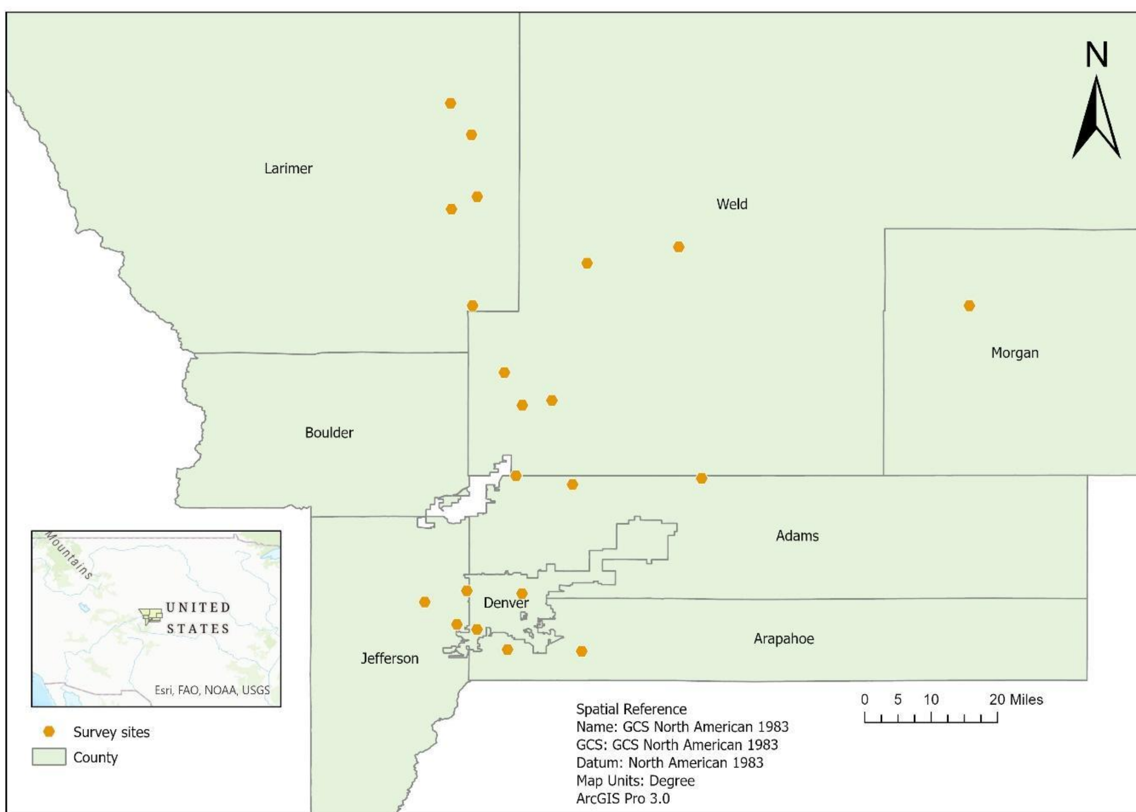


Figure 20: sampling sites of honey in Northern Colorado, USA, 2019-2020.

For heavy metals analysis, ten grams of honey were weighed for each sample and submitted to the Soil, Water, and Plant Testing Laboratory at Colorado State University for analysis. Heavy metals analysis was performed to detect the residues of Arsenic, Cadmium, Lead, and Selenium heavy metals using the Nitric and Perchloric Acids method (Soltanpour et al. 1982).

For pesticide analysis, 10 grams of samples were arranged in a cooler box at ~ 4° C and shipped overnight to the Chemical Ecology Core Facility at Cornell University, New York. Pesticide analysis was performed to detect the residues of 92 different types of pesticides, including some metabolites and breakdown products using the EN 15662 QuEChERS procedure (European Committee for Standardization 2018) by Liquid Chromatography-Mass Spectrometry (LC-MS/MS).

Chemicals

Lead Chloride ($PbCl_2$, Fisher Scientific, NY, USA) was diluted in DMSO as 100 mM stock solution and stored at 4 °C. Sodium Selenite (Na_2SeO_3, Sigma, Japan) was diluted in distilled water as 100 mM stock solution and stored at 4 °C. Imidacloprid (Sigma-Aldrich, USA) was diluted in DMSO as 100 mM stock solution and stored at –20 °C. Piperonyl butoxide,(Sigma-Aldrich, USA) was diluted in Ethanol as 100 mM stock solution and stored at –20 °C.

Cell Culture

Chinese Hamster Ovary CHO cells and CHO mutant BL10 cells (Giaccia et al. 1991) were routinely grown in 75-cm^2 culture flasks in DMEM medium supplemented with 10% Fetal Bovine Serum (FBS), 1% anti-anti, and 1% MEM nonessential amino acid. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C, and the medium was refreshed every 3-4 days. Human B-lymphoblast TK6 (Prado et al. 2009) and its mutant NH32 (p53 deletion) (Dural et al. 2020) and WTK1 (p53 mutated) (Moller et al. 2018) cells were maintained in RPMI medium with 10% FBS and 1% anti-anti. Cells were cultured in a 25 cm^2 flask with an appropriate cell number of around 100,000 cells/ml media per flask and grown with 5% CO_2 in an incubator at 37°C.

Clonogenic Cell Survival

A colony formation assay was used to determine cell sensitivity and the synergistic effect of pesticide-heavy metal combinations (Adan et al. 2016). CHO cells and CHO variant BL-10 cells were seeded onto 12 well plates and were treated with varying concentrations of Piperonyl butoxide, Imidacloprid, Na_2SeO_3, $PbCl_2$, and their different combinations. The plated cells were incubated for one week. Then, colonies were fixed with 100% ethanol and allowed to dry for 20 min at room temperature before staining. Next, colonies were stained using 0.1% crystal violet and allowed to dry before counting. Surviving colonies were counted under a microscope. Colonies containing at least 50 cells were counted as survivors. Cell survival fraction and survival curves

were drawn using GraphPad Prism 9.3.1. Data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) by implementing the one-way ANOVA and Tukey's multiple comparison analysis. At least four independent experiments were conducted.

Oxidative stress analysis

Suspension of 300,000 cells/ml CHO cells in PBS were treated with 2.5 µM Carboxy-H2DCFDA (Life Technologies, Eugene, OR) for 30 minutes, then cells were treated with different combination of pesticides and heavy metals and stored in a 37 °C incubator for 30 minutes or 24 hours. Following incubation, cells were centrifuged at 1000 rpm for 5 min. The supernatant was discarded. Pelleted cells were resuspended in 8 mL PBS, and 2 ml of cell suspension was transferred into new tubes. Ten µM of H_2O_2 was used for positive control. The green fluorescent signal was measured with Versa Fluor Fluorometer (Bio-Rad, Hercules, CA) and was then blanked with negative control.

Sister Chromatid Exchange Assay

SCE assay was used to determine the genotoxic effect of pesticide and heavy metals combinations. To do this analysis, G1-synchronized CHO cells were prepared by the mitotic shake-off method (Zwanenburg 1983). Cells were treated with different concentrations of Piperonyl butoxide, Imidacloprid, Na_2SeO_3, $PbCl_2$, and their different combinations, and 10 µM of BrdU (thymidine analogous) for 20-24 hours. After that, 10 µg/ml colcemid was added to block cells in the "second metaphase." Trypsinized cells were treated with 75 mM KCl hypotonic solution and fixed in Methanol: Acetic acid (3:1) fixative solution. Metaphase spreads were prepared over glass slides, and slides were treated with 10 µg/ml Hoechst 33258 solution, then slides were exposed to UV light and by UV trans-illuminator for 20 minutes. After that, slides

were transferred into a Coplin jar and treated with 2xSSC at 80℃ for 20 min. Then the slides were stained with 5% Giemsa stain for 5 min. Slides were rinsed with tap water and left to dry before analyzing under the microscope. Numbers of SCE per cell were scored and analyzed by implementing the one-way ANOVA analysis using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Thirty metaphases were analyzed per data point, and at least three independent experiments were conducted.

Apoptosis analysis

Apoptosis induction was assessed using DAPI staining and apoptotic body measurement (Alshiraihi and Kato 2023). We treated 300,000 cells/ml CHO cells with a variety of concentrations of pesticide-heavy metals. After 48 hours of incubation, cells were suspended in 3 ml of 75 mM KCl solution added to that 1 ml of methanol: acetic acid (3:1) fixative solution. The cell suspension was dropped on glass slides and left to dry. Then one drop of DAPI solution was added over the slide, covered with a cover slide, and examined under a fluorescence microscope. Approximately 100 cells from each slide were counted. Apoptosis ratios were also determined by scoring condensed nuclei and apoptotic bodies.

In Vitro Cell Growth Inhibition Assay

Human B-lymphoblastoid cells were plated at a density of 50,000 cells/ml onto a 15 ml centrifuge tube with varying concentrations of pesticide-heavy metals combinations; cell numbers were counted and scored as the number of proliferating cells after treatments at different time points (24, 48, and 72 h) using a Coulter Counter Z1 (Beckman-Coulter Z1 Coulter Particle Count Counter and Size Analyzer, Brea, CA, USA). Data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) using the two-way ANOVA analysis. At least four independent experiments were conducted.

STATISTICAL ANALYSIS

The statistical significance of the results in this study was analyzed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) for one-way ANOVA analysis and Tukey's multiple comparisons. A p-value of less than 0.05 was considered statistically significant for all analyses.

RESULTS

Heavy metals in honey

Twenty-one honey samples were collected and analyzed for four different heavy metals (Se, Pb, Cd, and As). Se and Pb were present at the highest concentrations in honey samples, for this, they have been selected for this study, with mean concentrations of 1034.86 ppb and 367.52 ppb, respectively (Figure 21). Test samples included 67% that were contaminated with more than 100 ppb of Pb, while 57% of samples were contaminated with higher than 100 ppb of Se.

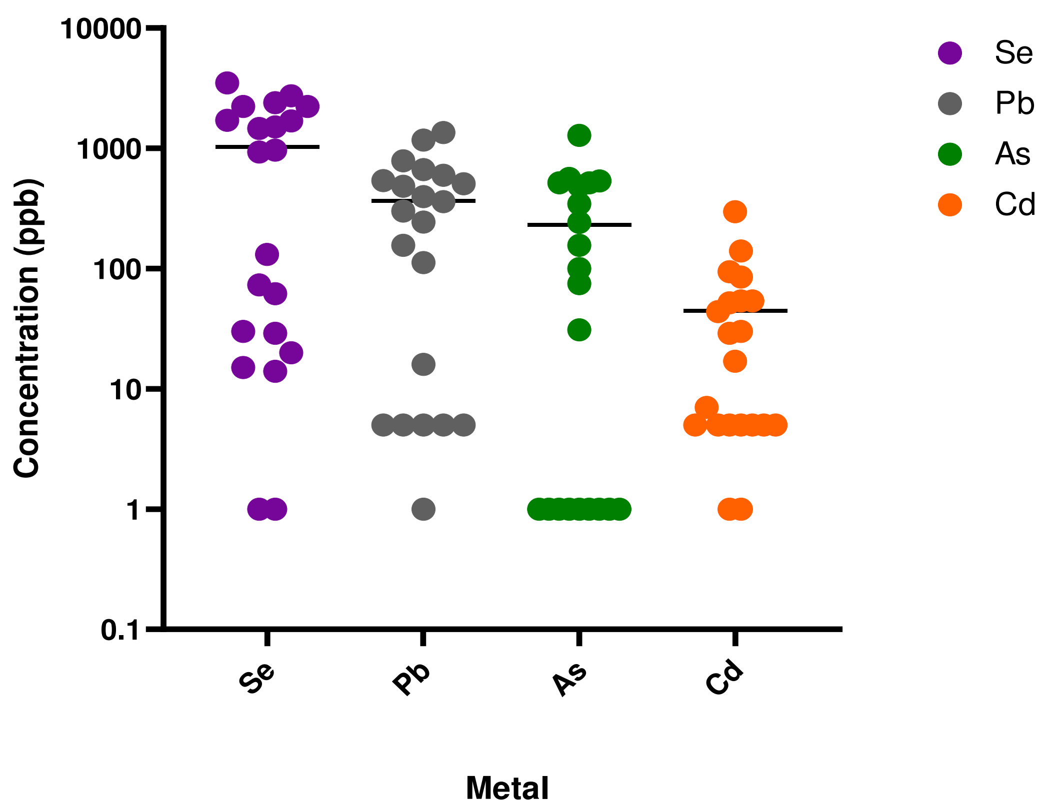


Figure 21: Distribution of heavy metal concentrations (As, Cd, Pb, and Se) (ppb) detected in honey samples. Black line represents the concentration mean.

Pesticides in honey

Sixteen chemicals were detected in honey samples, of which 9 were insecticides, 3 were herbicides, 2 were fungicides, 1 was an acaricide, and 1 was a pesticide synergist. Figure 22 shows the mean of different pesticide concentrations (ppb) found in the collected honey samples. Piperonyl butoxide was found in 52% of samples in a mean concentration of 0.15 ppb, while Imidacloprid was found in 29% of samples in a mean concentration of 0.2 ppb. The average concentrations for Piperonyl butoxide and Imidacloprid were below the tolerance established by federal regulations. The range of tolerance for Piperonyl butoxide residues in food was 0.1-20 ppm, while the range of tolerance for Imidacloprid residues was 0.05-48 ppm (The U.S. Code of Federal Regulations, 2020). Those were selected in the next biological study because of the

minimal information about their interaction and their possible synergistic genotoxic and cytotoxic effect in mammalian cells, as well as the claimed low toxicity of Imidacloprid (Neonicotinoid insecticide) compared to Coumaphos and Acephate (Organophosphate insecticides) (Selvam and Srinivasan 2019). 2,4-DMPF was not selected because it is a metabolite for Amitraz acaricide, which was reported to have a short half-life in honey (Shimshoni et al. 2019).

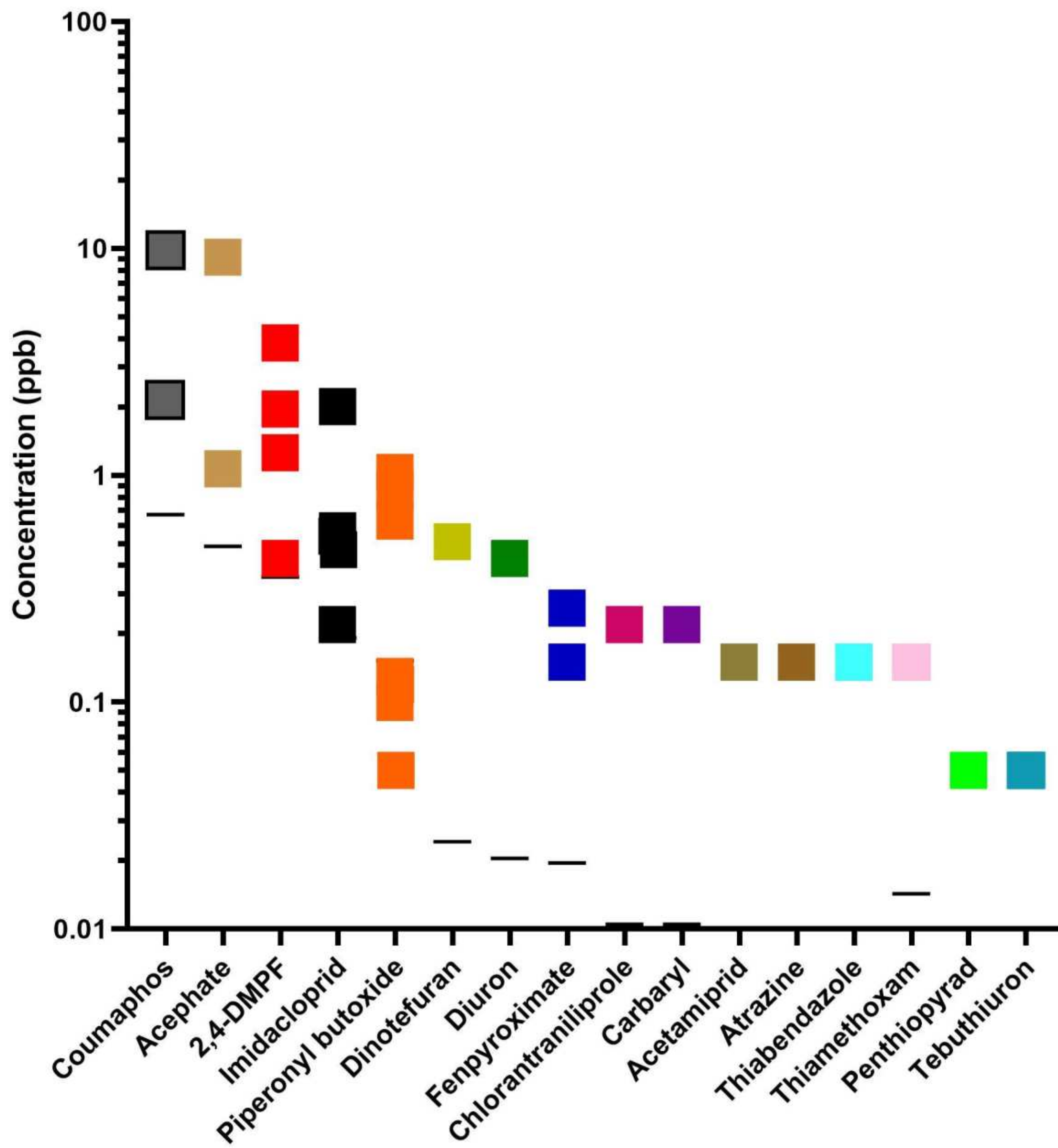


Figure 22: Distribution of pesticide concentrations (ppb) detected in honey samples. Black line represents the concentration mean.

Clonogenic cell survival-analysis

Based on the honey analysis results, Imidacloprid, Piperonyl butoxide, Sodium Selenite, and Lead Chloride were determined appropriate for our use in biological experiments. Cytotoxic effects were tested by colony formation assay (Figure 23). Piperonyl butoxide treatment showed a cell-killing effect above 50 µM for CHO cells. On the other hand, Imidacloprid treatment did not show a significant cell-killing effect in the tested concentrations ranged from 100 µM up to 500 µM. When 1µM of Na_2SeO_3 or $PbCl_2$ were combined, the cytotoxicity of 50 µM and µM Piperonyl butoxide was significantly enhanced (p-values were less than 0.05 for both treatments). Moreover, when 100 µM Piperonyl butoxide was added to Imidacloprid treatment, the cytotoxicity of Imidacloprid was significantly enhanced (p-value < 0.05).

CHO cell mutant BL10 was used to test the effect of glutathione S-transferase. If the cytotoxicity of metals or pesticides has heavily relied on reactive oxygen species, BL10 cells might present hypersensitivity to testing agents. Contrary to our assumption, BL10 did not show hypersensitivity to testing agents compared to CHO wild-type cells. Therefore, we assumed that cytotoxicity might not be strongly associated with glutathione S-transferase.

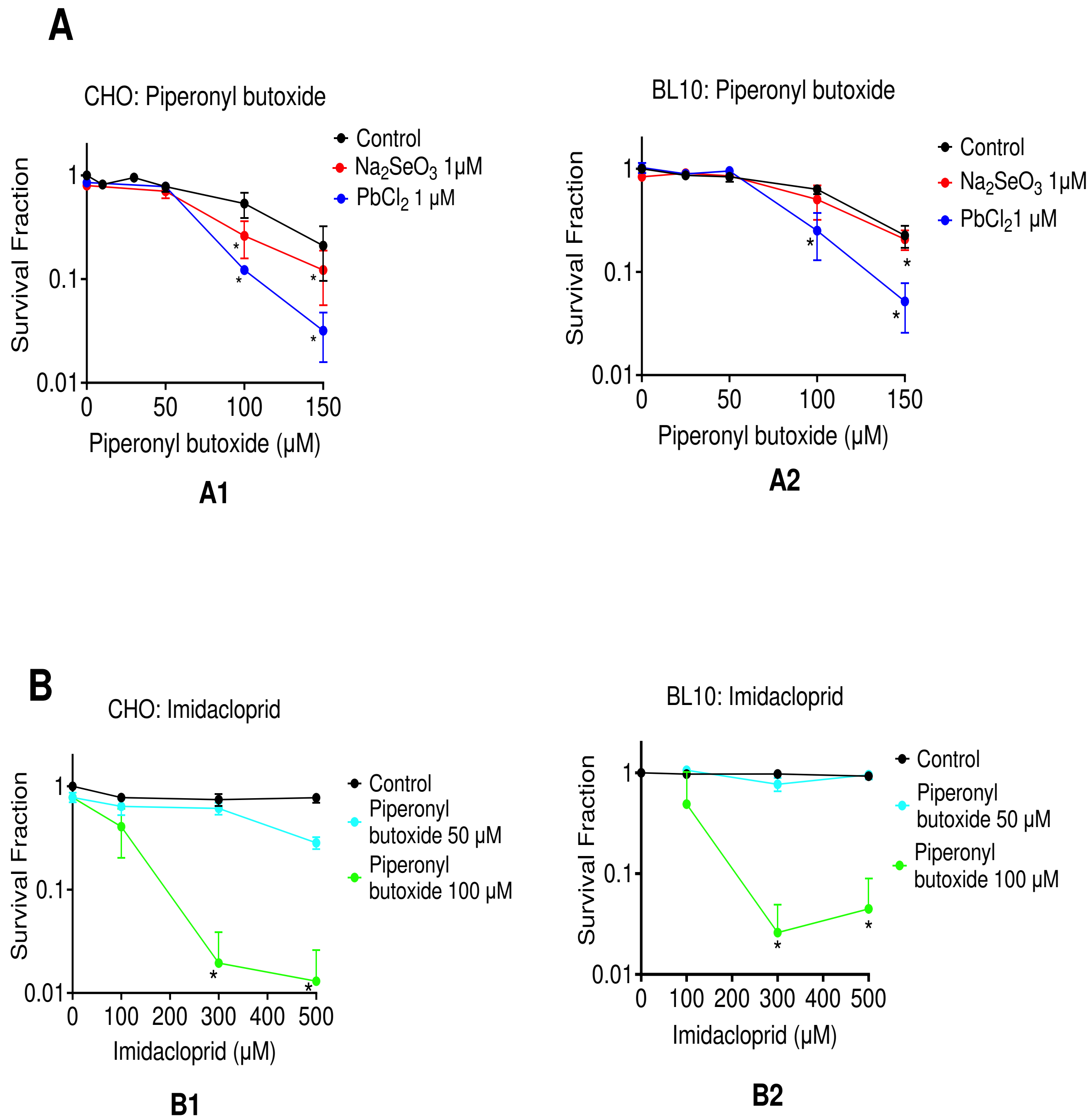


Figure 23: Single and combined treatment of pesticides and heavy metals on colony proliferation at different concentrations for CHO and BL10 cells. One-way ANOVA analysis was performed to determine the effect's significance. (A1) Piperonyl butoxide with $PbCl_2$ or Na_2SeO_3 single vs. combination effect on CHO colony proliferation fraction. (A2) Piperonyl butoxide with $PbCl_2$ or Na_2SeO_3 single vs. combination effect on BL10 colony proliferation fraction. (B1) Piperonyl butoxide and Imidacloprid single vs. combination effect on CHO colony proliferation fraction. (B2) Piperonyl butoxide and Imidacloprid single vs. combination effect on BL10 colony proliferation fraction. * Indicate $P<0.05$.

Oxidative stress analysis

The oxidative stress indicator, carboxy-H2DCFDA, a non-fluorescent reagent, was used to detect reactive oxygen species (ROS) in cells. When ROS are present, the carboxyl-H2DCFDA is oxidized and emits a green fluorescence. Our findings revealed that oxidative stress did not significantly enhance with time, comparing the results after 30 minutes of treatment with 24 hours as shown in Figures 24a and 24b. None of Piperonyl butoxide-heavy metal, Piperonyl butoxide-Imidacloprid combinations showed any significant impact on the level of ROS with all p-values higher than 0.05.

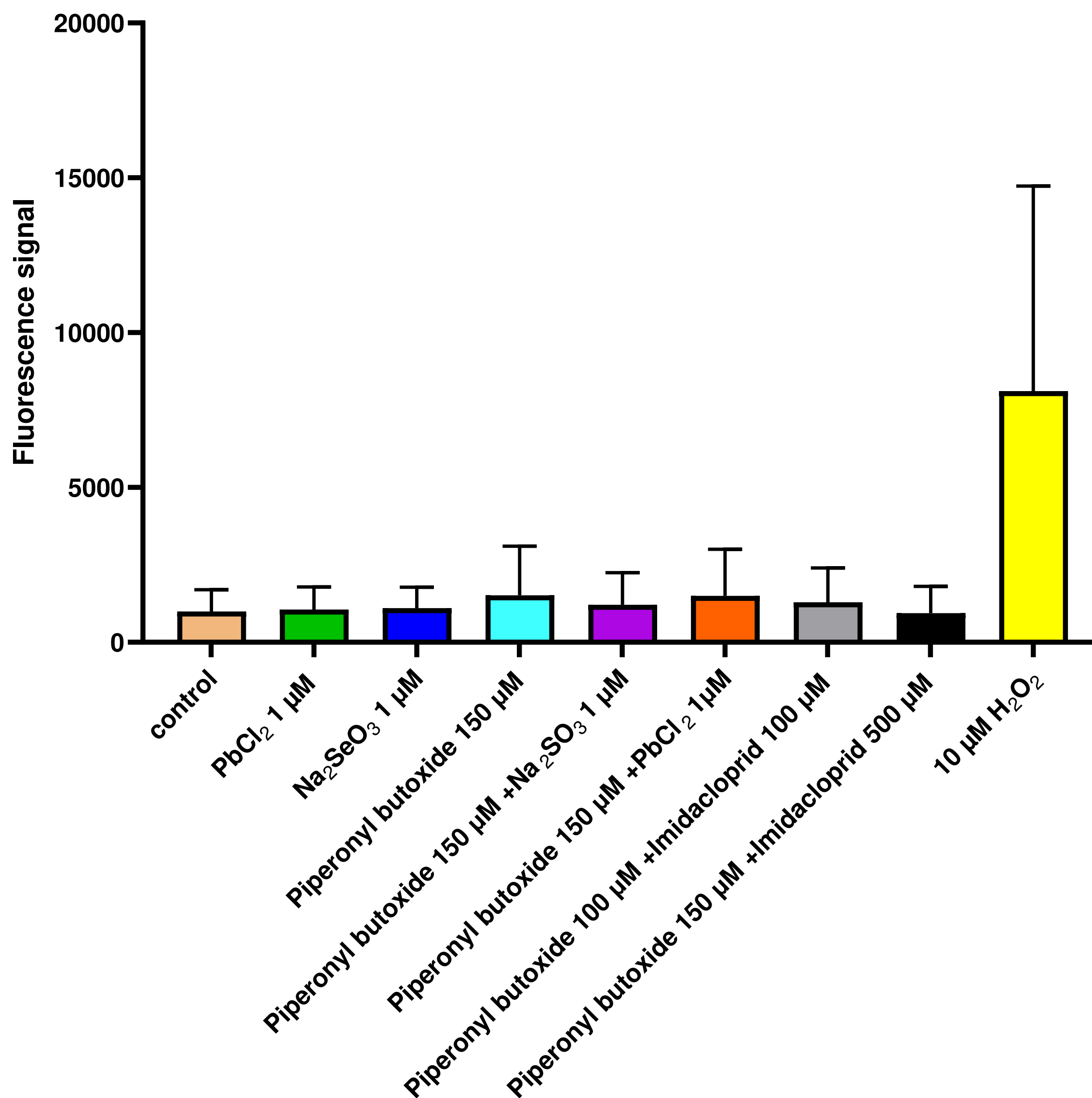


Figure 24a: Oxidative stress following 30 minutes of pesticide-heavy metal treatments of CHO cells. Error bars indicate the standard error of the mean of three independent experiments. Statistically significant differences were determined by using one-way ANOVA and Tukey's Multiple Comparison Test. Four independent experiments were conducted. Error bars represent the distribution of data around the mean. All p-values were > 0.05.

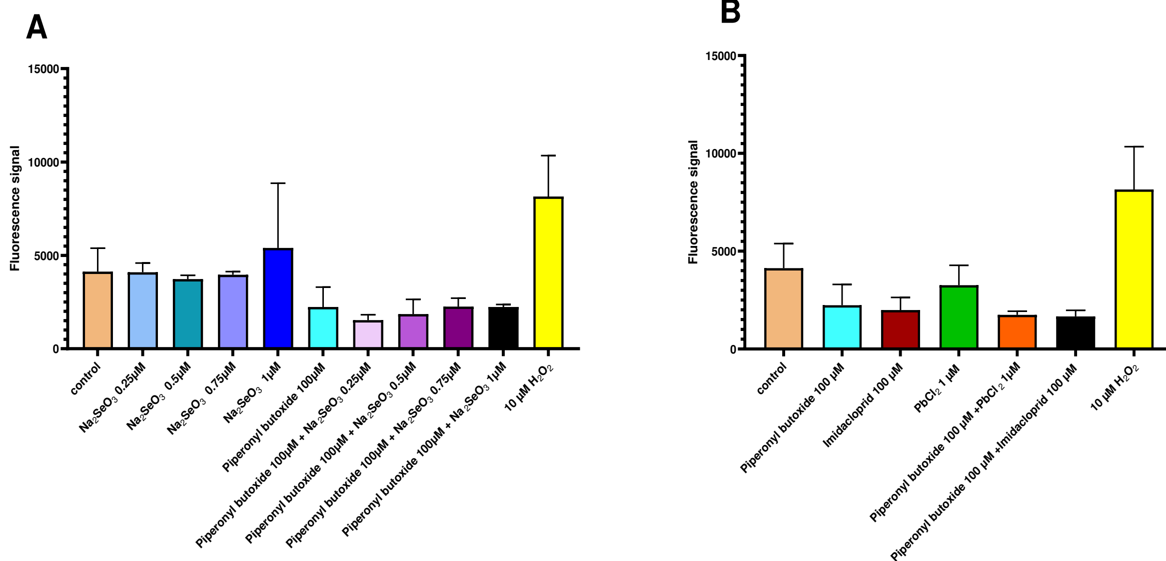


Figure 24b: Oxidative stress following 24 hours of pesticide-heavy metal treatments of CHO cells. A. Piperonyl butoxide with Na_2SeO_3 single vs. combination effect on ROS. B. Piperonyl butoxide with $PbCl_2$ or Imidacloprid single vs. combination effect on ROS. Error bars indicate the standard error of the mean of at least three independent experiments. Statistically significant differences were determined by using one-way ANOVA and Tukey's Multiple Comparison Test. All p-values were > 0.05.

Sister Chromatid Exchange analysis

To assess potential DNA damage, we conducted a sister chromatid exchange assay to investigate whether a pesticide-heavy metal combination has a synergistic genotoxicity effect due to the number of exchanged DNA fragments between two sister chromatids. Cell cultures were exposed to different concentrations of pesticides Imidacloprid (100, 300, and 500 µM) and Piperonyl butoxide (50, 100 µM) and heavy metals $PbCl_2$ (1 µM) and Na_2SeO_3 (1µM). The results did not show a significant enhancement of SCE induction, neither as single agent treatments nor as combinatorial treatments. However, we observed that a combination treatment of Piperonyl butoxide at a concentration of 100 µM combined with either 1 µM $PbCl_2$ or 1 µM Na_2SeO_3, were causing tangled chromosomes as well as insufficient metaphase chromosomes to analyze as shown in Figure 26, which we suggest was caused by the synergistic toxicity impact of Piperonyl butoxide that enhanced the toxicity of metals.

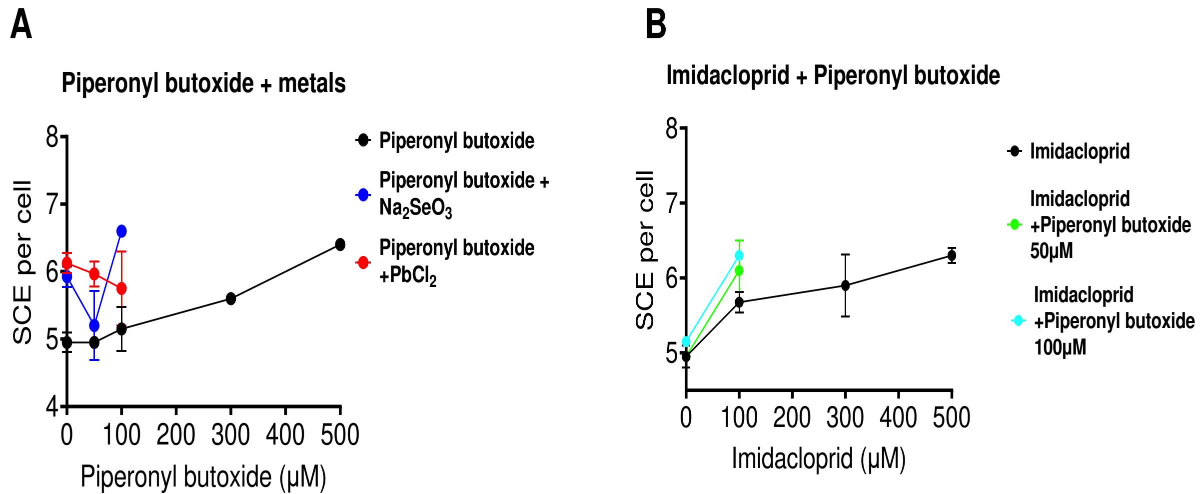


Figure 25: Sister chromatid exchange (SCE) analysis of CHO cells after pesticide-heavy metal treatments. Error bars indicate the standard error of the mean of at least three independent experiments. A. Piperonyl butoxide with $PbCl_2$ or Na_2SeO_3 single vs. combination effects on SCE. B. Piperonyl butoxide and Imidacloprid single vs. combination effects on SCE. Statistically significant differences were determined by using one-way ANOVA and Tukey's Multiple Comparison Test. All p-values were > 0.05.

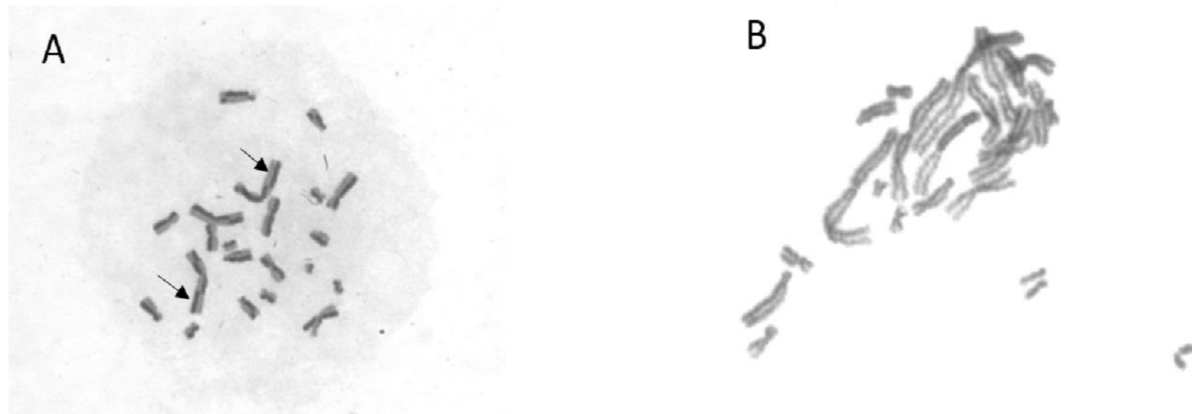


Figure 26: (A) SCE induction after treatment with a combination of 100 µM Imidacloprid and 1 µM $PbCl_2$, arrows show sister chromatid exchange fragments. (B) Tangled shape of chromosomes due to the synergistic cytotoxicity of Piperonyl butoxide and Na_2SeO_3combinations.

Apoptosis analysis

To test whether pesticide-heavy metal combinations induce apoptosis in CHO cells, we performed an apoptosis assay using DAPI staining analysis. After 48 hours of treatment with different combinations of pesticide-heavy metals, results showed an increase in the number of

apoptotic cells after treatment with Imidacloprid and Piperonyl butoxide as single agents compared with the control. However, the percentage of live cells decreased up to 70% under Piperonyl butoxide and $PbCl_2$ combination, 55% under Piperonyl butoxide and Na_2SeO_3 combination, and 71% under Imidacloprid and Piperonyl butoxide combination (Figure 27). Piperonyl butoxide with $PbCl_2$ or with Imidacloprid treatments showed a significant synergistic effect on the number of apoptotic cells compared with single-agent treatment, the P-values for Piperonyl butoxide-$PbCl_2$, Piperonyl butoxide -Imidacloprid treatments were < 0.05 (Figure 27).

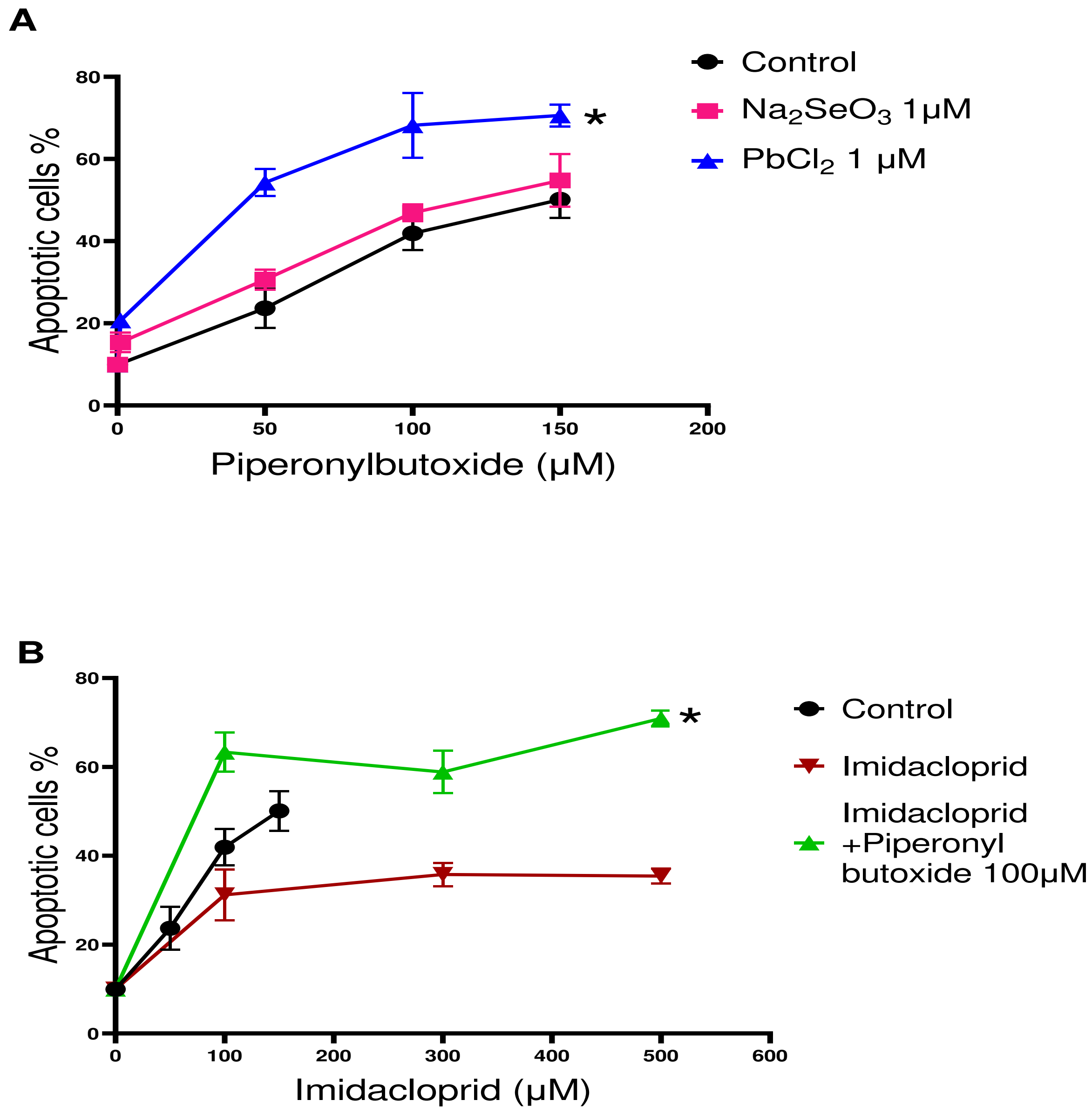


Figure 27. Cell apoptosis was assessed using DAPI staining and Fluorescence microscopy. One-way ANOVA analysis was performed to determine the effect's significance. (A) Piperonyl butoxide with $PbCl_2$ and Na_2SeO_3 single vs. combination effect on cell apoptosis; P-value for Piperonyl butoxide-$PbCl_2$ and Piperonyl butoxide-Na_2SeO_3 treatments are = 0.0388 and 0.191, respectively. (B) Imidacloprid and Piperonyl butoxide single vs. combination effect on cell apoptosis; P-value for Imidacloprid and Piperonyl butoxide treatment = 0.0018. Three independent experiments were conducted. * Indicate $P<0.05$.

Cell Growth Inhibition analysis

For single agents, 1 µM of Lead Chloride ($PbCl_2$) or Sodium Selenite (Na_2SeO_3) did not show cell growth inhibition for three days. For 100 µM of Piperonyl butoxide treatment, WTK6, TK6, and NH32 cells showed growth inhibition.

Piperonyl butoxide showed toxicity as a single agent through inhibiting cell growth.

Piperonyl butoxide toxicity was significantly enhanced when combined with $PbCl_2$ or Na_2SeO_3 as well as Imidacloprid in TK6 cells (P-values for Piperonyl butoxide-$PbCl_2$, Piperonyl butoxide-Na_2SeO_3 and Piperonyl butoxide-Imidacloprid combinations in TK6 were 0.008, 0.028, and 0.0032 respectively). Imidacloprid also caused a decrease in cell growth when applied as a single agent. Piperonyl butoxide and Imidacloprid combination did not show a drastic inhibition of cell growth in WTK, or NH32 cells. The cell proliferation fraction for each treatment is reported in Figure 28.

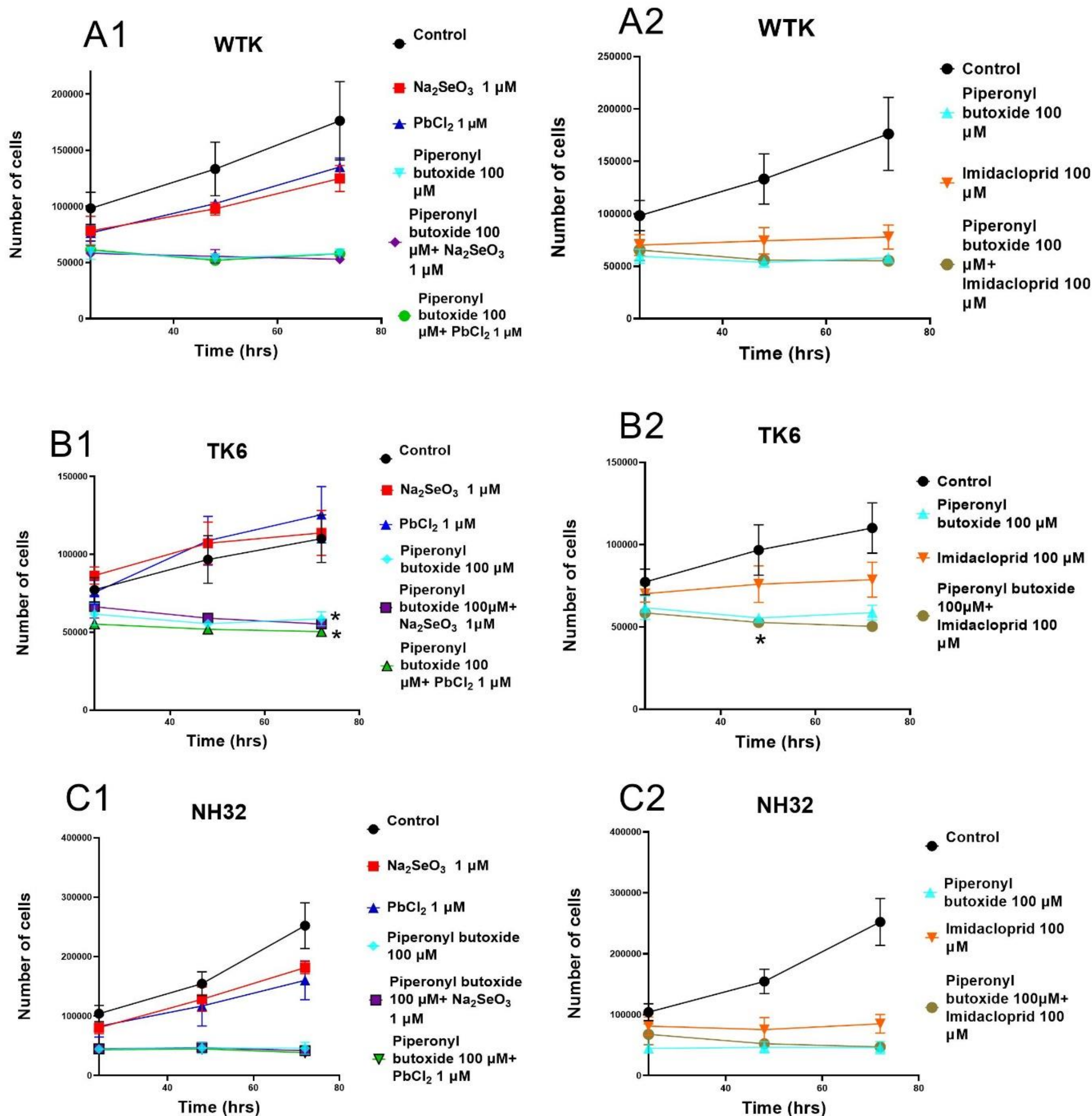


Figure 28: Single and combined effects of pesticides and heavy metals on cell survival. One-way ANOVA analysis was performed to determine significance. (A1) Piperonyl butoxide-$PbCl_2$ and Piperonyl butoxide-Na_2SeO_3 single vs. combination effect on WTK cell proliferation fraction. (A2) Piperonyl butoxide-Imidacloprid single vs. combination effect on WTK cell proliferation fraction. (B1) Piperonyl butoxide- $PbCl_2$ and Piperonyl butoxide- Na_2SeO_3 single vs. combination effect on TK6 cell proliferation fraction. (B2) Piperonyl butoxide-Imidacloprid single vs. combination effect on TK6 cell proliferation fraction. (C1) Piperonyl butoxide- $PbCl_2$ and Piperonyl butoxide- Na_2SeO_3 single vs. combination effect on NH32 cell proliferation fraction. (C2) Piperonyl butoxide-Imidacloprid single vs. combination effect on NH32 cell proliferation fraction. At least three independent experiments were conducted. * Indicate $P < 0.05$.

DISCUSSION

This study aimed to assess the potential synergistic cytotoxic and genotoxic effects of the combinatorial treatment of Piperonyl butoxide with Imidacloprid or heavy metals in honey. Initially, we identified the presence of Piperonyl butoxide, Imidacloprid and metals in honey samples (Figures 21 and 22). Further biological research was conducted to assess the potential synergistic effect. The cytotoxicity test indicated that each of the following combinations had a synergistic cytotoxic impact (Piperonyl butoxide-$PbCl_2$, Piperonyl butoxide-Na_2SeO_3, and Piperonyl butoxide-Imidacloprid), as shown in Figure 23. Nevertheless, the cytotoxicity was not depending on glutathione S-transferase (Figure 23).

Piperonyl butoxide was found to have a significant impact on cell proliferation (for TK6 cells) when combined with $PbCl_2$; Na_2SeO_3, or Imidacloprid (Figure 28, B1 and B2). Piperonyl butoxide showed to have a synergistic effect on cell apoptosis when it was combined with $PbCl_2$ or Imidacloprid (Figure 27); Nevertheless, it was not the case with Piperonyl butoxide-Na_2SeO_3 mixture (Figure 27). None of the Piperonyl butoxide- metals/Imidacloprid mixture induced any toxicity toward oxidation stress or SCE (Figures 24a, 24b, and 25).

As a single agent, Piperonyl butoxide has been found to induce genotoxicity and cause liver and kidney inflammation in rabbits (Vardavas et al. 2016) and have an adverse effect on the reproduction and orientation of mice (Tanaka 1992). In previous literature, Piperonyl butoxide had been found to induce the toxicity of Imidacloprid in different insects (Roy et al. 2009; Bao et al. 2015; Zhange et al. 2016). The suggested toxicity mechanism that explains cell death after cells were exposed to different combinations of Piperonyl butoxide-heavy metals/Imidacloprid is through inducing oxidative stress in cells. These responses happen through generating reactive oxygen species (ROS) or alteration in the antioxidant enzyme systems, this process contributes to increasing the genetic instability, where the ability of cells to detoxify or repair the damage is

disrupted and induces DNA strand breaks (Kryston et al. 2011). However, our results showed that cell treatment with different combinations of Piperonyl butoxide-pesticide-metals did not induce the generation of ROS. Yet, the results showed that Piperonyl butoxide had suppressed the oxidative effects of Na_2SeO_3. Nevertheless, Piperonyl butoxide-Imidacloprid combination did not impose a drastic effect in the oxidation stress treatments. Our results are partially consistent with the findings of Yardimci et al. (2014), as they reported that oxidative stress caused by Piperonyl butoxide-Imidacloprid can vary between rat sexes, tissue types and exposure duration, where oxidative stress was induced by Piperonyl butoxide-Imidacloprid in the liver of male rate but attenuated in the kidney in female rats. These similarities suggest another pathway of toxicity or other factors that would antagonize mixture toxicity.

Our results contradict the protective role of selenium as an antioxidant, whether it was in the form of selenocysteine or sodium selenite as reported in Battin et al. (2006) and Yousef et al. (2007) and the ameliorating role of selenium (as sodium selenate) against pesticides (Adesiyan et al. 2011), and the role of sodium selenite as an oxidation stress inducer in Hepatoma HepG2 cells (Shen et al. 1999). These discrepancies about the role of selenium as an antioxidant versus an oxidation stress agent open the gate for more investigations on its toxicity pathway.

Our present study, along with prior findings, underscores the value of a more comprehensive examination of pesticides' and heavy metals' combinatorial impacts to assign the lower thresholds more accurately for exposure. More research is needed to investigate different types of pesticides and heavy metals, the fluctuating effect of heavy metals, and the toxicity mechanisms behind the exposure to different combinations at various concentrations, including concentrations defined under the allowable limit of consumption or the level of concern.

LITERATURE CITED

Adan, A., Kiraz, Y., & Baran, Y. (2016). Cell proliferation and cytotoxicity assays. Current pharmaceutical biotechnology, 17(14), 1213-1221.

Adesiyan, A. C., Oyejola, T. O., Abarikwu, S. O., Oyeyemi, M. O., & Farombi, E. O. (2011). Selenium provides protection to the liver but not the reproductive organs in an atrazine-model of experimental toxicity. Experimental and toxicologic pathology, 63(3), 201-207.

Alharthi, W. A., Hamza, R. Z., Elmahdi, M. M., Abuelzahab, H. S., & Saleh, H. (2020). Selenium and L-carnitine ameliorate reproductive toxicity induced by cadmium in male mice. Biological trace element research, 197(2), 619-627.

Alimba, C. G., Dhillon, V., Bakare, A. A., & Fenech, M. (2016). Genotoxicity and cytotoxicity of chromium, copper, manganese and lead, and their mixture in WIL2-NS human B lymphoblastoid cells is enhanced by folate depletion. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 798, 35-47.

Alshiraihi, I., & Kato, T. A. (2023). Apoptosis Detection Assays. In Chromosome Analysis (pp. 53-63). Humana, New York, NY.

Al-Waili, N., Salom, K., Al-Ghamdi, A., & Ansari, M. J. (2012). Antibiotic, pesticide, and microbial contaminants of honey: human health hazards. The scientific world Journal, 2012.

Bao, W. X., Kataoka, Y., Fukada, K., & Sonoda, S. (2015). Imidacloprid resistance of melon thrips, Thrips palmi, is conferred by CYP450-mediated detoxification. Journal of Pesticide Science, 40(2), 65-68.

Barrón Cuenca, J., de Oliveira Galvão, M. F., Ünlü Endirlik, B., Tirado, N., & Dreij, K. (2022). In vitro cytotoxicity and genotoxicity of single and combined pesticides used by Bolivian farmers. Environmental and molecular mutagenesis, 63(1), 4-17.

Bassil, K. L., Vakil, C., Sanborn, M., Cole, D. C., Kaur, J. S., & Kerr, K. J. (2007). Cancer health effects of pesticides: systematic review. Canadian Family Physician, 53(10), 1704-1711.

Battaglia, C. L., Gogal Jr, R. M., Zimmerman, K., & Misra, H. P. (2010). Malathion, lindane, and piperonyl butoxide, individually or in combined mixtures, induce immunotoxicity via apoptosis in murine splenocytes in vitro. International journal of toxicology, 29(2), 209-220.

Battin, E. E., Perron, N. R., & Brumaghim, J. L. (2006). The central role of metal coordination in selenium antioxidant activity. Inorganic chemistry, 45(2), 499-501.

Casida, J. E. (1970). MFO involvement in the biochemistry of insecticide synergists. J. Agric. Food Chem. 18, 753–772.

Cedergreen, N. (2014). Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PloS one, 9(5), e96580.

Dural, E., Shah, U. K., Pritchard, D., Chapman, K. E., Doak, S. H., & Jenkins, G. J. S. (2020). The effect of chronic dosing and p53 status on the genotoxicity of pro-oxidant chemicals in vitro. Mutagenesis, 35(6), 479-489.

Dwivedi, N., & Flora, S. J. (2011). Concomitant exposure to arsenic and organophosphates on tissue oxidative stress in rats. Food and chemical toxicology, 49(5), 1152-1159.

European Committee for Standardization. (2018). Foods of Plant Origin–Multimethod for the Determination of Pesticide Residues using GC–and LC–Based Analysis following Acetonitrile Extraction/Partitioning and Clean-up by Dispersive SPE–Modular QuEChERS–Method.

Giaccia, A. J., Lewis, A. D., Denko, N. C., Cholon, A., Evans, J. W., Waldren, C. A., ... & Brown, J. M. (1991). The hypersensitivity of the Chinese hamster ovary variant BL-10 to bleomycin killing is due to a lack of glutathione S-transferase-α activity. Cancer research, 51(16), 4463-4469.

Glynne-Jones, D. (1998). History of PBO In "PBO—The Insecticide Synergist" (D. Glynne Jones, ed.). Academic Press, San Diego.

Grigoryan, K. (2016). Safety of honey. In Regulating safety of traditional and ethnic foods (pp. 217-246). Academic Press.

Institóris, L., Ųndeger, Ų., Siroki, O., Nehéz, M., & Dési, I. (1999). Comparison of detection sensitivity of immuno-and genotoxicological effects of subacute cypermethrin and permethrin exposure in rats. Toxicology, 137(1), 47-55.

Irungu, J., Raina, S., & Torto, B. (2016). Determination of pesticide residues in honey: a preliminary study from two of Africa's largest honey producers. International Journal of Food Contamination, 3(1), 1-14.

Kılıç Altun, S., Dinç, H., Paksoy, N., Temamoğulları, F. K., & Savrunlu, M. (2017). Analyses of mineral content and heavy metal of honey samples from south and east region of Turkey by using ICP-MS. International Journal of Analytical Chemistry, 2017.

Kopp, B., Sanders, P., Alassane-Kpembi, I., Fessard, V., Zalko, D., Le Hégarat, L., & Audebert, M. (2020). Synergic toxic effects of food contaminant mixtures in human cells. Mutagenesis, 35(5), 415-424.

Mitchell, E. A., Mulhauser, B., Mulot, M., Mutabazi, A., Glauser, G., & Aebi, A. (2017). A worldwide survey of neonicotinoids in honey. Science, 358(6359), 109-111.

Møller, P., Wils, R. S., Jensen, D. M., Andersen, M. H. G., & Roursgaard, M. (2018). Telomere dynamics and cellular senescence: an emerging field in environmental and occupational toxicology. Critical Reviews in Toxicology, 48(9), 761-788.

Naccari, C., Macaluso, A., Giangrosso, G., Naccari, F., & Ferrantelli, V. (2014). Risk assessment of heavy metals and pesticides in honey from Sicily (Italy). Journal of Food Research, 3(2), 107.

Prado, G., Bhalli, J. A., & Marcos, R. (2009). Genotoxicity of heptachlor and heptachlor epoxide in human TK6 lymphoblastoid cells. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 673(2), 87-91.

Rai, A., Maurya, S. K., Khare, P., Srivastava, A., & Bandyopadhyay, S. (2010). Characterization of developmental neurotoxicity of As, Cd, and Pb mixture: synergistic action of metal mixture in glial and neuronal functions. Toxicological sciences, 118(2), 586-601.

Romero, A., Potter, M. F., & Haynes, K. F. (2009). Evaluation of piperonyl butoxide as a deltamethrin synergist for pyrethroid-resistant bed bugs. Journal of economic entomology, 102(6), 2310-2315.

Roy, S., Mukhopadhyay, A., & Gurusubramanian, G. (2009). The synergists action of piperonyl butoxide on toxicity of certain insecticides applied against Helopeltis theivora waterhouse (Heteroptera: Miridae) in the dooars tea plantations of north bengal, India. Journal of Plant Protection Research, 49(2).

Ru, Q. M., Feng, Q., & He, J. Z. (2013). Risk assessment of heavy metals in honey consumed in Zhejiang province, southeastern China. Food and chemical toxicology, 53, 256-262.

Ruiz-Toledo, J., Vandame, R., Castro-Chan, R. A., Penilla-Navarro, R. P., Gómez, J., & Sánchez, D. (2018). Organochlorine pesticides in honey and pollen samples from managed colonies

of the honey bee Apis mellifera Linnaeus and the stingless bee Scaptotrigona mexicana Guérin from Southern, Mexico. Insects, 9(2), 54.

Ruschioni, S., Riolo, P., Minuz, R. L., Stefano, M., Cannella, M., Porrini, C., & Isidoro, N. (2013). Biomonitoring with honeybees of heavy metals and pesticides in nature reserves of the Marche Region (Italy). Biological trace element research, 154(2), 226-233.

Selvam, V., & Srinivasan, S. (2019). Neonicotinoid poisoning and management. Indian Journal of Critical Care Medicine: Peer-reviewed, Official Publication of Indian Society of Critical Care Medicine, 23(Suppl 4), S260.

Şenyildiz, M., Kilinc, A., & Ozden, S. (2018). Investigation of the genotoxic and cytotoxic effects of widely used neonicotinoid insecticides in HepG2 and SH-SY5Y cells. Toxicology and Industrial Health, 34(6), 375-383.

Shen, H. M., Yang, C. F., & Ong, C. N. (1999). Sodium selenite-induced oxidative stress and apoptosis in human hepatoma HepG2 cells. International journal of cancer, 81(5), 820-828.

Shi, L., Wang, X., Duan, Y., Li, K., & Ren, Y. (2022). Antagonistic effects of selenium on lead-induced oxidative stress and apoptosis of Leydig cells in sheep. Theriogenology, 185, 43-49.

Shimshoni, J. A., Sperling, R., Massarwa, M., Chen, Y., Bommuraj, V., Borisover, M., & Barel, S. (2019). Pesticide distribution and depletion kinetic determination in honey and beeswax: Model for pesticide occurrence and distribution in beehive products. PLoS One, 14(2), e0212631.

Silici, S., & Atayoglu, A. T. (2015). Mad honey intoxication: A systematic review on the 1199 cases. Food and chemical toxicology, 86, 282-290.

Singh, N., Gupta, V. K., Kumar, A., & Sharma, B. (2017). Synergistic effects of heavy metals and pesticides in living systems. Frontiers in chemistry, 5, 70.

Sirianni, S. R., & Huang, C. C. (1983). Induction of sister chromatid exchange by various selenium compounds in Chinese hamster cells in the presence and absence of S9 mixture. Cancer Letters, 18(1), 109-116.

Smith, K. E., Weis, D., Amini, M., Shiel, A. E., Lai, V. W. M., & Gordon, K. (2019). Honey as a biomonitor for a changing world. Nature Sustainability, 2(3), 223-232.

Soltanpour, P.N., J. Benton Jones, Jr., and S.M Workman. Optical Emission Spectroscopy. in Methods of Soil Analysis. Part 2. A.L. Page, et al., ed., pp29-65, 1982.

Tayama, S. (1996). Cytogenetic effects of piperonyl butoxide and safrole in CHO-K1 cells. Mutation Research/Genetic Toxicology, 368(3-4), 249-260.

The Code of federal regulations (2022). GovInfo. Available at https://www.govinfo.gov/content/pkg/CFR-2020-title40-vol26/xml/CFR-2020-title40-vol26-part180.xml. (Accessed: January 30, 2023)

Wang, J. J., Wei, D., Dou, W., Hu, F., Liu, W. F., & Wang, J. J. (2013). Toxicities and synergistic effects of several insecticides against the oriental fruit fly (Diptera: Tephritidae). Journal of Economic Entomology, 106(2), 970-978.

Yaqub, G., Khalid, M., Ikram, A., & Sohail, A. (2020). Monitoring and risk assessment due to presence of metals and pesticides residues in honey samples from the major honey producing forest belts and different brands. Food Science and Technology, 40, 331-335.

Yardimci, M., Sevgiler, Y., Rencuzogullari, E., Arslan, M., Buyukleyla, M., & Yilmaz, M. (2014). Sex-, tissue-, and exposure duration-dependent effects of imidacloprid modulated by piperonyl butoxide and menadione in rats. Part I: oxidative and neurotoxic potentials. Arhiv za higijenu rada i toksikologiju, 65(4), 387-397.

Young, S. J., Gunning, R. V., & Moores, G. D. (2005). The effect of piperonyl butoxide on pyrethroid-resistance-associated esterases in Helicoverpa armigera (Hübner)(Lepidoptera: Noctuidae). Pest Management Science: formerly Pesticide Science, 61(4), 397-401.

Yousuf, S., Atif, F., Ahmad, M., Hoda, M. N., Khan, M. B., Ishrat, T., & Islam, F. (2007). Selenium plays a modulatory role against cerebral ischemia-induced neuronal damage in rat hippocampus. Brain research, 1147, 218-225.

Zhang, J., Zhang, Y., Wang, Y., Yang, Y., Cang, X., & Liu, Z. (2016). Expression induction of P450 genes by imidacloprid in Nilaparvata lugens: A genome-scale analysis. Pesticide Biochemistry and Physiology, 132, 59-64.

Zwanenburg, T. S. B. (1983). Standardized shake-off to synchronize cultured CHO cells. Mutation Research Letters, 120(2-3), 151-159.

CHAPTER 7: CONCLUSIONS

I have always been fascinated with ecology's cause and effect concept and how it is crucial to comprehend the relationships between stressors and ecological responses to seek explanations and reach practical solutions. More precisely, the relationships between humans and other parts of the ecosystem, the stressors humans create, how the ecosystem responds, how we process to understand it, and what we can do to overcome these issues fascinate me.

In this dissertation, I am attempting to put a brick in the wall of understanding the drivers behind the problem of honey bee population decline, considering one of many drivers that trigger this problem and how, from my point of view, it is closely related and connected with human health. Based on the fact that honey bees and the in-hive stored floral products can be used as a biomonitoring tool to detect ecosystem exposure to different contaminants, I was trying to understand one of the stressors that drive the Colony Collapse Disorder phenomenon, which is the chemical stressor. Pesticides have been investigated heavily as the leading cause of bee decline, with less focus on heavy metals as another chemical stressor. In chapter two, I decided to investigate both. I collected pollen and honey samples from different hives in Northern Colorado. I then got them analyzed for pesticides and heavy metals to understand how these toxins would affect the bee population. While pesticide levels were found to be below the accepted levels of concern for the health of honey bees, heavy metal residue levels pose severe threats to honey bees. However, future risk-based research will be required to consider the possibility of combinatorial effects brought on by the interaction of pesticides and heavy metals. The collected pollen and honey samples were acquired from hives located in urban and agricultural areas to understand spatial variability's effect on the quantity and quality of accumulated toxins in hives. Based on the

analysis results, there was no significant spatial variation in pesticide or heavy metal in the collected pollen and honey samples.

In chapter three, I examined pollen samples and sorted them for their botanical sources to determine the effect of landscape heterogeneity on pollen variety to comprehend another stressor that led to the collapse of the bee population, the nutritional stressor. I also calculated the most prevalent botanical families that will reveal the preferred protein sources for honey bees. The findings showed a geographical difference in Shannon-Weaver diversity, with urban locations having a higher diversity index and a greater variety of pollen taxa gathered than agricultural areas, which had a lower diversity index and less diversity in pollen taxonomic variety. Does having higher pollen diversity in urban areas mean that bees are nutritionally stressed in agricultural areas? This is one of the variables that I tried to elucidate in chapter four, along with the chemical stressor. By taking into account landscape fluctuations, pesticide breakdown, and honey bees' capacity to detoxify contaminants, I used agent-based modeling in an attempt to explain bees' foraging behavior under different stressors, like exposure to toxins, land variation, and nutritional deficits. The findings of this study showed that spatial variation had a substantial impact on the amounts of collected floral products that had accumulated in the hive, higher in agricultural areas, which in turn had a significant impact on the amounts of pesticides and heavy metals that had accumulated there. These discrepancies between experimental and model findings open the door for more research to study more variables collectively that would affect honey bee behavior and population decline.

Based on the honey sample tests in chapter two and the connection between how artificial toxins cause the loss of the bee population and how that affects human health, I had concentrations of contaminants quantified. Sixteen different pesticides and four heavy metals were found in

honey. These findings highlight the potential for increased toxicity due to widespread chemical use and the existence of chemical and metal residues in a variety of meals other than honey. From these chemicals found in honey samples, we selected a pesticide (Imidacloprid), a pesticide synergist (Piperonyl butoxide), and two heavy metals (Pb) and (Se) to examine any potential synergistic effects caused by combinatorial treatments with these chemicals. We observed distinct combinatorial toxicity effects of these chemicals at the cellular level, which opened the door for further investigation into the toxicological mechanisms underlying exposure to various combinations at various concentrations, including values below the permissible intake limit or the level of concern. This information will later help decision-makers, and regulatory bodies reevaluate whether these chemicals' allowable limits are safe under synergistic interaction.

APPENDIX I

Table A1: Dates of pollen and honey sample collection and quantities obtained.

Site	Description	Pollen (g)	Date	Honey (g)	Date
1	Agriculture	95.23	7/20/19	25.96	10/13/19
		79.00	8/25/19		
		238.94	9/15/19		
2	Agriculture	24.34	7/7/19	48.36	9/26/19
		91.00	8/5/19		
		100.20	9/2/19		
3	Agriculture	34.30	7/1/19	36.10	9/14/19
		68.47	7/30/19		
		73.12	8/25/19		
4	Agriculture	24.33	7/28/19	32.83	none
		21.93	8/16/19		
5	Agriculture	94.23	7/2/19	34.98	9/30/19
		21.53	7/30/19		
		22.91	8/20/19		
6	Urban	23.97	7/6/19	29.78	9/6/19
		62.26	8/1/19		
		75.51	9/1/19		
7	Urban	45.30	7/17/19	20.89	9/21/19

		52.57	8/9/19		
		38.46	9/1/19		
8	Urban	63.50	7/17/19	25.67	9/14/19
		25.22	8/20/19		
9	Urban	23.55	7/26/19	None	NA
		24.30	8/20/19		
10	Agriculture	126.56	6/27/20	32.00	10/1/20
		106.50	7/16/20		
		117.91	8/8/20		
11	Agriculture	25.27	6/26/20	26.03	9/1/20
		53.00	7/3/20		
		35.14	8/2/20		
12	Agriculture	201.00	7/2/20	None	NA
		24.51	9/12/20		
13	Agriculture	65.01	6/6/20	23.30	8/4/20
		59.13	7/22/20		
		60.56	8/5/20		
14	Agriculture	54.85	6/28/20	20.57	8/22/20
		90.01	7/14/20		
		31.41	8/22/20		

15	Agriculture	1020.00	6/23/20	22.65	9/24/20
		520.00	7/24/20		
		1035.00	8/18/20		
16	Urban	22.12	7/1/20	30.57	8/31/20
		67.73	8/10/20		
		98.69	9/1/20		
17	Urban	23.65	6/27/20	20.20	9/23/20
18	Urban	54.05	6/7/20	26.78	9/9/20
		61.77	7/22/20		
		55.56	8/5/20		
19	Urban	34.98	6/7/20	21.67	8/24/20
		64.09	7/12/20		
		69.62	8/20/20		
20	Urban	49.52	6/27/20	28.23	7/22/20
		66.95	7/9/20		
		65.43	8/5/20		
21	Urban	22.80	7/26/20	20.43	9/15/20
22	Urban	22.17	6/2/20	31.35	9/24/20
		54.10	7/16/20		
		120.30	8/13/20		
23	Urban	24.53	7/7/20	27.54	8/5/20

	28.01	8/4/20
	35.15	9/4/20

APPENDIX II

Table A2. Types and concentrations of pesticides surveyed in honey and pollen samples. LOD and LOQ are the Limit of Detection and Limit of Quantitation respectively, which represent the lowest concentrations of pesticides that can be detected.

Pesticide	Type	LOD-LOQ (ppb) honey	LOD-LOQ (ppb) pollen
2,4-DMPF	Insecticide	0.29 - 0.86	0.40 - 1.20
4-Hydroxy-chlorothalonil	Fungicide	1.43 - 4.29	2.00 - 6.00
Acephate	Insecticide	0.71 - 2.14	1.00 - 3.00
Acetamiprid	Insecticide	0.07 - 0.21	0.1 - 0.30
Ametryn	Herbicide	0.03 - 0.09	0.04 - 0.12
Atrazine	Herbicide	0.07 - 0.21	0.1 - 0.30
Avermectin B1a	Acaricide	0.43 - 1.29	0.60 - 1.80
Azoxystrobin	Fungicide	0.03 - 0.09	0.04 - 0.12
Bendiocarb	Insecticide	0.09 - 0.26	0.12 - 0.36
Boscalid	Fungicide	1.43 - 4.29	2.00 - 6.00
Bromuconazole	Fungicide	0.43 - 1.29	0.60 - 1.80
Carbaryl	Insecticide	0.14 - 0.43	0.20 - 0.60
Carbofuran	Insecticide	0.03 - 0.09	0.04 - 0.12
Chlorantraniliprole	Insecticide	0.14 - 0.43	0.20 - 0.60
Chlorpyrifos	Insecticide	4.29 - 12.86	6.00 - 18.00
Clomazone	Herbicide	0.11 - 0.34	0.16 - 0.48
Clothianidin	Insecticide	0.29 - 0.86	0.40 - 1.20
Coumaphos	Insecticide	1.43 - 4.29	2.00 - 6.00
Cyanazine	Herbicide	0.14 - 0.43	0.20 - 0.60
Cyantraniliprole	Insecticide	0.14 - 0.43	0.20 - 0.60
Cyflufenamid	Fungicide	0.14 - 0.43	0.20 - 0.60
Cyprodinil	Fungicide	0.03 - 0.09	0.04 - 0.12
Cyromazine	Insecticide	0.71 - 2.14	1.00 - 3.00
Difenoconazole	Fungicide	0.07 - 0.21	0.1 - 0.30

Diflubenzuron	Acaricide	2.86 - 8.57	4.00 - 12.00
Dimoxystrobin	Fungicide	0.03 - 0.09	0.04 - 0.12
Dinotefuran	Insecticide	0.14 - 0.43	0.20 - 0.60
Diuron	Herbicide	0.29 - 0.86	0.40 - 1.20
Fenamidone	Fungicide	0.07 - 0.21	0.1 - 0.30
Fenbuconazole	Fungicide	0.14 - 0.43	0.20 - 0.60
Fenhexamid	Fungicide	2.86 - 8.57	4.00 - 12.00
Fenpyroximate	Acaricide	0.07 - 0.21	0.10 - 0.30
Fipronil	Insecticide	0.14 - 0.43	0.20 - 0.60
Fluazifop	Herbicide	0.43 - 1.29	0.60 - 1.80
Fluazinam	Fungicide	0.14 - 0.43	0.20 - 0.60
Fludioxonil	Fungicide	0.43 - 1.29	0.60 - 1.80
Flufenacet	Herbicide	0.29 - 0.86	0.40 - 1.20
Flumioxazin	Herbicide	7.14 - 21.43	10.00 - 30.00
Fluometuron	Herbicide	0.29 - 0.86	0.40 - 1.20
Fluopicolide	Fungicide	0.14 - 0.43	0.20 - 0.60
Fluopyram	Fungicide	0.03 - 0.09	0.04 - 0.12
Fluoxastrobin	Fungicide	0.03 - 0.09	0.04 - 0.12
Flupyradifurone	Insecticide	0.29 - 0.86	0.40 - 1.20
Fluxapyroxad	Fungicide	0.29 - 0.86	0.40 - 1.20
Fumagillin	Fungicide	1.43 - 4.29	2.00 - 6.00
Hexaflumuron	Insecticide	2.86 - 8.57	4.00 - 12.00
Imidacloprid	Insecticide	0.14 - 0.43	0.20 - 0.60
Indoxacarb	Insecticide	0.43 - 1.29	0.60 - 1.80
Malaoxon	Insecticide	0.03 - 0.09	0.04 - 0.12
Mandipropamid	Fungicide	0.06 - 0.17	0.08 - 0.24
Metalaxyl	Fungicide	0.07 - 0.21	0.10 - 0.30
Metazachlor	Herbicide	0.03 - 0.09	0.04 - 0.12
Metconazole	Fungicide	0.29 - 0.86	0.40 - 1.20
Methiocarb	Insecticide	0.29 - 0.86	0.40 - 1.20
Methoprotryne	Herbicide	0.03 - 0.09	0.04 - 0.12

Methoxyfenozide	Insecticide	0.07 - 0.21	0.10 - 0.30
Metobromuron	Herbicide	0.43 - 1.29	0.60 - 1.80
Metolachlor	Herbicide	0.14 - 0.43	0.20 - 0.60
Mevinphos	Insecticide	0.14 - 0.43	0.20 - 0.60
Myclobutanil	Fungicide	0.07 - 0.21	0.10 - 0.30
Napropamide	Herbicide	0.03 - 0.09	0.04 - 0.12
Penthiopyrad	Fungicide	0.03 - 0.09	0.04 - 0.12
Phenmedipham	Herbicide	0.14 - 0.43	0.20 - 0.60
Phosmet	Insecticide	1.43 - 4.29	2.00 - 6.00
Picoxystrobin	Fungicide	0.03 - 0.09	0.04 - 0.12
Piperonyl butoxide	pesticide synergist	0.03 - 0.09	0.04 - 0.12
Profenophos	Insecticide	0.57 - 1.71	0.80 - 2.40
Prometon	Herbicide	0.03 - 0.09	0.04 - 0.12
Prometryn	Herbicide	0.03 - 0.09	0.04 - 0.12
Propazine	Herbicide	0.03 - 0.09	0.04 - 0.12
Propiconazole	Fungicide	0.29 - 0.86	0.40 - 1.20
Pyraclostrobin	Fungicide	0.03 - 0.09	0.04 - 0.12
Pyrimethanil	Fungicide	0.14 - 0.43	0.20 - 0.60
Spinetoram	Insecticide	0.07 - 0.21	0.10 - 0.30
Spinosad	Insecticide	0.07 - 0.21	0.10 - 0.30
Spirotetramat	Insecticide	0.07 - 0.21	0.10 - 0.30
Sulfentrazone	Herbicide	2.86 - 8.57	4.00 - 12.00
Sulfoxaflor	Insecticide	1.43 - 4.29	2.00 - 6.00
Tebuconazole	Fungicide	0.29 - 0.86	0.40 - 1.20
Tebufenozide	Insecticide	0.03 - 0.09	0.04 - 0.12
Tebuthiuron	Herbicide	0.03 - 0.09	0.04 - 0.12
Terbutryn	Herbicide	0.03 - 0.09	0.04 - 0.12
Tetraconazole	Fungicide	0.29 - 0.86	0.40- 1.20
Tetramethrin	Insecticide	0.43 - 1.29	0.60 - 1.80
Thiabendazole	Fungicide	0.07 - 0.21	0.10 - 0.30
Thiacloprid	Insecticide	0.07 - 0.21	0.10 - 0.30

Thiamethoxam	Insecticide	0.07 - 0.21	0.10 - 0.30
Thiobencarb	Herbicide	0.43 - 1.29	0.60 - 1.80
Thiophanate-methyl	Fungicide	0.07 - 0.21	0.10 - 0.30
Triadimefon	Fungicide	0.29 - 0.86	0.40 - 1.20
Trifloxystrobin	Fungicide	0.03 - 0.09	0.04 - 0.12
Triflumizole	Fungicide	0.07 - 0.21	0.10 - 0.30

APPENDIX III

Table A3. Statistical data summary of pesticide residues detected in pollen samples.

Pesticide	Agriculture	Urban	Multiple t-test			
	Mean (ppb)	Mean (ppb)	SE of Difference	P-value	t	df
2,4-DMPF	ND	0.12	0.12	0.33	1.00	11.00
Atrazine	3.44	2.03	1.00	0.28	1.12	13.19
Azoxystrobin	0.40	0.63	0.342	0.45	0.78	12.31
Bendiocarb	0.00	0.02	0.02	0.33	1.00	11.00
Carbaryl	0.76	16.28	7.97	0.07	1.96	11.05
Chlorantraniliprole	1.65	11.06	10.64	0.38	0.89	11.39
Chlorpyrifos	17.30	26.01	27.98	0.72	0.36	14.38
Clothianidin	0.88	0.10	0.38	0.08	1.89	11.73
Cyanazine	0.03	ND	0.03	0.33	1.00	11.00
Cyprodinil	0.02	ND	0.02	0.33	1.00	11.00

Diuron	3.21	2.14	1.21	0.48	0.71	14.92
Fenpyroximate	0.06	0.01	0.04	0.36	0.94	13.05
Fluoxastrobin	0.00	0.01	0.01	0.33	1.00	11.00
Imidacloprid	0.10	1.37	0.52	0.03	2.44	11.51
Indoxacarb	0.00	0.24	0.24	0.33	1.00	11.00
Malaoxon	0.01	0.02	0.01	0.14	1.52	17.76
Mandipropamid	0.01	0.00	0.01	0.33	1.00	11.00
Metalaxyl	0.01	0.01	0.02	>0.99	0.00	22.00
Metconazole	1.59	0.73	1.28	0.50	0.68	19.64
Metolachlor	1.12	0.15	0.49	0.06	2.00	11.52
Methoxyfenozide	0.04	0.03	0.03	0.63	0.48	21.52
Myclobutanil	0.00	0.03	0.02	0.16	1.48	11.00
Penthiopyrad	0.07	0.01	0.04	0.21	1.33	11.53
Picoxystrobin	0.01	0.05	0.05	0.46	0.75	12.19

Piperonyl butoxide	1.44	3.83	1.84	0.21	1.30	12.07
Prometon	0.02	0.08	0.05	0.24	1.23	12.31
Propiconazole	0.75	0.10	0.48	0.19	1.37	11.45
Pyraclostrobin	0.20	0.12	0.08	0.38	0.89	21.55
Pyrimethanil	0.02	0.00	0.03	0.33	1.00	11.00
Spinosad	0.06	0.05	0.08	0.87	0.16	22.00
Spirotetramat	0.01	0.00	0.01	0.33	1.00	11.00
Tebuthiuron	0.13	0.08	0.11	0.65	0.46	17.65
Tebuconazole	0.35	3.76	3.43	0.33	0.99	11.12
Thabendazole	0.01	0.01	0.18	>0.99	0.00	22.00
Thiamethoxam	0.13	0.24	0.13	0.41	0.84	14.94
Thiophanate-methyl	0.19	105.2	104.4	0.33	1.01	11.00
Trifloxystrobin	0.00	0.02	0.01	0.08	1.915	11.00

APPENDIX IV

Table A4. Statistical data summary of pesticide residues detected in honey samples.

Pesticide	Agriculture	Urban	Multiple t-test			
	Mean (ppb)	Mean (ppb)	SE of Difference	P-value	t	df
2,4-DMPF	0.04	0.64	0.38	0.14	1.57	10.26
Acephate	0.00	0.92	0.82	0.28	1.12	10.00
Acetamiprid	0.00	0.02	0.02	0.34	1.00	10.00
Atrazine	0.00	0.02	0.02	0.34	1.00	10.00
Carbaryl	0.00	0.02	0.02	0.34	1.00	10.00
Chlorantraniliprole	0.00	0.02	0.02	0.34	1.00	10.00
Coumaphos	0.36	0.22	0.92	0.36	0.94	11.15
Dinotefuran	0.00	0.04	0.04	0.34	1.00	10.00
Diuron	0.00	0.04	0.04	0.34	1.00	10.00
Fenpyroximate	0.00	0.04	0.03	0.17	1.47	10.00

Imidacloprid	0.00	0.37	0.18	0.06	2.05	10.00
Penthiopyrad	0.00	0.00	0.00	0.34	1.00	10.00
Piperonyl butoxide	0.11	0.19	0.13	0.53	0.64	18.78
Tebuthiuron	0.00	0.01	0.01	0.08	1.94	10.00
Thiabendazole	0.02	0.00	0.02	0.34	1.00	9.00
Thiamethoxam	0.08	0.00	0.05	0.17	1.63	4.00

APPENDIX V

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Undergraduate Research as a High-impact Practice for Engaging Students of Color in Ecology

Mai M. Awad[1] and Mark A. Brown[1,2,3,4,5]
[1]Graduate Degree Program in Ecology, Colorado State University, Fort Collins, Colorado 80523 USA
[2]Department of Ethnic Studies, Colorado State University, Fort Collins, Colorado 80523 USA
[3]Department of Clinical Sciences, Colorado State University, Fort Collins, Colorado 80523 USA
[4]Global Health and Health Disparities, Colorado School of Public Health, Fort Collins, Colorado 80523 USA
[5]Cell and Molecular Biology Program, Colorado State University, Fort Collins, Colorado 80523 USA

Introduction

The number of Hispanic, African American, Alaskan Native, and American Indian students who graduate in science disciplines and find employment in science careers is unacceptably low, as are the retention rates of the small number of students of color who matriculate into graduate-level science programs. While approximately 25,000 PhDs in STEM disciplines are awarded each year by universities in the United States, students enrolled as underrepresented minorities (predominantly Black and Hispanic) earn less than 5% of those degrees despite comprising ~30% of the total population (Fabio et al. 2008, National Science Foundation 2018). Students of color face many obstacles and barriers to science engagement, including cultural differences and lack of exposure to mentors and role models (Hall and Post-Krammer 1987, Maton et al. 2000, House 2000, Armstrong et al. 2007). These barriers limit the ability of underrepresented minorities to succeed in STEM pathways.

Such small numbers of students of color in these areas can have a variety of negative impacts on issues that range from the US economy and the conceptualization of graduate degrees to careers that some students consider as realistic options. Diverse mentors/role models are critical for successful science recruitment and retention programs (Council of Graduate Schools 2003). If science faculty of color are a rarity, how are undergraduate and graduate students of color able to find role models? Likewise, if persons of color are a rarity among the ranks of science professionals, how are recent graduates of color able to find role models in science careers? This situation may partially be caused by the cultural and family values of persons of color, which may lead to decreased participation in STEM fields from the start. In order to change this trend and move traditionally underrepresented students into graduate programs and, ultimately, senior positions in science disciplines, we need to establish a pipeline of programs that support the engagement of students of color in science education.

The essential role of faculty mentor contact and engagement is a common theme across models for student engagement and retention (Tinto 2012). Engagement methods that have been shown to improve retention include faculty-mentored research, virtual reality, active learning, internships, and reverse classroom structures (Ryan 2019, Sultan et al. 2019, Stranford et al. 2020, Cottone 2020). Faculty-mentored research is an ideal engagement strategy because it gives students a tangible, hands-on application of

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classroom concepts while also preparing them with practical problem-solving skills (Kardash 2000, National Research Council 2000, Boyd et al. 2009). The intersection between theory (introduced in the classroom) and practice (obtained through mentored research experiences) improves learning outcomes and an early definition of career goals (Kuh 2011). Through research experiences, a student's first-hand exposure and response to real-world problems, along with real-time reflection upon the products of their actions, facilitate the development and evolution of knowledge across their discipline (National Research Council 2000). Undergraduate research is particularly well suited for bridging intersecting disciplines in highly complex fields like ecology (Itin 1999, Kuh and Kinzie 2018, Emery et al. 2019, Hernandez et al. 2018, Martinez et al. 2018, Chelberg and Bosman 2019, Morton 2020). At Colorado State University (CSU), as highlighted below, mentored research has proven especially effective at increasing academic performance, retention, and realization of career opportunities for science students of color. This underscores the value of undergraduate research as a high-impact practice for engaging students of color in ecology.

Discussion

In 2009, CSU established its first all-university office for undergraduate research. At that time, university surveys indicated that fewer than 1400 of CSU's approximately 26,000 undergraduates participated in faculty-mentored research. Starting as early as freshman year, the office invites undergraduates to participate in faculty-mentored research, presents students with the benefits of undergraduate research, and works with students and faculty to place students in research positions. The number of undergraduate researchers at CSU is now pushing 6000, despite research remaining voluntary for all students. Notably, the increase in participation of CSU students of color in research has significantly outpaced that of the general student population by a margin of 560%, compared to 326%, respectively. More importantly, science students of color participating in formal research programs at CSU far outperform other students of color and the general student population based on several key metrics, including STEM persistence, retention, and entry into STEM graduate programs and/or careers. While it is possible that the students that opt for extracurricular research experience may not be a true "random" sample of the general student population, it remains true that CSU's increased emphasis on student research has provided greater opportunity for all students and that retention has also increased. Participants of CSU's Rocky Mountain Scholars Program (RMSP), which provides participants with cohort-based research placements, place emphasis on the recruitment and success of STEM students of color. Since 2011, participants have had a 100% rate of retention in higher education and a 99% rate of persistence in STEM programs. These rates are dramatically higher than our institutional average of 68%. Among the graduates of this program, 89.6% have either elected to pursue a STEM graduate degree or have entered the STEM workforce.

Among a range of focus areas, the RMSP added an Ecology Cohort in 2019 that provides undergraduate research opportunities. The current project for this cohort of 15 students (ranging from freshman to seniors) involves a cross-disciplinary study that spans ecological health, animal health, and public health. Briefly, the project samples regional honey and pollen samples from a network of managed beehives to identify contaminants, including heavy metals, trace metals, insecticides, fungicides, and herbicides. In silico modeling is conducted to predict broader impacts on bee health, ecological health, and human health. For participants, what begins as in-field sampling later leads to chemical analyses, pollen identification, computer modeling, and, ultimately, cell-based studies of the impacts each

identified adulterating agent (both individually and combinatorically) has on human breast, colorectal, and hepatic cells using proliferation and tumorigenicity assays. Thus, participants are able to focus on a specific aspect of this project while being exposed to a wide range of meaningful procedures in studying the cascade of intersecting impacts related to interconnected ecosystems. These are highly impactful, career-defining experiences for most participants.

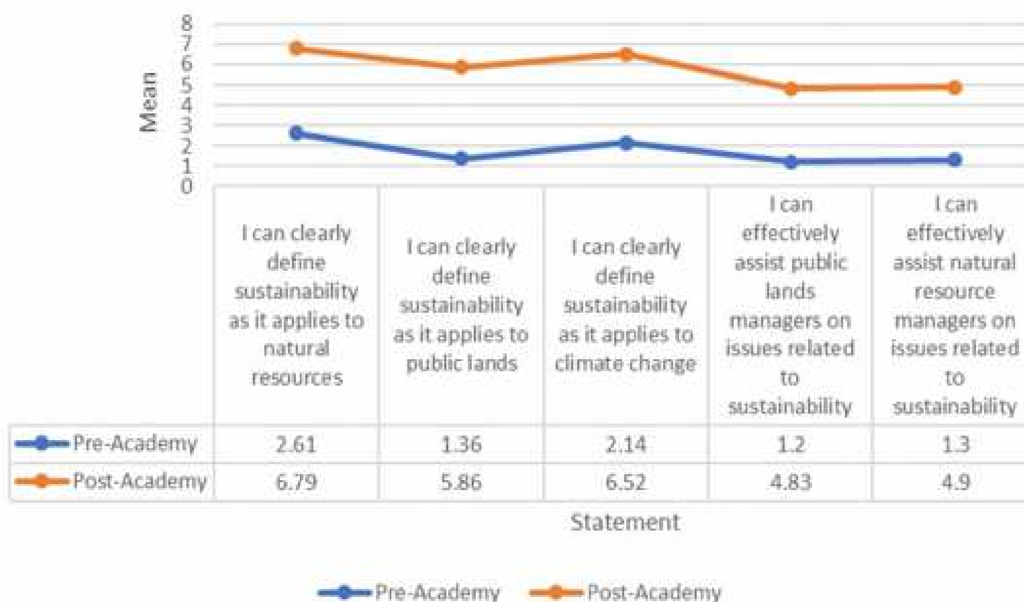

Fig. 1. Sustainability: Define and assist self-efficacy survey results (Bowser et al. 2014).

Among CSU's formal undergraduate research programs, the RMSP is a program that emphasizes the engagement of students of color in ecology-focused experiential learning that has shown some of the most profound impacts. The Rocky Mountain Science and Sustainability Network (RMSSN) was established at CSU based upon the overarching vision to help train the next diverse generation of public lands leaders. The year-to-year average of students of color in the RMSSN is approximately 75%. Based on a battery of self-efficacy measures, as well as a range of qualitative indices (Davis et al. 2012, Bowser et al. 2014, Gretzel et al. 2014, Halliwell et al. 2020), the RMSSN now includes a network of hundreds of program graduates who are rapidly ascending the ranks of public lands agencies throughout the world. Figs. 1 and 2 below manifest a shift toward stronger self-efficacy following the experience of RMSSN Academy. In Fig. 1, the collected student responses after engaging with the RMSSN activities reveal the shift toward more confidence in defining and assisting with global environmental sustainability. In Fig. 2, the collected responses after the academy experience display a significant shift toward confidence, scientific knowledge, and the ability to

define, explain, or assist in climate change efforts. According to student surveys, the leading and overarching cause of these positive results was research experience, although mentorship and cohort support also played a role.

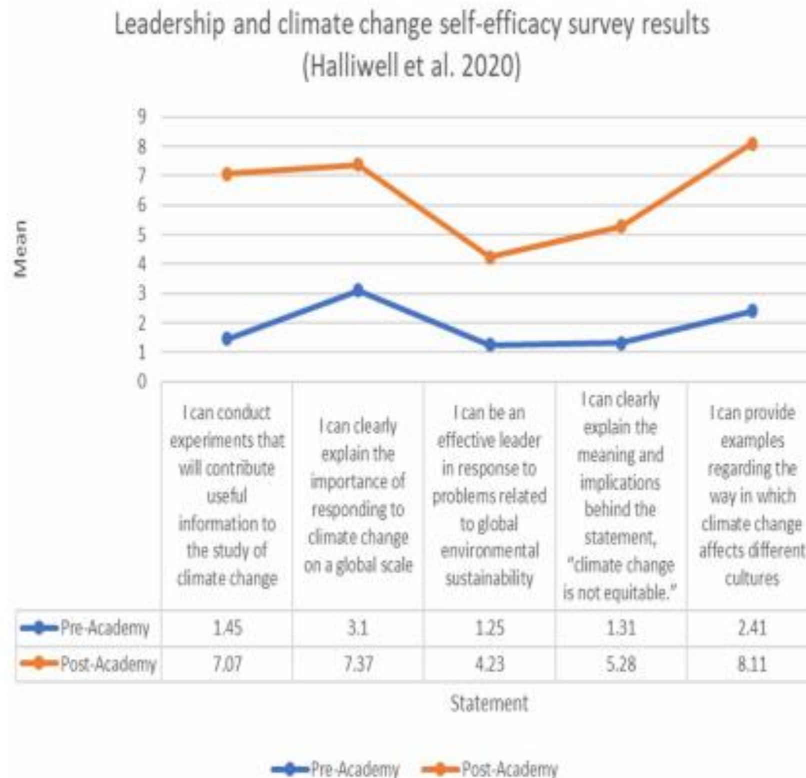

Fig. 2. Leadership and climate change self-efficacy survey results (Halliwell et al. 2020).

Conclusion

Underrepresentation of students of color in the ecological sciences has far-reaching socioeconomic impacts and severely limits the diversity and breadth of influence and experience brought to bear on ecological issues. Student engagement is a powerful tool for both recruitment and retention, and it is the most important factor in retention programs for undergraduate students of color (Upcraft et al. 2005, Pascarella et al. 2008, Tinto 2012). Undergraduate research has been previously proven to have the most positive outcomes, among all high-impact practices, with regard to promoting student engagement, and we have repeated those results through a combination of formal and informal programs at Colorado State University. In particular, the RMSSN provides a framework for implementing mentored research as a high-impact practice in undergraduate ecology education.

For more information about RMSSN or the Rocky Mountain Scholars Program, see https://rmssn.wordpress.com/ and https://tilt.colostate.edu/OURA/RMSP.

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Literature Cited

Armstrong, M. J., A. R. Berkowitz, L. A. Dyer, and J. Taylor. 2007. Understanding why underrepresented students pursue ecology careers: a preliminary case study. Frontiers in Ecology and the Environment 5:415–420.

Bowser, G., U. Gretzel, E. Davis, and M. Brown. 2014. Educating the future of sustainability. Sustainability 6:692–701.

Boyd, M. K., J. L. Wesemann, and K. A. Frederick. 2009. Broadening participation in undergraduate research: Fostering excellence and enhancing the impact. Council on Undergraduate Research: Washington, D.C., USA.

Chelberg, K. L., and L. B. Bosman. 2019. The role of faculty mentoring in improving retention and completion rates for historically underrepresented STEM students. International Journal of Higher Education 8:39–48.

Cottone, A. M., and S. Yoon. 2020. Improving the design of undergraduate biology courses toward the goal of retention: The case of real-world inquiry and active learning through metagenomics. J Microbiol Biol Educ. 21:1–10.

Council of Graduate Schools. 2003. Achieving an inclusive graduate community (Inclusiveness Series, vol1, p22) Washington, D.C.

Davis, E., G. Bowser, and M. Brown. 2012. Creating the global leader and global mind-set: Engaging multicultural students in multidimensional learning. Pages 891-899 *in* D. R. Gallagher, editor. Environmental leadership: A reference handbook. SAGE Publishers, Thousand Oaks, California, USA.

Emery, N. A., A. Hund, R. Burks, M. Duffy, C. Scoffoni, and A. Swei. 2019. Students as ecologists: Strategies for successful mentorship of undergraduate researchers. Academic Practice in Ecology and Evolution 9:4316–4326.

Fabio, D., N. Brandi, and L. Frehill. 2008. Professional Women and Minorities: A total human resources data compendium. Commission on Professionals in Science and Technology, Washington, D.C., USA.

Graham, M. J., J. Frederick, A. Byars-Winston, A. B. Hunter, and J. Handelsman. 2013. Increasing persistence of college students in STEM. Science 341:1455–1456.

Gretzel, U., E. B. Davis, G. Bowser, J. Jiang, and M. Brown. 2014. Creating global leaders with sustainability mindsets–Insights from the RMSSN summer academy. Journal of Teaching in Travel & Tourism 14:164–183.

Hall, E. R., and P. Post-Kammer. 1987. Black mathematics and science majors: Why so few? Career Development Quarterly 35:206–219.

Halliwell, P., S. Whipple, K. N. Hassel, G. Bowser, D. W. Husic, and M. A. Brown. 2020. Twenty-first-century climate education: Developing diverse, confident, and competent leaders in environmental sustainability. Bulletin of the Ecological Society of America 101:e01664.

Hernandez, P. R., A. Woodcock, M. Estrada, and P. W. Schultz. 2018. Undergraduate research experiences broaden diversity in the scientific workforce. BioScience 68:204–211.

House, J. D. 2000. Academic background and self-beliefs as predictors of student grade performance in science, engineering and mathematics. International Journal of Instructional Media 27:207.

Itin, C. M. 1999. Reasserting the philosophy of experiential education as a vehicle for change in the 21st century. Journal of Experiential Education 22:91–98.

Kardash, C. M. 2000. Evaluation of undergraduate research experience: Perceptions of undergraduate interns and their faculty mentors. Journal of Educational Psychology 92:191.

Kuh, G. D. 2011. Student success in college: The promise of high impact practices. Association of American Colleges and Universities, Washington, D.C., USA.

Kuh, G. D., and J. Kinzie. 2018. What Really Makes a 'High-Impact' Practice High Impact? Inside Higher Education 1.

Martinez, T., N. Bloom, A. V. Whipple, and S. Chischilly. 2018. Partnerships, participation, and equity in undergraduate research. Scholarship and Practice of Undergraduate Research; Washington 1:4:40–45.

Maton, K. I., F. A. III Hrabowski, and C. L. Schmitt. 2000. African American college students excelling in the sciences: College and postcollege outcomes in the Meyerhoff Scholars Program. Journal of Research in Science Teaching: The Official Journal of the National Association for Research in Science Teaching 37:629–654.

Morton, T. R. 2020. A phenomenological and ecological perspective on the influence of undergraduate research experiences on Black women's persistence in STEM at an HBCU. Journal of Diversity in Higher Education. Advance online publication. https://doi.org/10.1037/dhe0000183.

National Research Council. 2000. How people learn: Brain, mind, experience, and school: Expanded edition. National Academies Press, Washington, D.C., USA.

National Science Foundation. 2018. Earned doctoral degrees, by citizenship, field, race, and ethnicity: 2000–15. Science and Engineering Indicators, Appendix Table 2–32. nsf.gov/statistics/2018/nsb20181/assets/561/tables/at02-32.pdf

Pascarella, E. T., T. A. Seifert, and E. J. Whitt. 2008. Effective instruction and college student persistence: Some new evidence. New Directions for Teaching and Learning 2008:55–70.

Ryan, E., and C. Poole. 2019. Impact of Virtual Learning Environment on Students' Satisfaction, Engagement, Recall, and Retention. Journal of Medical Imaging and Radiation Sciences 50:408–415.

Stranford, S. A., J. A. Owen, F. Mercer, and R. R. Pollock. 2020. Active learning and technology approaches for teaching immunology to undergraduate students. Frontiers in Public Health 8:114.

Sultan, L., W. Abuznadah, H. Al-Jifree, M. A. Khan, B. Alsaywid, and F. Ashour. 2019 . An experimental study on usefulness of virtual reality 360° in undergraduate medical education. Advances in Medical Education and Practice 10:907–916.

Tinto, V. 2012. Completing college: Rethinking institutional action University of Chicago Press, Chicago, Illinois, USA.

Upcraft, M. L., J. N. Gardner, and B. O. Barefoot. 2005. Challenging and supporting the first-year student: A handbook for improving the first year of college. Volume 254. Jossey-Bass, San Francisco, California, USA.