QUANTIFYING SUGAR LEVELS IN BUMBLE BEE HEMOLYMPH IN RELATION TO SUBLETHAL DOSAGES OF PESTICIDES

By

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A capstone project submitted for Graduation with University Honors

May 06, 2021

University Honors

University of California, Riverside

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ABSTRACT

Bumble bees (genus *Bombus*) use sugars from nectar as their primary source of energy; these sugars are essential for the collection, utilization, and distribution of energy within bees. Bumble bees must exist in a rapid state of absorbing and breaking down sugars into usable energy to fuel their flight; thus, bumble bees are ideal organisms for studying energetics and the process by which sugars are biochemically redistributed throughout body systems. A deeper understanding of bumble bee energetics segues to further comprehension of bumble bees' functional ability and impact on various ecosystems as a critical pollinator. I hypothesize that the neonicotinoid family of insecticides may adversely impact the bee energetic state. I analyzed the sugar concentration of bumble bee hemolymph (i.e., insect blood) under separate conditions (bees fed a normal diet, bees starved, bees exposed to sublethal dosages of pesticides). I anticipate that exposing bees to sublethal doses of insecticides will decrease the concentration of sugars in their hemolymph, subsequently decreasing their functional ability, which may ultimately impact pollination where bumble bees are critical to the ecosystem.

ACKNOWLEDGEMENTS

I would like to thank my faculty mentor and principal investigator, Dr. S. Hollis Woodard, for her unwavering support, mentorship, and compassion throughout the entirety of my undergraduate career. I would also like to thank my graduate student mentor, Natalie Fischer, for her continued patience, understanding, and guidance over the past four years. Dr. Woodard and the other members of the Woodard Lab have transformed me as a student, as a researcher, and as a person, and I am beyond grateful for the continued support that I have received from my colleagues and mentors in the lab. The Woodard Lab has been a home away from home for me, and I am beyond grateful for the wonderful experiences, memories made, and lessons learned through my research journey. I am honored to be a part of the Woodard Lab, and I will take with me all that I have learned for the remainder of my professional career.

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INTRODUCTION

Bumble bees exist in a constant state of absorbing and breaking down sugars into usable energy [1-2]; thus, bumble bees are ideal organisms for studying energetics and the process by which sugars are biochemically redistributed throughout the body. Bumble bees consume nectar, which is high in carbohydrate concentration; sucrose, the most common carbohydrate found in nectar, is then broken down into the simpler sugars glucose and fructose once ingested by the bee [1]. These sugars act as the primary energy source for bumble bees [4], that can be stored in various tissues or exist in a free-floating state within hemolymph, a fluid contained within the open circulatory systems of invertebrates [1, 3]. It is currently unclear how varying nutritional conditions impact sugar levels in bumble bee hemolymph, and on what timescale sugars are used for energy. The effects of neonicotinoid pesticides on bumble bee sugar levels are also largely unknown. Neonicotinoids, such as imidacloprid, are widely used pesticides that have been implicated in the overall decline of pollinator populations worldwide [2]. Neonicotinoids are insect neurotoxins that block acetylcholine receptors and, at high exposure levels, lead to paralysis and eventual death of exposed insects [1-4]. These pesticides also have sublethal effects on bees, such as a reduction in individual bee and colony health through weakened immune systems[3] and abnormal foraging behaviors [1, 4]. Additional studies have demonstrated that imidacloprid exposure, along with other neonicotinoid pesticides, can result in neurodegeneration within the insect central nervous system as a result of mitochondrial dysfunction [5-6]. The mitochondria are cellular organelles which house the tricarboxylic acid cycle and the electron transport chain. These biochemical processes are responsible for the majority of ATP production, and thus cellular energy. As such, mitochondrial dysfunction and neurodegeneration can be expected to produce several downstream physiological effects on the insect body and brain [5]. I predict that

exposing bees to sublethal, field-realistic doses of pesticide will decrease the concentration of sugars in the hemolymph, which may decrease their functional ability. This prediction is based on the notion that neonicotinoids disrupt a bee's central nervous system, which may have adverse effects on the distribution of sugars for energy. This study is the first to analyze the effects that imidacloprid has on the energetic state of bumble bees in the frame of hemolymph sugar concentrations. Quantifying the sugar levels of bee hemolymph in relation to sublethal doses of imidacloprid is important for better understanding both the energetic and physiological states of bumble bees exposed to important stressors involved in their decline.

Sugars play an integral role in central carbon metabolism, which constitutes several biochemical pathways by which sugars, amino acids, and other biological macromolecules are converted into precursors of energy metabolism [4-5]. These biological precursors include those involved in glycolysis, the tricarboxylic acid cycle, and the electron transport chain [8], all of which are essential for energy production in bees. Hemolymph constantly circulates and remains in direct contact with the tissues of bees, serving a transportive role [1, 4, 9]. The sugars and metabolites required to meet the physiological demands of bumble bees are directly transported to tissues where the majority of central carbon metabolism takes place [3-4], including the thoracic tissue containing flight muscles [4-5]. Muscles within the thoraces of bumble bees power flight, which is an extremely energetically demanding activity[5] necessary for foraging, and these muscles depend on sugars and other metabolites transported in hemolymph to power central carbon metabolism. Better understanding the effects of imidacloprid on the metabolic pathways which power flight, a key component of bumble bee functional ability, is essential to assessing the role that imidacloprid plays in bumble bee population decline; this study is the first to examine the effects of imidacloprid on central carbon metabolism, specifically energy metabolism, in bumble bee thoracic tissue.

To test the effects of imidacloprid on bumble bee hemolymph sugar levels and energy metabolites, workers were exposed to field-realistic, sublethal levels of imidacloprid (5 ppb)[1, 2, 7, 9] according to four paradigms of pesticide exposure. Bumble bees were exposed to either a *chronic* treatment (consistent exposure for seven days), an *acute-early* treatment (exposure for two days at the beginning of the exposure period), or an *acute-late* treatment (exposure for two days at the end of the exposure period). These groups were used to determine whether relatively short exposure times (*acute-late*) are sufficient to elicit any changes in sugar metabolism and if the history of exposure will have any effects on sugar or metabolite levels (*acute-early*). Bees were subsequently collected at eight-hour intervals within a 24-hour period, then hemolymph was collected and glucose concentrations were quantified. An additional experiment was performed using the same exposure paradigms in order to examine the effects of pesticide exposure on the central carbon metabolism within thoracic tissue. Bumble bee thoraces were separated following exposure to imidacloprid, and metabolomic approaches were then used to quantify the relative abundances of key metabolites within bumble bee tissues.

Methods

Bumble Bee Rearing

Bumble bee colonies provided by Koppert Biological Systems (Howell, MI) were used in this study; for each experiment, three colonies were maintained at 27 °C and 60% RH under dim red light, which is not visible to bumble bees. Colonies were fed honey bee collected pollen (Brushy Mountain Bee Farm) and a nectar substitute supplied by Koppert Biological Systems. The experimental sampling design included a balanced representation of bees from these colonies equally distributed across our treatment groups. The selected bees used in the

experiment were removed from their original colonies and placed in individual cages (W7.5 x D7.5 x H4.5 cm) maintained in an incubator at 25 °C and 70% RH.

Imidacloprid Preparation

A 5 parts per billion (ppb; or micrograms/L) imidacloprid solution was prepared by adding dilutions of an imidacloprid stock solution to 1000 mL of 50% sucrose solution (w/v). This concentration of imidacloprid was used based on a survey of the available literature on neonicotinoid levels used in lab and field experiments or estimated from environmental screenings; this represents a field-realistic, sublethal exposure level for bumble bees [1, 7, 9]. Stock pesticide solution was prepared by dissolving 1 mg of pure imidacloprid into 100 mL of distilled water, and then subsequently mixing the two together to produce a 10 ppm imidacloprid stock. Then, 0.5 mL of the 10 ppm imidacloprid stock was aliquoted into 99.5 mL of distilled water, achieving a 100 mL solution. The 100 mL imidacloprid solution was then added to the sucrose solution. Concentration of all imidacloprid solutions were validated using EnviroLogix Quantiplate Kits (EP006) and all fell within 5-5.5 ppb imidacloprid.

Experimental Design

Bees were placed in one of four treatment groups: 1) *chronic*, 2) *acute-early*, 3) *acute-late* and 4) *untreated* (control) (**Figure 1**). *Untreated* bees were fed an untreated 50% sucrose solution (w/v) whereas all of the treated bees were fed a 50% sucrose solution (w/v) with 5 ppb of imidacloprid. All bumble bees in the study then underwent a seven-day exposure period, where they were provided with either treated or untreated sucrose solution according to their treatment group. *Chronic* bees were exposed to the treated sucrose solution for the full seven

days. *Acute-early* (Acute 1) bees were provided with an imidacloprid treated sucrose solution treatment for the first two days of the feeding period, and then were switched to the untreated sucrose solution for the remaining five days. *Acute-late* (Acute 2) bees were administered an untreated sucrose solution for the first five days and then switched to the imidacloprid sucrose solution for the final two days of the feeding period. *Untreated* bees were fed untreated sucrose solution for the full seven days. To prevent mold growth, all sucrose solutions were changed out every two to three days.

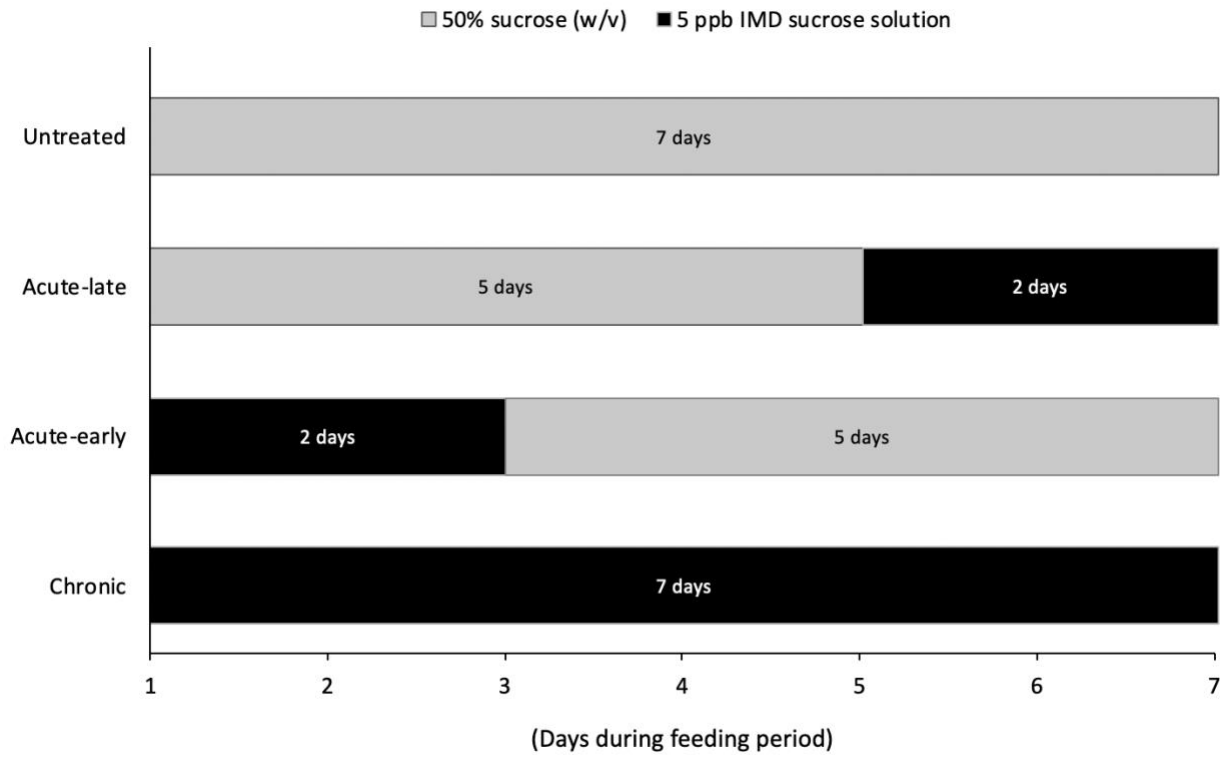

Figure 1. *Experimental treatments.* For each experimental trial, bees were exposed to four different treatments of imidacloprid-treated or untreated sucrose solution. Sucrose treatments were either 50% sucrose (w/v) (*untreated*) or 5 ppb imidacloprid sucrose solution (*chronic*, *acute-early*, or *acute-late*).

Total Bees	0 Hour	8 Hours	16 Hours	24 Hours
Chronic	11	11	12	11
Acute 1	12	11	12	11
Acute 2	11	11	11	9
Untreated	11	11	12	10
n=177				

Table 1. *Total bees collected for metabolomic analysis*. A total of 177 bees across three colonies were used in the experiment. Three trials were run, with balanced representation from each of the three colonies across the treatment groups.

Following the exposure period, all bees underwent a brief starvation period of four hours, during which sucrose solutions were removed from the cages to prevent feeding. This was then followed by a brief refeeding period of one hour with the administration of an untreated sucrose solution (for all treatment groups) as a means to increase the likelihood that the bees would have sufficient sugar in their system prior to hemolymph collection. All bees in the experiment were deprived of pollen after removal from their natal colonies. This was to limit variation in levels of sugars and other metabolites introduced from pollen consumption [10], and studies have suggested that pollen deprivation has negligible effects on overall bumble bee survivability within the initial ten days of treatment [10-11].

The nectar-feeders were weighed both before and after the one-hour refeeding period in order to confirm that all bees consumed sucrose solution during the refeeding period. Directly after the one-hour refeeding period, the sucrose solution was removed from all cages, and then

bee hemolymph was collected either 0, 8, 16, or 24 hours following the refeeding period. These eight-hour timepoints were established to examine how sugar and metabolite levels change over a given 24-hour time period. Throughout the experiment, all bees remained in the incubator with the conditions described above.

For both experiments, hemolymph and thoracic tissue samples were collected every eight hours over a 24-hour period. Samples were collected at standardized timepoints; across both experiments, the 0-hour bees were collected at 2:00 PM, the 8-hour bees were collected at 10:00 PM, the 16-hour bees were collected at 6:00 AM, and the 24-hour bees were collected at 2:00 PM the following day. Throughout this study, the protocols for imidacloprid preparation and treatment remained consistent across both experiments, while only the tissue collection (hemolymph or thoracic tissue) and subsequent analysis (glucose concentration assay or metabolomic analysis, respectively) differed.

Sample Collection and Metabolomic Analyses

Hemolymph was collected via the antennae method of hemolymph extraction [26], which was optimized in my previous research. For this procedure, bumble bees were physically manipulated so that the head was exposed and hemolymph could be extracted efficiently (**Figure 2**). Once the head was exposed, the head and antennal areas were disinfected with 70% ethanol and sterilized tweezers were used to detach the antennae from the head. Light pressure was applied to the abdomen and thorax in order to increase hemolymph flow, which manifested as a drop of hemolymph from the antennal extraction point. Approximately two microliters of hemolymph were collected per bumble bee, and samples were immediately placed on dry ice to prevent melanization and the degradation of carbohydrate metabolites in hemolymph [12], and then

subsequently frozen in -80ºC storage. The samples were stored at -80 °C until they were ready for further analysis.

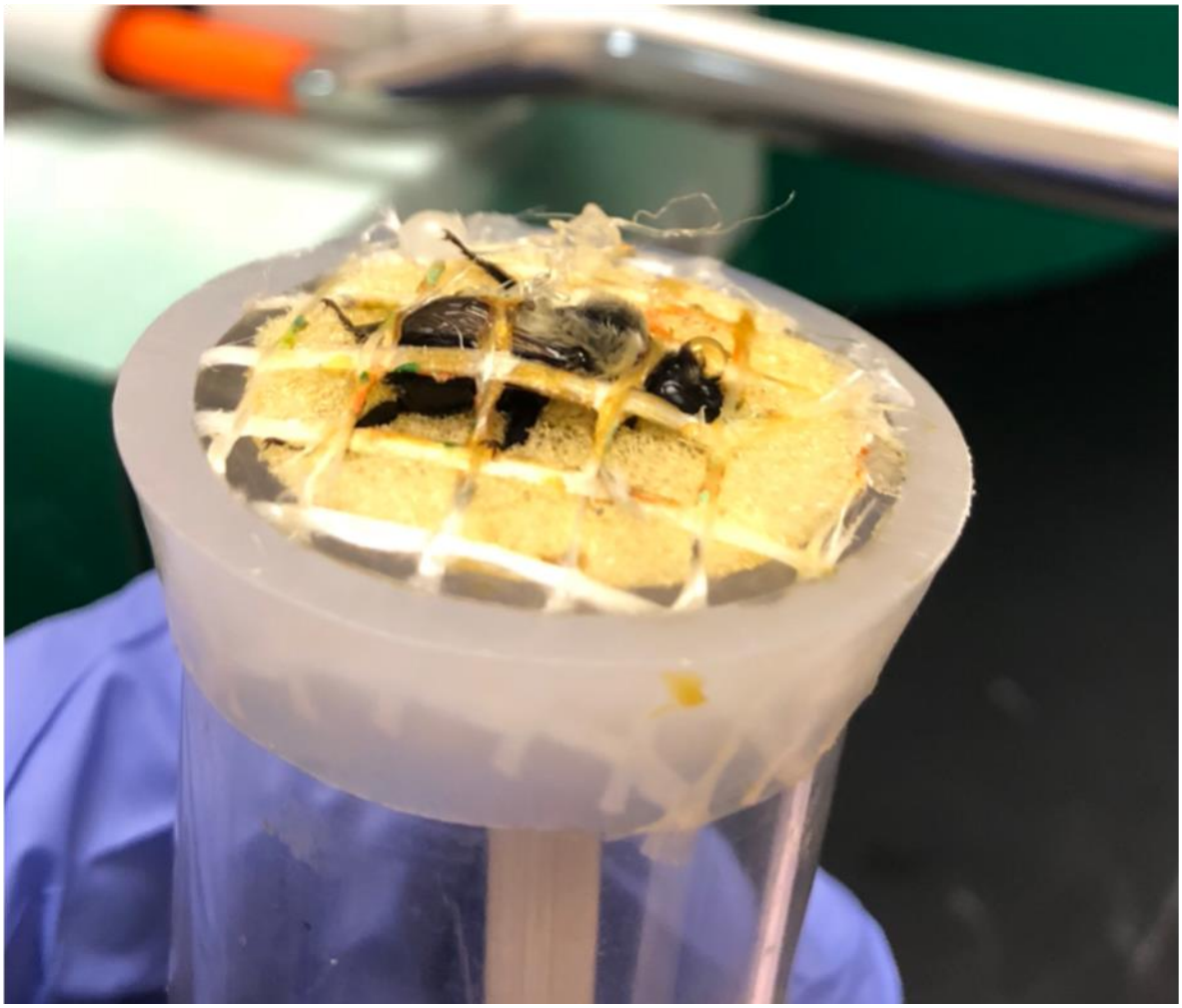

Figure 2. *Hemolymph isolated from the antennae of a bumble bee worker*. Exhibited is a drop of pure hemolymph extracted via the antennae method of hemolymph extraction. The bee was suspended in place via a tagging apparatus; this chamber is not necessary for the antennae method of hemolymph extraction provided that the bee is held in place and is in a position to be physically manipulated for extraction of hemolymph.

Glucose concentrations within the extracted hemolymph was quantified via a pre-prepared glucose assay kit (Sigma-Aldrich Glucose Assay Kit) and then processed by a Varioskan Lux microplate reader set to quantify glucose concentrations at an absorbance of 340 nm [12]. Glucose concentrations were calculated based on standard curves via ScanIt computer software. Quantified glucose levels were compiled and statistically analyzed via R v.3.3.1 software (R Core Team).

Bee thoraces were utilized for the analysis of the central carbon metabolome. The bodies were collected and snap frozen in liquid nitrogen in order to prevent the degradation of metabolites [12]. Thoraces were then separated from the rest of the bodies over dry ice. Thoraces were specifically used for metabolomic analysis as the thorax contains flight muscles, which have high metabolic demands [4, 13]. Energy and carbohydrate metabolism is essential within the thoracic muscles as they must maintain high levels of ATP in order to activate muscle contraction for flight and activity [14]. The bumble bee thoraces were stored at -80°C prior to analysis at the UC Riverside Metabolomics Core Facility.

A targeted assay was employed to detect the constituents of the central carbon metabolism, which encompasses several sugar phosphates involved in glycolysis, organic acids involved in the tricarboxylic acid cycle, and amino acid metabolism. It is important to note that the central carbon metabolism analysis also detects some neutral sugars, but does not specifically identify which sugar is present due to the use of liquid chromatography (for instance the assay cannot differentiate hexose from glucose) [12]. We used an interactive Pathway Analysis module developed specifically for metabolomics studies to analyze the central carbon metabolome; this

module performed both a pathway enrichment analysis and a topology analysis to further examine central carbon metabolism. (MetaboAnalyst 4.0).

Results

Hemolymph glucose concentrations

The data from measuring hemolymph glucose concentrations suggests that there was no significant difference among imidacloprid treatment groups, but due to small sample size (n = 24), these results may not be conclusive. The imidacloprid treatments resulted in a trend of lower glucose concentrations, while untreated bees represented the highest overall concentrations of glucose in collected hemolymph (**Figure 3**). While the results are not fully conclusive, the trends in data exhibit that pesticide treatments may potentially negatively impact the sugar levels of bees, and by extension their energetic states. Additional sample analysis is needed to further quantify the sugar levels of the extracted hemolymph, but due to temporal and physical restraints imposed by the COVID-19 pandemic, this was impossible.

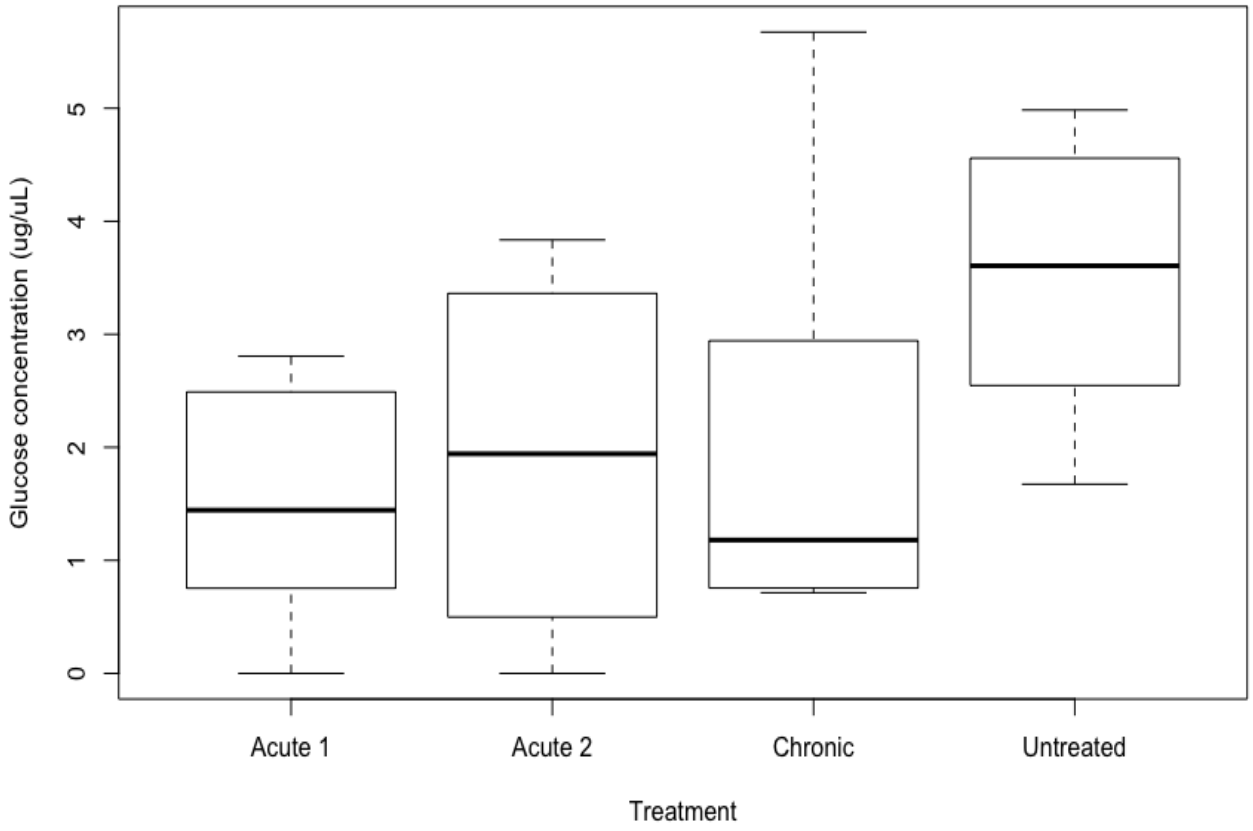

Figure 3. *Glucose concentration by imidacloprid treatment*. These data demonstrate glucose concentrations (ug/uL) per treatment group. A generalized linear model test was employed; only the untreated group was significant with a p-value of 0.01 ($p = 0.01$). This may be due to the small sample size per treatment group.

Central carbon metabolism

In total, 78 metabolites were identified as being present within our thoracic tissue samples. Of these, 37 metabolites were impacted by imidacloprid treatment groups. Significant metabolites include hexose (a six-carbon monosaccharide such as glucose), cysteinesulfinic acid (also known as 3-Sulfino-L-alanine), nicotinamide adenine dinucleotide (NAD), and adenosine diphosphate

(ADP). The key metabolites NAD and ADP play significant roles in the central carbon metabolism and are highly relevant to the biochemical utilization and redistribution of sugar and metabolites for energy [12, 14]. The relationships between these key metabolites and pesticide exposure treatments are displayed below (**Figures 4-9**); these selected figures are sourced from our collaborators Dr. Jay Kirkwood and Manhoi Hur at the UCR Metabolomics Core Facility.

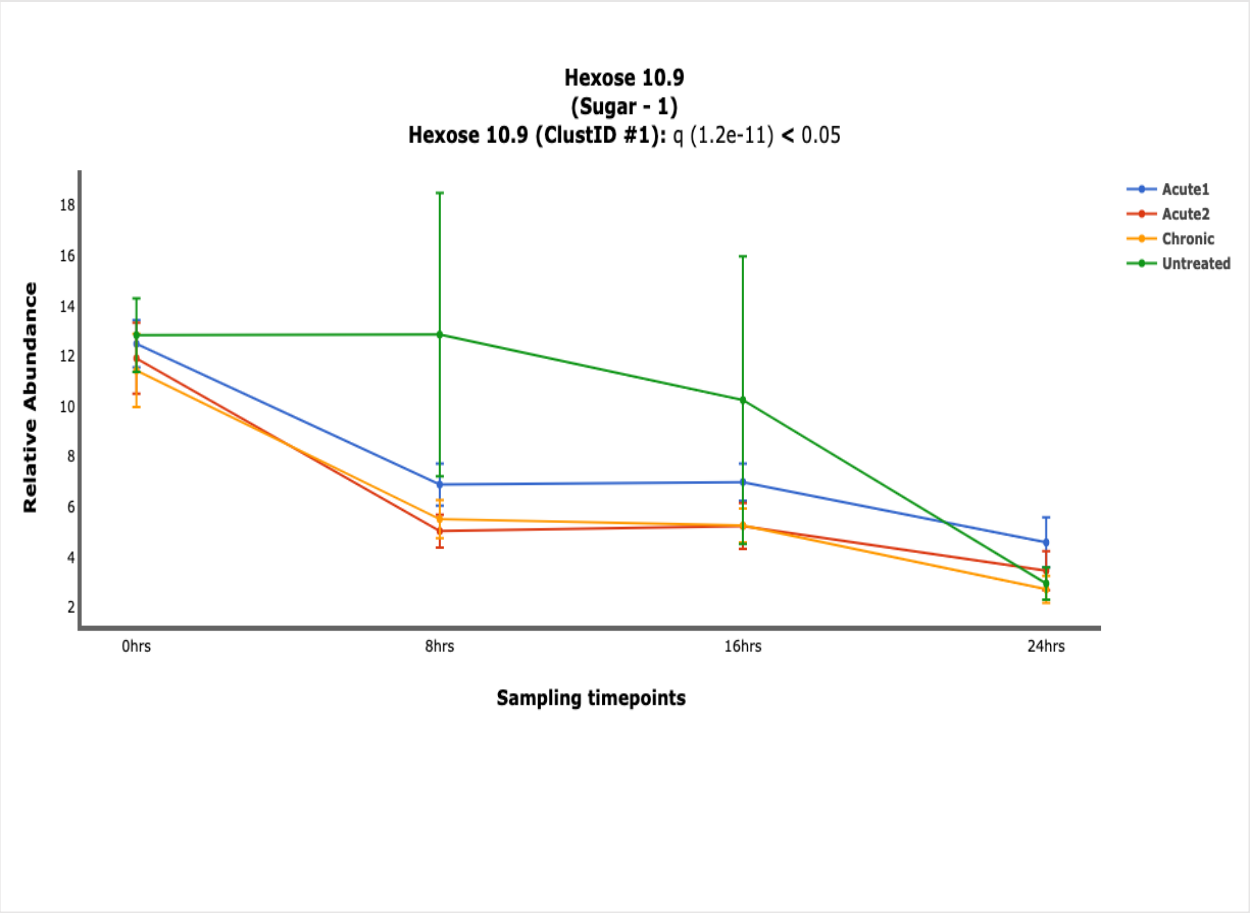

Figure 4. *Key Metabolite – Hexose relative abundance across imidacloprid treatments*. Across the 24-hour period, the hexose abundance in all imidacloprid treated bees decreased at a faster rate than *untreated* bees. The large error bars within the *untreated* group may be the result of natural biological variation in sugar levels due to differences in nectar consumption.

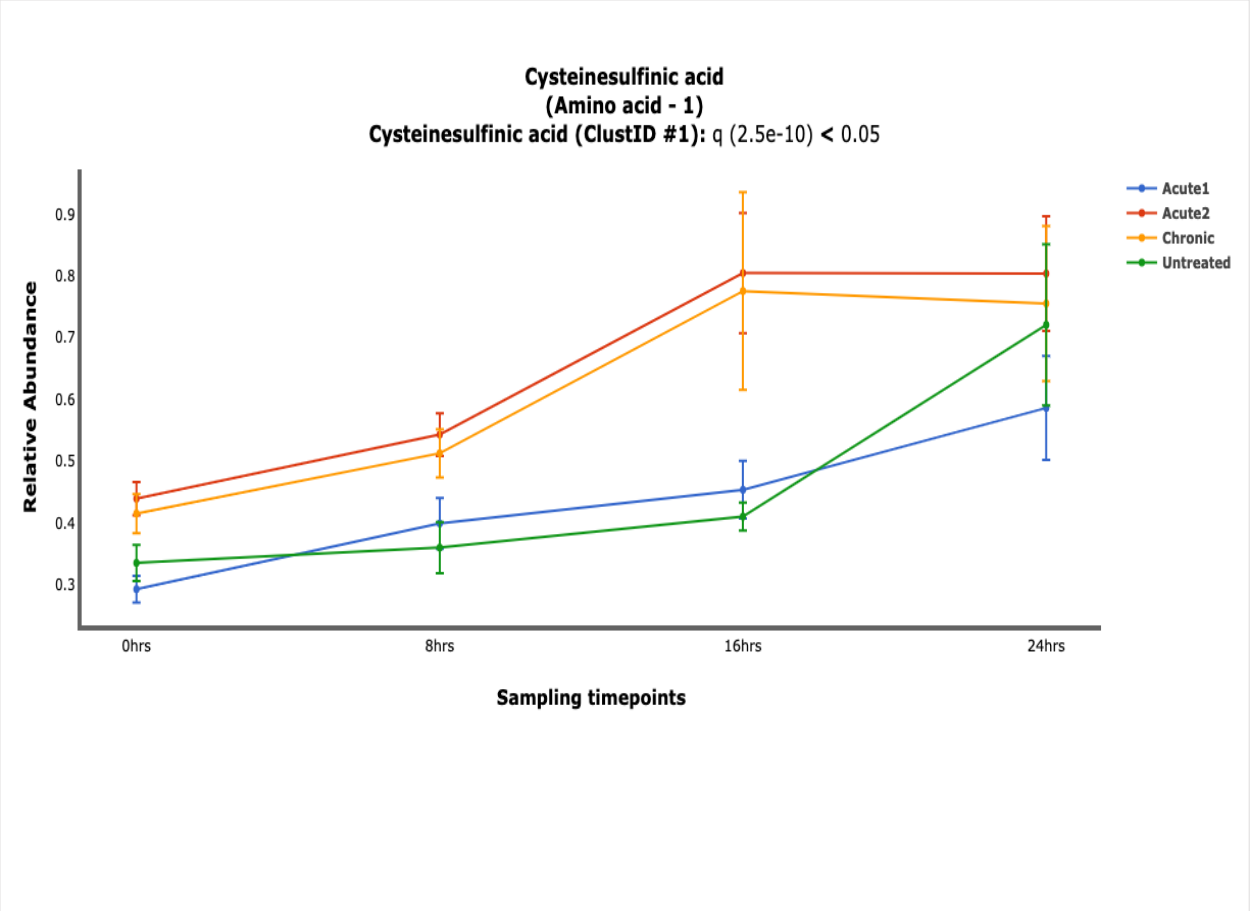

Figure 5. *Key Metabolite – Cysteinesulfinic acid relative abundance across imidacloprid treatments*. Through the 24-hour period, each group followed similar trends in metabolite changes with relative abundance being generally consistent at different sampling timepoints. The differences occur in the rates at which cysteinsulfinic acid increases.

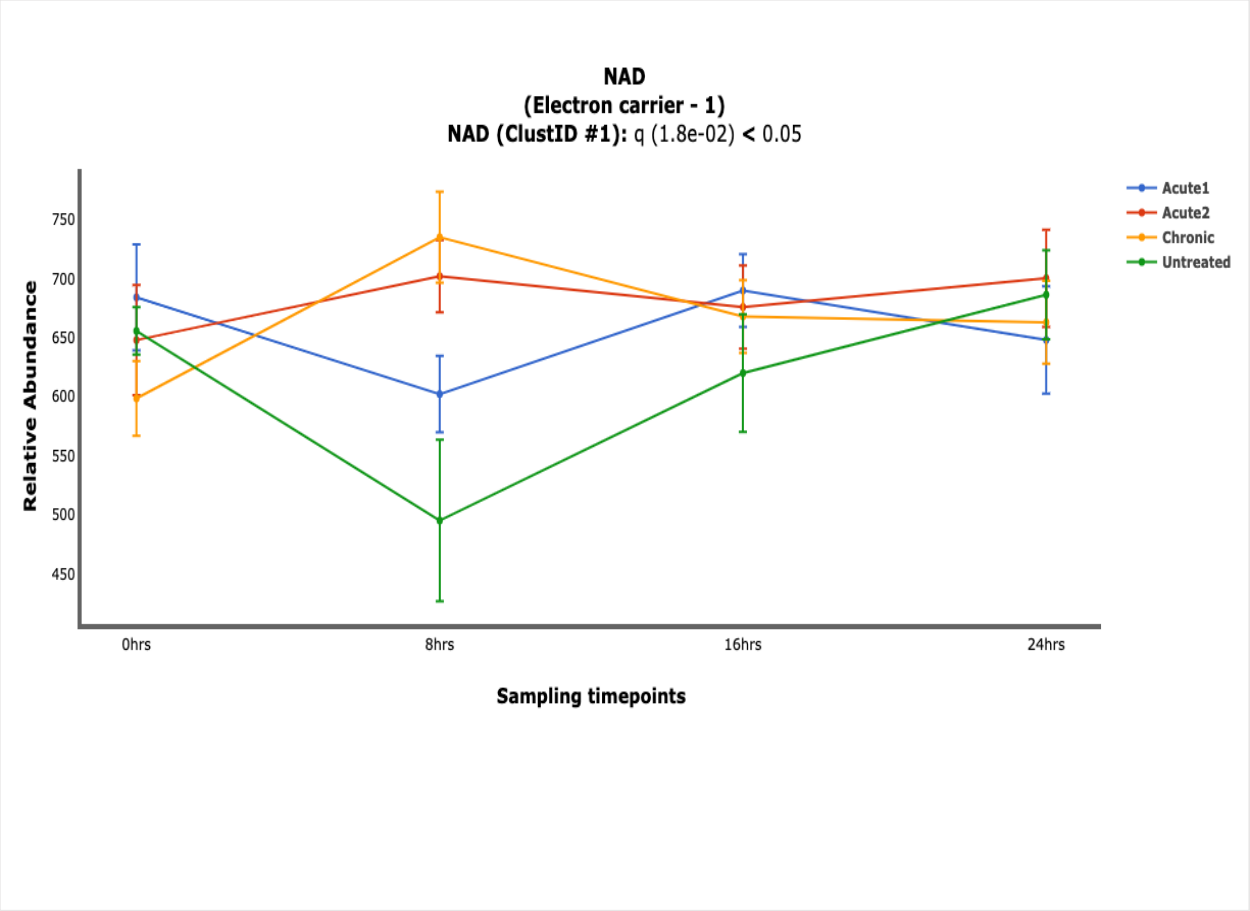

Figure 6. *Key Metabolite – NAD relative abundance across imidacloprid treatments*. Abundance of NAD can indicate free energy availability or energy being used. The relative abundance of NAD across treatment groups varies the most at the 8-hour time point.

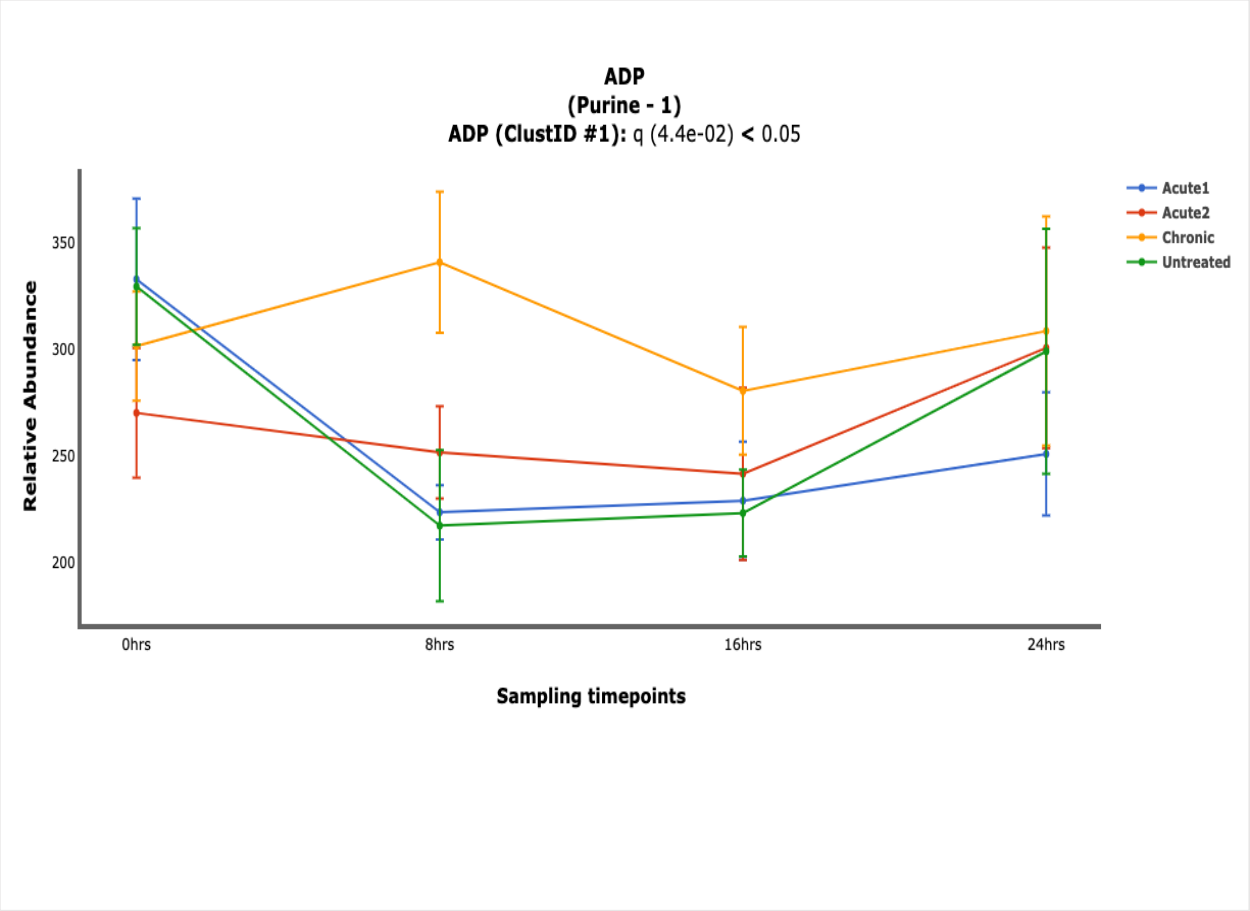

Figure 7. *Key Metabolite – ADP relative abundance across imidacloprid treatments.* Abundance of ADP can indicate present free energy or energy being used. The largest variance in relative abundance is observable at the 8-hour timepoint, which is similar to NAD.

The key metabolites NAD and ADP are involved in the production of energy (NAD is an electron carrier in the electron transport chain and ADP is the precursor for ATP) [22]. To better visualize the impact that pesticide exposure has on the relative abundance of key metabolites involved in the central carbon metabolome, only the *chronic* and *untreated* treatment groups are visualized in the figures below. **Figure 8** and **Figure 9** represent the relative abundances of key metabolites, NAD and ADP across *chronic* and *untreated* treatment groups to demonstrate areas of significance in the graphs.

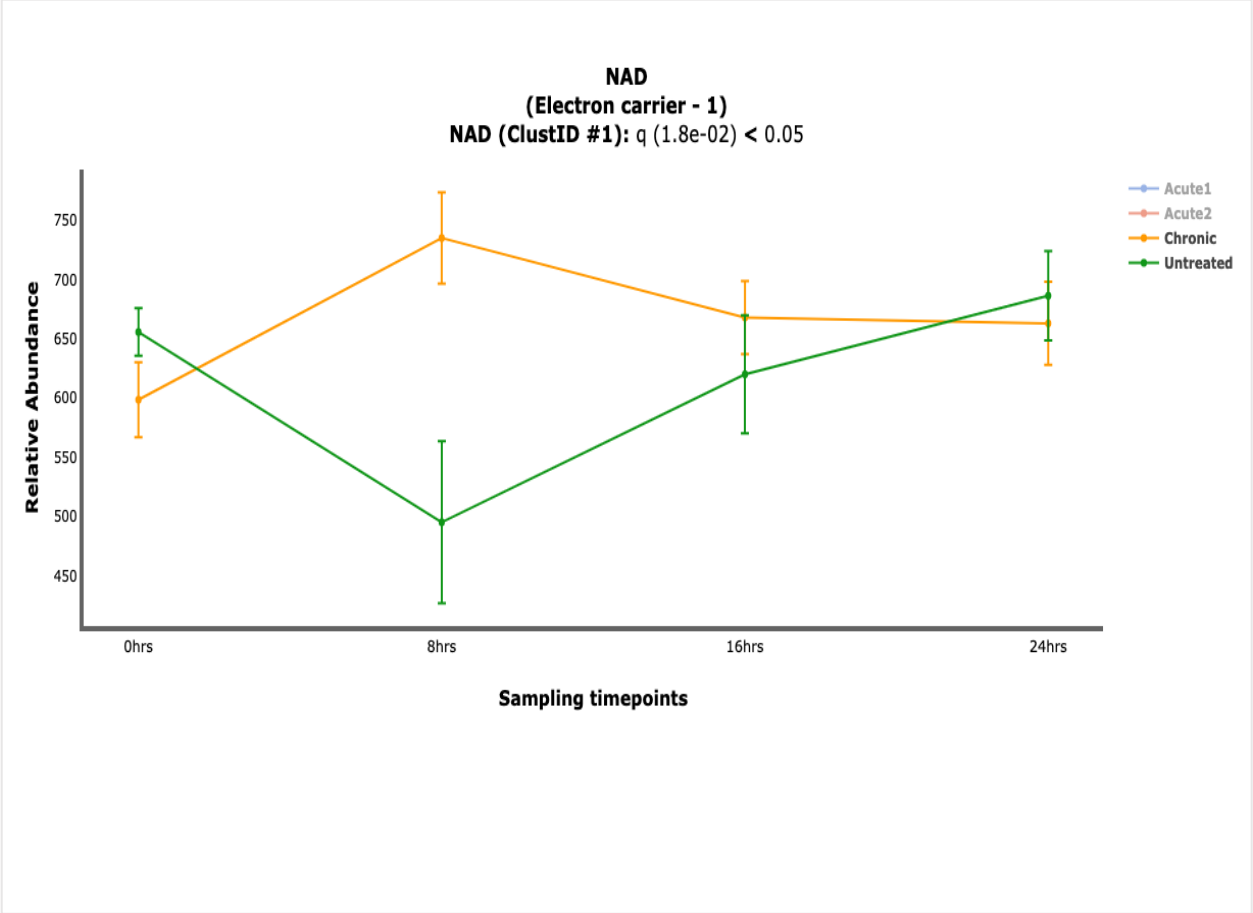

Figure 8. *Key Metabolite – NAD relative abundance across chronic and untreated treatments*. Only the *chronic* and *untreated* treatment groups are visualized. The largest amount of variation in relative abundance of NAD is observed at the 8-hour timepoint.

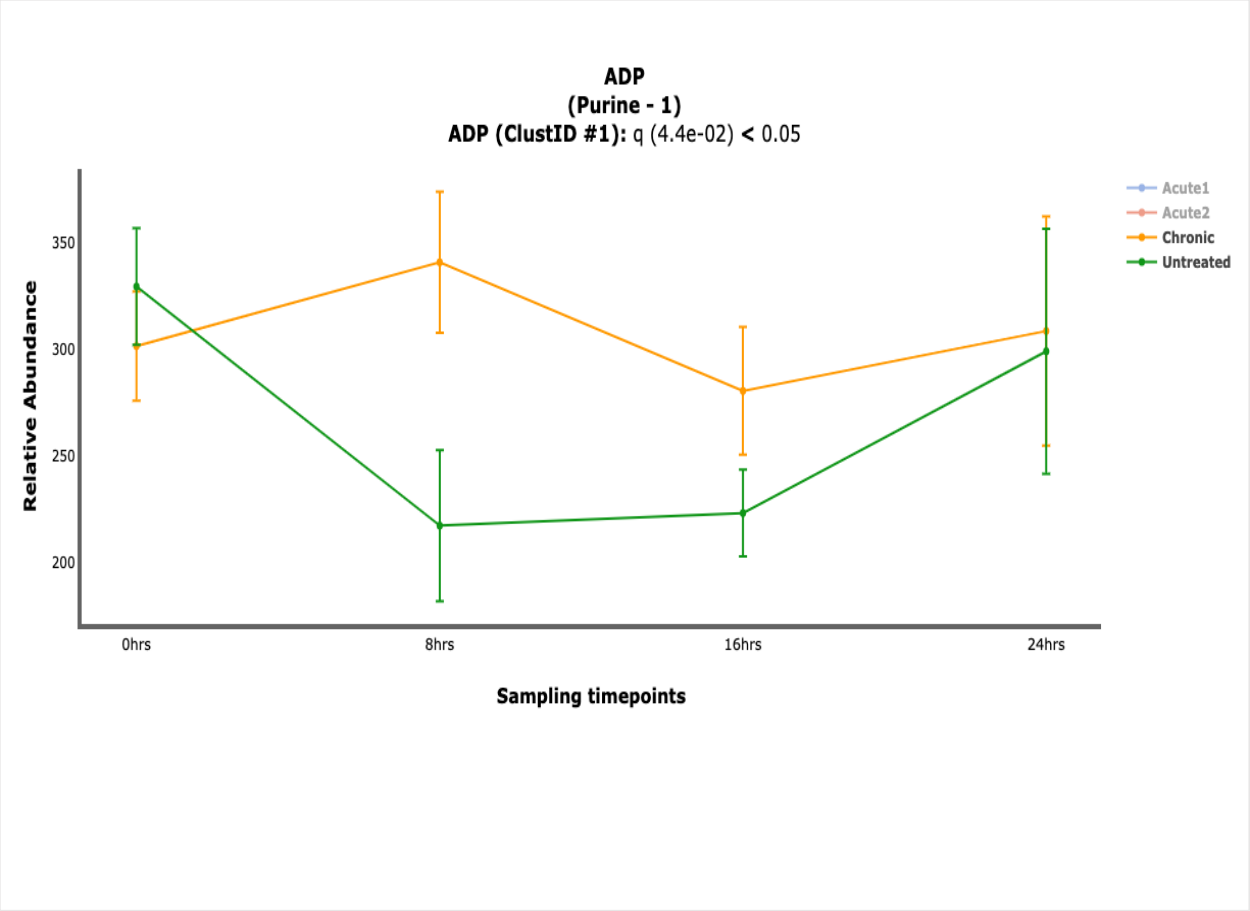

Figure 9. *Key Metabolite – ADP relative abundance across chronic and untreated treatments*. Only the *chronic* and *untreated* treatment groups are visualized. The largest amount of variation in relative abundance of ADP is observed at the 8-hour timepoint.

The central carbon metabolome was further analyzed using the Pathway Analysis module on MetaboAnalyst, which is a web-based platform developed specifically for metabolomic studies. The purpose of MetaboAnalyst is to provide a free, user-friendly, and easily accessible tool for analyzing data arising from high-throughput metabolomics analysis. MetaboAnalyst provides tools for the identification of compounds and pathway mapping for annotating significant features. Pathway enrichment analysis uses the metabolite relative abundance values and has the potential to identify subtle but consistent changes in the compounds involved in biological pathways.

Pathway topology analysis uses degree centrality and betweenness centrality to estimate the local connectivities and global network topology, respectively [15]. These measures aid in better understanding which pathways are changed or modified under the experimental conditions, especially when considering the complex relationships between molecules within the target organism's biological pathways [15].

To generate these data, pathway analyses of all of the samples collected from the imidacloprid-treated bees were examined, and joint pathway analyses were conducted in order to compare experimental groups with one another. MetaboAnalyst only allows for two treatments to be analyzed at a time; thus, comparative pathway analyses were run multiple times in order to do pairwise analyses of all the treatment groups. Data were displayed using an interactive visualization system, in which the graphical output contained the view of the metabolomic pathways. Through MetaboAnalyst, the metabolic pathways were visualized (**Figure 10**), and a detailed table describing the pathway analysis was generated, describing the compounds and their significance (**Table 2**).

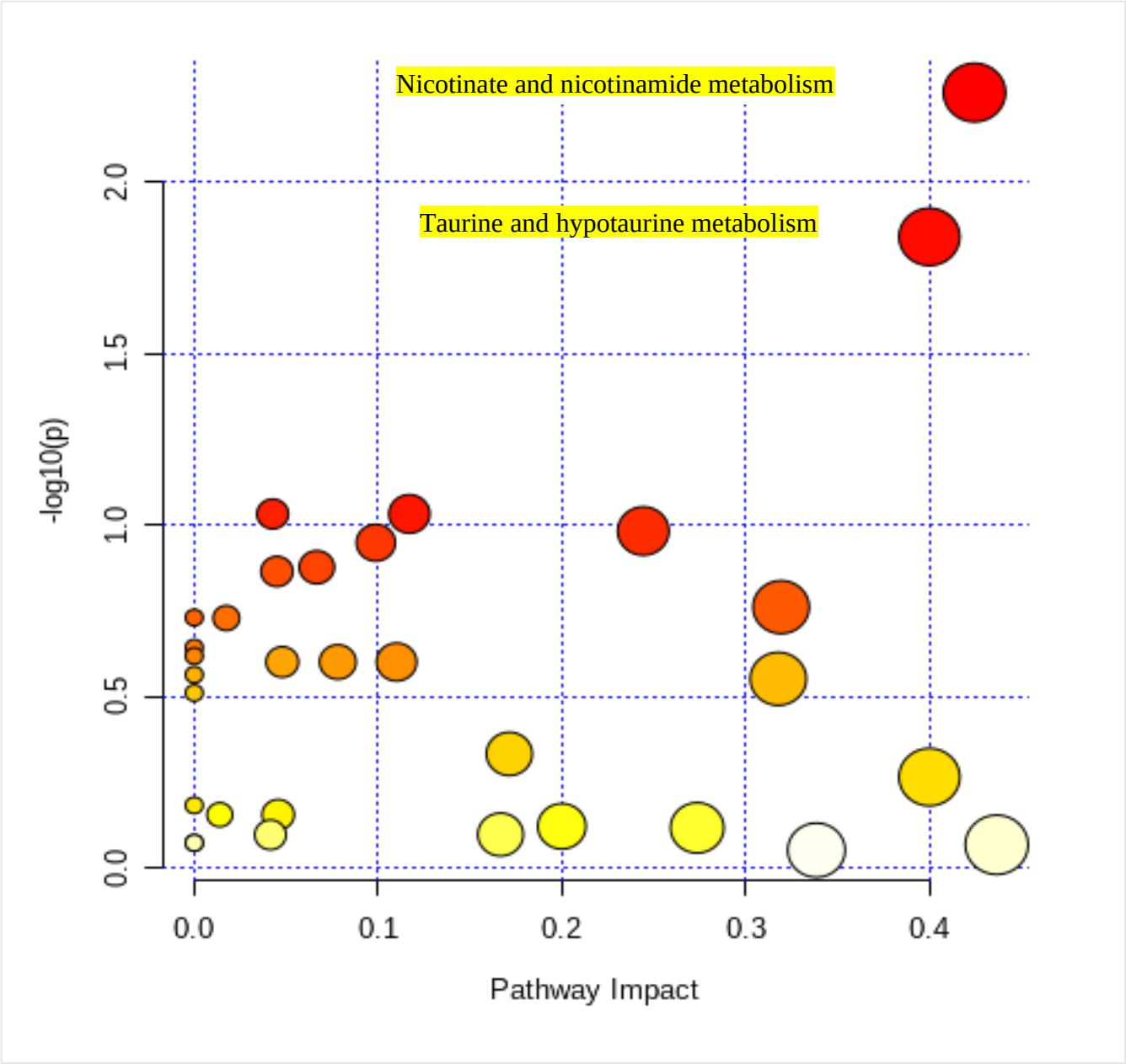

Figure 10. *Pathway Analysis of the 8-hour untreated versus chronic treatment groups.* This analysis shows all matched pathways according to the p-values derived from the pathway enrichment analysis and the pathway impact values derived from the pathway topology analysis. The colors (ranging from yellow to red) indicate the different levels of significance of the metabolites within the data, with yellow being the least significant and red being the most significant.

Table 2: Result from Pathway Analysis

	Total Cmpd	Hits	Raw p	-log10(p)	Holm adjust	FDR	Impact
Nicotinate and nicotinamide metabolism	9	3	5.50E-03	2.26E+00	1.76E-01	1.76E-01	0.42
Taurine and hypotaurine metabolism	7	1	1.45E-02	1.84E+00	4.49E-01	2.32E-01	0.40
Inositol phosphate metabolism	28	1	9.31E-02	1.03E+00	1.00E+00	5.00E-01	0.12
Phosphatidylinositol signaling system	28	1	9.31E-02	1.03E+00	1.00E+00	5.00E-01	0.04
Cysteine and methionine metabolism	32	5	1.04E-01	9.81E-01	1.00E+00	5.00E-01	0.24
Purine metabolism	63	8	1.13E-01	9.47E-01	1.00E+00	5.00E-01	0.10
Glyoxylate and dicarboxylate metabolism	24	2	1.33E-01	8.76E-01	1.00E+00	5.00E-01	0.07
Citrate cycle (TCA cycle)	20	1	1.37E-01	8.64E-01	1.00E+00	5.00E-01	0.04
Arginine and proline metabolism	31	6	1.74E-01	7.60E-01	1.00E+00	5.00E-01	0.32
Sphingolipid metabolism	18	1	1.87E-01	7.29E-01	1.00E+00	5.00E-01	0.00
Galactose metabolism	27	3	1.87E-01	7.28E-01	1.00E+00	5.00E-01	0.02
Thiamine metabolism	7	2	2.28E-01	6.42E-01	1.00E+00	5.00E-01	0.00
Ascorbate and aldarate metabolism	6	2	2.42E-01	6.17E-01	1.00E+00	5.00E-01	0.00
Amino sugar and nucleotide sugar metabolism	34	1	2.51E-01	6.00E-01	1.00E+00	5.00E-01	0.11
Pentose and glucuronate interconversions	16	1	2.51E-01	6.00E-01	1.00E+00	5.00E-01	0.08
Starch and sucrose metabolism	14	1	2.51E-01	6.00E-01	1.00E+00	5.00E-01	0.05
Glutathione metabolism	26	3	2.74E-01	5.62E-01	1.00E+00	5.00E-01	0.00
Glycine, serine and threonine metabolism	30	4	2.82E-01	5.51E-01	1.00E+00	5.00E-01	0.32
Valine, leucine and isoleucine biosynthesis	8	1	3.09E-01	5.09E-01	1.00E+00	5.21E-01	0.00
Arginine biosynthesis	12	4	4.66E-01	3.32E-01	1.00E+00	7.45E-01	0.17
Histidine metabolism	9	1	5.44E-01	2.64E-01	1.00E+00	8.30E-01	0.40
Propanoate metabolism	21	1	6.58E-01	1.82E-01	1.00E+00	8.85E-01	0.00
Pyrimidine metabolism	40	3	7.01E-01	1.55E-01	1.00E+00	8.85E-01	0.05
Pantothenate and CoA biosynthesis	18	2	7.01E-01	1.54E-01	1.00E+00	8.85E-01	0.01
Butanoate metabolism	14	1	7.58E-01	1.21E-01	1.00E+00	8.85E-01	0.20
Alanine, aspartate and glutamate metabolism	23	4	7.65E-01	1.16E-01	1.00E+00	8.85E-01	0.27
Aminoacyl-tRNA biosynthesis	48	8	8.01E-01	9.66E-02	1.00E+00	8.85E-01	0.17
Glycerophospholipid metabolism	32	3	8.02E-01	9.56E-02	1.00E+00	8.85E-01	0.04
Biotin metabolism	10	1	8.47E-01	7.22E-02	1.00E+00	8.85E-01	0.00
Lysine degradation	21	2	8.47E-01	7.22E-02	1.00E+00	8.85E-01	0.00
beta-Alanine metabolism	14	4	8.58E-01	6.68E-02	1.00E+00	8.85E-01	0.44
Tryptophan metabolism	30	2	8.89E-01	5.10E-02	1.00E+00	8.89E-01	0.34

Table 2. *Result from Pathway Analysis (in 8-hour untreated vs. chronic treatment groups).* Total Cmpd is the total number of compounds in the pathway; Hits represent the number of metabolites from the data within that pathway; Raw p is the p-value calculated from the enrichment analysis; the Holm p is the p-value adjusted by Holm-Bonferroni method; FDR is the p-value adjusted using False Discovery Rate; Impact is the pathway impact value calculated from pathway topology analysis.

An analysis of the central carbon metabolome indicated that key metabolites involved in central carbon metabolism were impacted by the imidacloprid treatments. **Figure 10** visualizes the pathway impact of metabolites impacted by imidacloprid exposure; both nicotinate and nicotinamide metabolism as well as taurine and hypotaurine metabolism were most significantly impacted by the imidacloprid treatments. **Table 2** displays the results of the treatment group pathway analysis and reaffirms that nicotinate and nicotinamide metabolism and taurine and hypotaurine metabolism are impacted by the imidacloprid treatments. In both nicotinamide adenine dinucleotide (NAD) and taurine metabolisms, the enrichment analysis confirmed statistical significance with p-values of 5.5E-3 and 1.45E-2, respectively.

Cysteinesulfinic acid is a non-coded amino acid that is produced post-translationally within the cell; it plays a large role in amino acid metabolic pathways and exists downstream of the taurine pathway. Our analysis shows that cysteinesulfinic acid is impacted by imidacloprid exposure [19]. **Figure 11** demonstrates the biochemical pathways in which cysteinesulfinic acid (also known as 3-Sulfino-L-alanine), hypotaurine, and taurine are involved.

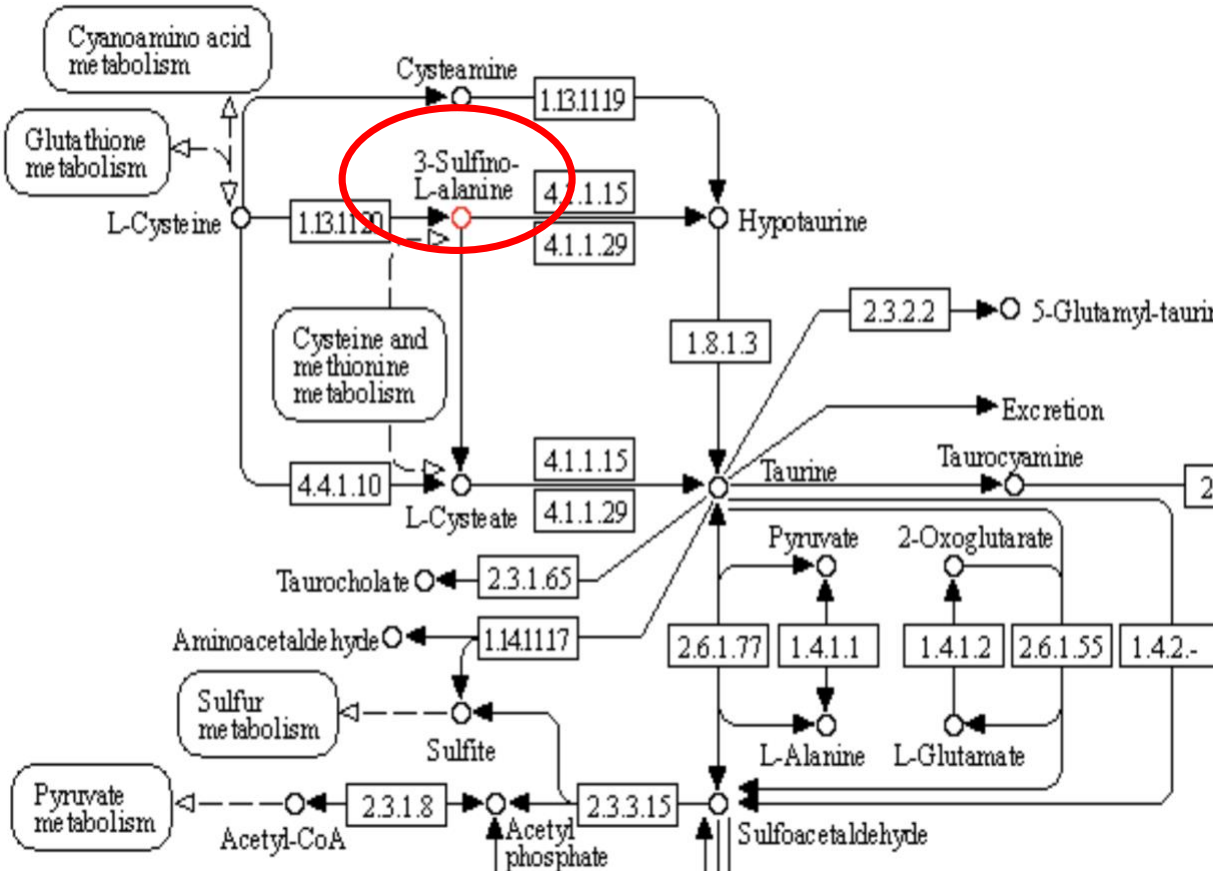

Figure 11. *Taurine and Hypotaurine metabolism*. Taurine has an excitation-reducing neuromodulatory role within the flight-motor system [19-20]. 3-Sulfino-L-alanine, also known as cysteinesulfinic acid, is an important precursor in taurine metabolism [19].

Discussion

Here, we demonstrate that sublethal, field-realistic doses of the neonicotinoid pesticide, imidacloprid, negatively impact bumble bee sugar levels as well as their central carbon metabolome, which constitutes several biochemical pathways essential for metabolic activity [5, 16]. The effects of sublethal, field realistic doses on bees were observed in the data provided by our quantitative sugar and metabolite assays. Our preliminary data suggests that as bumble bees

were exposed to imidacloprid, concentrations of glucose in hemolymph appear to decrease, though not significantly, which may have been due to small sample size. Neonicotinoids disrupt a bee's central nervous system, therefore halting bodily function and the distribution of sugars for energy [1-4], so there is a possibility that increased exposure to the pesticide negatively impacted their abilities to metabolize sugar and other metabolites involved in the central carbon metabolome [17-18].

Each of the four key metabolites selected from the metabolomic analysis are heavily involved in a variety of biochemical functions and metabolic pathways. Hexose represents any class of simple sugars that contain a six-carbon structure, which are essential for energy metabolism [27]; hexose relative abundance represents many of the simple sugars found in bumble bee tissues, such as glucose and fructose. At the start of the 24-hour sampling period, the relative abundance of hexose across all four treatment groups are very similar, however abundance noticeably drops off at a quicker rate in all three imidacloprid treatment groups when compared to the *untreated* group **(Figure 4)**. These data indicate that relative abundance of hexose in bumble bee tissue is impacted by imidacloprid exposure, which further affirms the trend we see in our results from hemolymph analysis.

In the insect central nervous system, the taurine pathway is upregulated in order to reduce excitation [19-20]; imidacloprid, on the other hand, is an agonist which causes stimulation and overexcitation within the nervous system [20]. Cysteinesulfinic acid, being a precursor for taurine metabolism, is impacted by imidacloprid exposure. Seeing that the relative abundance of cysteinesulfinic acid increases when exposed to the neonicotinoid imidacloprid (**Figure 5**), there may be a relationship between taurine metabolism and imidacloprid [18, 20], as taurine abundance may potentially increase with cysteinesulfinic acid. It may be within the realm of possibility that

taurine is being upregulated by the cells of exposed bees as a means to reduce or modulate the levels of excitatory imidacloprid, which would explain the increase in cysteinesulfinic acid and the significantly impacted metabolism of taurine.

Nicotinamide adenine dinucleotide (NAD) is an important electron carrier involved in the electron transport chain and aids in the production of large quantities of ATP [22]. NAD is essential in energy production, and yet in *untreated* bees there is a drop in the abundance of NAD at the 8-hour timepoint, which is followed by a gradual increase of NAD relative abundance over time (**Figure 6** and **Figure 8**). A potential explanation for this trend would be the influence of circadian rhythm on the energy production within bees [23]. At the 8-hour timepoint, samples were collected at night, a time when there may be less energetic demands on bees relative to daytime activity. However, in the *chronic* treatment group, this trend is not followed. The pathway analysis confirmed that nicotinate and nicotinamide metabolism is the most significantly impacted metabolic pathway at the 8-hour timepoint of *chronic* vs. *untreated* exposure groups (**Table 2**). Further, the increase in NAD at the 8-hour timepoint in the *chronic* treatment group, could be attributed to the excitatory effects of imidacloprid increasing the demand for cellular energy [25].

Adenosine diphosphate, or ADP, is the biological precursor to ATP, which is a fundamental unit of cellular energy. ADP presence is indicative of the amount of energy available, as well as the amount of energy being used for metabolism [5, 22]. ADP is similar to NAD in that it is essential for energy production, while also experiencing a drop off in relative abundance at the 8-hour timepoint followed by a gradual increase in abundance in the *untreated* bees (**Figure 7** and **Figure 9**). This may be due to circadian rhythm reducing the demand for

energy at night when bees are not out foraging, however, this requires further testing and analysis in order to make a formal observation.

Overall, our observations and analyses of the central carbon metabolome represent yet another aspect of the complexity of imidacloprid's interactions with the energetic state of bees. Changes in the metabolites of the central carbon metabolome generally follow a trend irrespective of treatment groups, and the differences all fall within their relative abundances and rates of change at the different experimental timepoints. While it is difficult to come to a definite conclusion as analysis has not yet been finalized, we know that hexose, cysteinesulfinic acid, NAD, and ADP are all essential metabolites that are impacted by exposure to sublethal doses of imidacloprid.

Our examination of central carbon metabolites impacted by sublethal doses of imidacloprid indicate that an increase in pesticide exposure impacts sugar levels and metabolite levels, which may result in an increase or decrease in the bee functional ability [4, 23, 24]. Central carbon metabolism consists of many biochemical pathways involved in the breakdown and synthesis of sugars, amino acids, lipids, and other metabolic precursors used in cellular processes essential for energy production and physiological function [5, 17, 22]. These experiments will not only serve as a comparison for data between varying pesticide studies but also to improve an overall understanding of bumble bee functional abilities when exposed to pesticides. This research will aid in making clear the intersection between the effects of pesticide exposure on the physiology and the functional ability of bees. Future work includes continuing experimental trials and observing the effects that neonicotinoid pesticides have on bumble bee metabolism through continued analysis of the central carbon metabolome.

This project is highly relevant to bumble bee conservation and the further management of ecosystems as bumble bees are essential pollinators in both natural and agricultural environments. It is essential to understand not only the nutritional biology and energetic state of bumble bees, but also the effects of pesticides implicated in the global decline of bumble bee populations. Through these experiments, a primary factor in the decline of bumble bee populations may be better understood to advance both bumble bee conservation and the improved management of the ecosystem.

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