Identifying the Role of Glyphosate-Containing Herbicides on Honeybee Mortality Rates and Colony Collapse Disorder

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An Environmental Science Paper
Presented to
Junior Science, Engineering, and Humanities Symposium
University of Missouri – St. Louis

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September 1, 2008 – February 9, 2009

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TITLE: Identifying the Role of Glyphosate-Containing Herbicides on Honeybee Mortality Rates and Colony Collapse Disorder

The world food supply and economy are directly dependent upon the pollination of crops and plants by honeybees. Due to the recently discovered condition known as Colony Collapse Disorder, the world honeybee population has declined significantly since 2006 (Kaplan 2008). It is estimated that one-third of all food in the human diet is dependent upon the work of honeybees; therefore, it can be assumed that the decline in honeybee population caused by Colony Collapse Disorder could lead to an acute decline in the world food supply (Delaplane 2000). Scientists have hypothesized that several possible factors may contribute to Colony Collapse Disorder, including the use of herbicides (Kaplan 2008). The purposes of this research are to determine whether there is a correlation between the use of Roundup QuikPro®, a glyphosate-containing herbicide, and the occurrence of Colony Collapse Disorder in bee populations from the State of Missouri, and to determine whether there is an increased mortality rate in honeybees that have been exposed to glyphosate-supplemented diets when compared to those that have been fed pure sugar water.

Samples of bees and honey of hives from colonies that have and have not been affected by Colony Collapse Disorder were collected from beekeepers located throughout the State of Missouri. These samples were analyzed using a liquid-chromatography mass spectrometer to detect traces of glyphosate and its metabolite aminomethylphosphonic acid. Nine hives, built according to a design described by Dr. Marion Ellis of the University of Nebraska-Lincoln, were filled with bees, and the nine hives were divided into three groups of three hives each. Each group was fed one of the following: sugar water, sugar water with glyphosate, or sugar water with Roundup QuikPro®. The amount of glyphosate added was based on a study performed by Blackburn and Boutin (2003). All hives were kept in a dark, humidified room located in the science department in Camdenton High School, Camdenton, MO. Data on mortality were collected and subjected to a two-way Analysis of Variance (ANOVA). Samples of bees and comb from each experimental hive were analyzed using a liquid-chromatography mass spectrometer (USGS Water Research Facility, Lawrence, KS).

The results of this study indicate that the mortality rate of bees that were fed glyphosate-supplemented diets, both pure glyphosate and Roundup QuikPro®, were significantly greater than that of hives without glyphosate supplemented diets ($p < 0.001$). Results from the LS/MS are pending development of a new testing protocol by the USGS research facility for this project.

Contents

Purpose

The purpose of this research was to:

1. Determine whether hives with glyphosate-supplemented diets exhibit a greater rate of mortality compared to hives with no supplementation.

2. Determine whether hives with Roundup QuikPro®-supplemented diets exhibit a greater rate of mortality compared to hives with no supplementation

3. Determine whether the herbicide glyphosate can be detected in samples of honey and bees collected from hives with glyphosate-supplemented diets.

4. Determine whether samples from hives with glyphosate-supplemented diets have greater levels of glyphosate than samples from hives with no supplementation.

5. Determine whether samples from hives with Roundup QuikPro®-supplemented diets have greater levels of glyphosate than samples from hives with no supplementation.

6. Determine whether samples that have been affected by Colony Collapse Disorder have greater levels of glyphosate than samples from hives that have not been affected by Colony Collapse Disorder.

Hypotheses

It was hypothesized that:

1. Hives with glyphosate-supplemented diets would exhibit a greater rate of mortality than hives with no supplementation.

2. Hives with Roundup QuikPro®-supplemented diets would exhibit a greater rate of mortality than hives with no supplementation.

3. It would be possible to detect glyphosate in samples of honey and bees collected from hives with glyphosate-supplemented diets.

4. If glyphosate could be detected in samples of honey and bee from hives with glyphosate-supplemented diets, then there would be significantly greater levels of glyphosate in samples of such hives when compared to samples of hives with no supplementation.

5. If glyphosate could be detected in samples of honey and bees from hives with Roundup QuikPro®-supplemented diets, then there would be significantly greater levels of glyphosate in samples from such hives when compared to samples from hives with no supplementation.

6. If glyphosate could be detected in samples of honey and bees collected from hives with glyphosate-supplemented diets, then there would be significantly greater levels of glyphosate in samples from hives that have been affected by Colony Collapse Disorder when compared to samples from hives that have not been affected by Colony Collapse Disorder.

Variables

Lab Study:

1. The independent variable in this portion of the study is the chemical used to supplement the diet of each honeybee hive (glyphosate and Roundup QuikPro®).

2. The dependent variables were the levels of glyphosate detected in honey and bees, honeybee behavior, and mortality of honeybees in each hive.

3. Constants included the hive construction, air temperature, water and sugar water diet, lighting, size of bee sample, duration of experiment, genetic origin of the bee population, size of bee population, and proximity of hives to one another.

Field Study:

1. The independent variable in this portion of the study is the historical presence of colony collapse disorder in hives of donated honey samples.

2. The dependent variable was the level of glyphosate detected in the samples of honey and bees donated by Missouri beekeepers.

3. Constants for this portion of the study were very difficult to control but included such factors as size of sample, approximate date on which sample was collected, and growing season for which samples were produced.

Literature Review

Colony Collapse Disorder

Apis mellifera, the honeybee, is vital to a healthy, balanced environment and the economy of the world. Honeybees pollinate nearly 130 species of plant life (Kaplan 2008). In addition to directly affecting the food supply by enabling growth of fruits, vegetables, nuts, grains, and seeds, honeybees also pollinate feed sources, such as clover, for livestock. Thus, honeybees are indirectly responsible for an estimated one-third of the world food supply (Delaplane 2000). In the United States alone, honeybees contribute fifteen million dollars worth of value each year to the agricultural economy (Organic Consumers Association 2007). The absence of honeybees would create not only a worldwide famine, but also dramatic economic inflation.

The disappearance of honeybees is a plausible possibility. In 2006, the population of honeybees in the United States began to decline rapidly (Kaplan 2008). According to Pennsylvania State University, Pennsylvania beekeeper David Hackenberg was the first to report major honeybee losses after the disappearance of approximately two thousand of his 2,900 hives in the Fall of 2006. Also in 2006, a beekeeper from the Midwest reported the loss of ninety six percent of his thirteen thousand beehives (Organic Consumers Association 2007). In the spring of 2007, an eighty percent hive loss was reported by an Ohio beekeeper (Organic Consumers Association 2007). After countless other reports of devastating honeybee losses during the winter of 2006-2007, it was estimated that over one-fourth of the United States' 2.4 million honeybee colonies had disappeared (PBS 2008). Since then, losses have also been reported throughout the world, in countries such as Italy, Australia, France, Brazil, Canada, Germany, and Great Britain (eWorldVu 2008). The British Beekeepers Association has even predicted that the honeybee population in Great Britain could completely disappear by 2018 if the crisis continues at its current rate (eWorldVu 2008).

In an effort to define and explain this new and unusual phenomenon, researchers coined the term Colony Collapse Disorder (Kaplan 2008). Definitions of Colony Collapse Disorder vary from source to source because the specific signs of the disorder are not always the same. Colony Collapse Disorder is always characterized by the unexplained death of honeybees within a colony. Most sources maintain that the queen, undeveloped honeybees, honey, and comb remain in the hive while most worker bees, including dead ones, disappear. A few sources also note a delay in the work of robber bees—opportunistic bees that plunder the unguarded honey of a weak or empty hive—and hive pests such as wax moths, hive beetles, and others. For the purposes of this paper, Colony Collapse Disorder will be defined simply as the unexplained death of honeybees within a colony.

Beekeepers and scientists have been unable to explain the reason for Colony Collapse Disorder (Kaplan 2008). Typical causes of minor hive loss might include starvation, parasites, or freezing; however, these causes are insufficient to explain the massive hive losses that began in 2006 (Kaplan 2008). Since the recognition of Colony Collapse Disorder, entomologists and other scientists have attempted to identify possible causes. Proposed causes have included pesticides, genetically modified crops, pathogens, varroa mites, viruses, stress due to excess pollinating, the pollination of plants with little nutritional value, or herbicides (Kaplan 2008, USDA 2008). While many of these proposed causes are known to have adverse effects on honeybees, no single factor seems to be responsible for Colony Collapse Disorder (Kaplan 2008). Therefore, many experts, including entomologist Jeff Pettis of the U.S. Department of Agriculture, believe that Colony Collapse Disorder could be caused by a group of factors, rather than just a single factor (Kaplan 2008)

Glyphosate and Glyphosate-based Herbicides

Glyphosate [N-(phosphonomethyl) glycine] is an organic compound that was introduced in 1971 to serve as the active ingredient in Monsanto's Roundup®. Roundup® is a non-selective, post-emergent, and broad-spectrum herbicide (Byer et al. 2008). This means that Roundup® is effective on any germinated plant life and can be applied anywhere on the plant. These qualities made Roundup® different from other herbicides at the time of Roundup®'s introduction. When applied to a plant, the acid form of glyphosate reacts to form a water-soluble salt, which is transported to the root system of the plant (Blackburn and Boutin 2003). There, the herbicide kills the plant by disrupting the biosynthesis of aromatic amino acids, which are necessary for the production of plant proteins (Perez et al. 2007).

The post-emergent quality of Roundup® reduces soil erosion by enabling farmers to kill weeds without disturbing the soil (Cornel [no date]). Glyphosate degrades relatively quickly in soil, as its average half-life is forty-seven days; the actual figure, though, varies greatly with soil anatomy (Byer 2008). Aminomethylphosphonic acid (AMPA) is the chief metabolite of glyphosate; generally, AMPA takes longer to degrade than glyphosate (Environmental Monitoring & Pest Management 1998).

Though Roundup® is used for its effects on plants, the herbicide also has effects on other organisms (Cornell [no date]). Glyphosate has been detected in the urine of farmers who have used Roundup® (Byer 2008). Similar studies have found that glyphosate inhibits steroid hormone production in men. Exposure to glyphosate is associated with an increased chance of premature births and miscarriages (Byer 2008 and Cox 2000). In other studies, glyphosate has been reported to decrease the sperm count and increase the occurrence of liver tumors in male rats (Cox 2000). One study indicated that after exposure to dried Roundup®, the population of beneficial insects such as ladybugs was reduced by more than half (Cox 2000). As a result of these studies, concern has been raised about the effects of Roundup® on non-target organisms (Blackburn and Boutin 2003). Adding to the concern is the significant increase in the use of Roundup® since Monsanto's 1996 introduction of Roundup Ready® crops, which are genetically modified to be unaffected by Roundup® (Byer 2008). As recently as 2005, the use of Roundup® in the United States was an estimated 103 to 113 million pounds per year (Organic Consumers Association 2005).

Method

Field Study

At the Missouri Beekeepers Association Fall Meeting in Osage Beach, Missouri, beekeepers were asked to participate in honeybee research. Those who volunteered were asked to provide a sample of seven to ten honeybees and a two-inch piece of comb from the same hive. The volunteers were mailed a package containing instructions for sample collection, sample collection containers, and a questionnaire asking about the location, health, and age of the beehives from which samples were taken. Most of the beekeepers mailed one or more samples, providing a total of ten samples.

Lab Component

Based on literature review, Dr. Marion Ellis from the University of Nebraska-Lincoln was contacted to discuss his previous honeybee experiment designs. Nine cages were constructed according to a design described by Dr. Ellis. Each cage was eight inches high by eight inches wide by five inches deep. The two larger faces of the cube were made of screen wire, and the other faces were made of wood. One two-inch circle was drilled on the top face of each cage. A mason jar with a flat and ring lid was placed upside down in each hole. Previously, a drill had been used to puncture each lid with fifteen small holes. A triangular piece of comb foundation was attached to the inside of the rear screen panel using a soldering iron. A water source was created by filling a test tube with water, attaching the test tube to the front screen panel with wire, and placing a strip of cotton fabric into the test tube to act as a wick; one water source was attached to each cage.

Equal volumes of sugar and water were combined and heated until the sugar dissolved completely to create a sugar solution. The solution was allowed to cool and was kept in a standard refrigerator for one day. The solution was divided into nine 236-mL portions and poured into the Mason jars to be placed in the hive. The test tubes were filled with approximately 7.5 mL water. Approximately three hundred live Italian honeybees were measured using a one-cup dry measuring cup and placed into each hive. All honeybees were from the same hive that belonged to the researcher and her family. After the bees were added, the hives were transported for about three miles where they were moved indoors, into a dark, temperature-controlled, noise-free room with an ionizing humidifier. Each hive was randomly assigned to a group: control, glyphosate, or Roundup QuikPro®. The hives were arranged in two rows, and cages from each group were arranged in an alternating pattern to eliminate extraneous variables.

All honeybees were allowed to acclimate to their new environment for four days, during which time all groups were fed plain sugar water. Additional sugar water of the previous concentration was created and divided into two 355-mL portions. After cooling, chemicals were added to each portion of sugar water using quantities described by a study performed by Blackburn and Boutin in 2003. In the study, the researchers planted seeds from mature plants that had been previously sprayed with 100% label rate of Roundup® (Blackburn and Boutin 2003). A standard of glyphosate was made to correspond with the 100% label rate of Roundup® by combining glyphosate and water at a concentration of 400 μg/mL (Blackburn and Boutin 2003).

In this study, a concentrated solution of 400 micrograms glyphosate per 100 mL sugar water was prepared using one of the 354-mL portions of sugar water: 0.144 grams of glyphosate (molar mass: 169.07g/mol) was added. 0.216 grams of Roundup QuikPro® were added to the second 354-mL solution of sugar water. Because Roundup QuikPro consists of 66.6% glyphosate and 33.4% inert ingredients, the amount was adjusted so that the ratio of glyphosate to sugar water would be the same as the previous solution. The sugar water solutions were stirred and heated briefly over a hot plate to ensure the dissolution of the solute. The solutions were allowed to cool and stored in a standard refrigerator until needed.

The original sugar water was removed from each cage in the Roundup QuikPro® and glyphosate groups. Each cage in the Roundup QuikPro® group received 118-mL of the Roundup QuikPro® sugar water, and each cage in the glyphosate group received 118-mL of the glyphosate sugar water.

Because the honeybees consumed more sugar water than the expected amount, more sugar water was mixed in the same way it had been mixed on the first day. The control group received sugar water with no supplementation, the glyphosate group received sugar water with glyphosate supplementation, and the Roundup group received sugar water with Roundup QuikPro® supplementation.

Observations were recorded about mortality, general behavior, and water consumption; each time the sugar water was replaced, the amount of leftover sugar water and amount of added sugar water was measured and recorded. Water was added to the water source every day using a syringe. The quantity of water was recorded each time water was added.

The bees were left undisturbed, with the exception of data collection, watering, and feeding maintenance, to function as a normal hive for four days before the chemicals were introduced to the experimental groups. Ten days after the chemicals were introduced to the experimental groups the final data were collected. At least four bees from each cage were collected to be used for testing, and the comb from each cage was collected for testing. Because the honeybees built less comb than expected, the comb was not used for testing.

Mass Spectrometry & Data Analysis

The samples from beekeepers in the State of Missouri, as well as the samples from the lab component of the experiment were analyzed with a liquid-chromatography mass spectrometer. Originally, Dr. Nathan Leigh of the University of Missouri-Columbia (MU) was contacted to analyze samples for the research. However, it was discovered that MU had only a gas-chromatography mass spectrometer (GS/MS), and that a GS/MS is not sensitive enough to detect the glyphosate in honeybees and comb. It is more useful when detecting a broad variety of chemicals rather than one specific chemical. Therefore, Dr. Mike Meyer was contacted at the United States Geological Survey Water Research Facility in Lawrence, Kansas, where a liquid-chromatography mass spectrometer (LS/MS) was available. An LS/MS is more appropriate for detection of glyphosate because an LS/MS can detect aminomethylphosphonic acid (AMPA), the metabolite of glyphosate, and is more sensitive than a GS/MS. The LS/MS used in this research can detect as little as 17 ppb. When all the samples had been collected, they were delivered to the Dr. Meyer in person. Dr. Meyer briefly explained how the LS/MS operated and then analyzed the samples that were given to him using the LS/MS. Mortality data were also analyzed. All data were subjected to a two-factor analysis of variance that used an alpha-level of 0.05 to determine significance.

Discussion and Results

Mortality Rate

In the lab component of this research, it was determined that the average mortality rate was significantly greater in both groups with supplemented diets when compared to the group with no supplementation. Both experimental groups, which were the glyphosate and Roundup QuikPro® groups, were found to exhibit significantly greater mortality when compared to the non-supplementation group ($p < 0.001$). The mortality rate for the group that was given sugar water with glyphosate was an average of 0.66 honeybees per day, whereas the average mortality rate for group that was given sugar water with Roundup was an average of 1.06 honeybees per day. These numbers compare to a mortality rate of only 0.26 honeybees per day for the control group. The Roundup QuikPro® group was also determined have significantly greater mortality when compared to the glyphosate only supplementation group ($p = 001$). This indicates that not only did pure glyphosate increase mortality, but something else, perhaps some inert ingredient(s) within Roundup QuikPro®, also contributed to honeybee mortality.

Given the results of this study, it is impossible to categorically state that glyphosate products cause Colony Collapse Disorder. However, the data indicates that it is certainly plausible that glyphosate herbicides may contribute to the phenomenon. The hypothesis that there is no one single factor that causes Colony Collapse Disorder, as suggested by others (Kaplan 2008), supports the possibility that glyphosate may play a role. It is important to understand that the results of this study should be considered preliminary results with a relatively small sample set ($n = 63$). Further studies should be conducted to validate these results given the importance of honeybees to a healthy ecosystem and agricultural economy.

Glyphosate Analysis

It was the original intention of this research to determine if it were possible to detect glyphosate and its metabolite, AMPA, in samples of honey, comb, and bees. Because this type of analysis has never been conducted, a new protocol is required to detect glyphosate in samples of honey. Dr. Meyer, of the U.S.G.S Water Research Facility, is currently developing a new protocol to detect glyphosate from samples of honey, comb, and honeybee tissue. Preliminary results look promising but analysis has not yet been completed.

Statistical Analysis

Honeybee Mortality Data

Anova: Two-Factor Without Replication

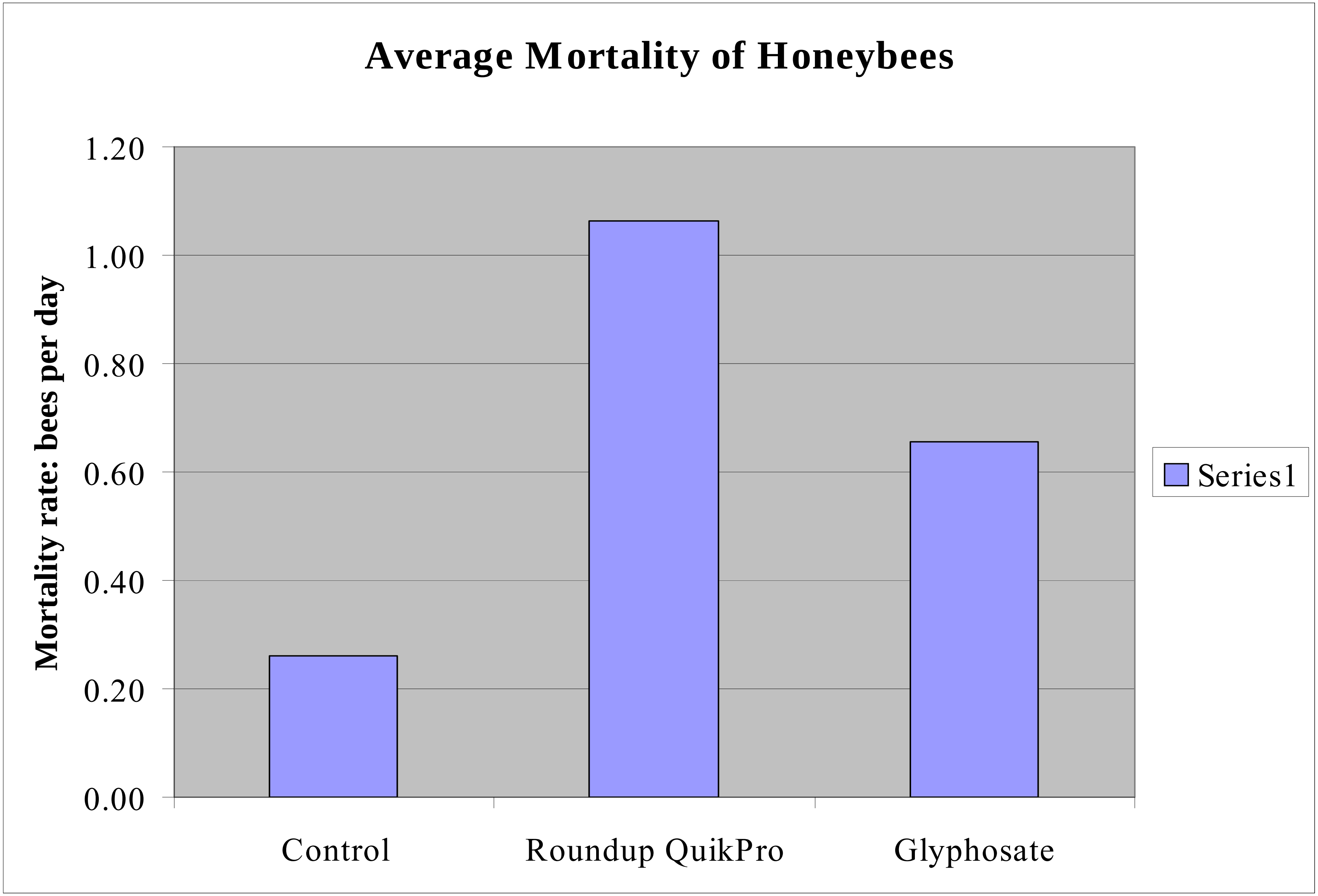
SUMMARY	*Count*	*Sum*	*Average*	*Variance*
replicate number	63	126	2	0.677419
Day	63	630	10	21.48387
Mortality	63	52.33333	0.830688	3.778613

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	597.958	62	9.644484	1.183705	0.213072	1.42094
Columns	3138.315	2	1569.158	192.5888	9.25E-39	3.069289
Error	1010.316	124	8.14771			
Total	4746.589	188				

$n = 63$ $p < 0.001$

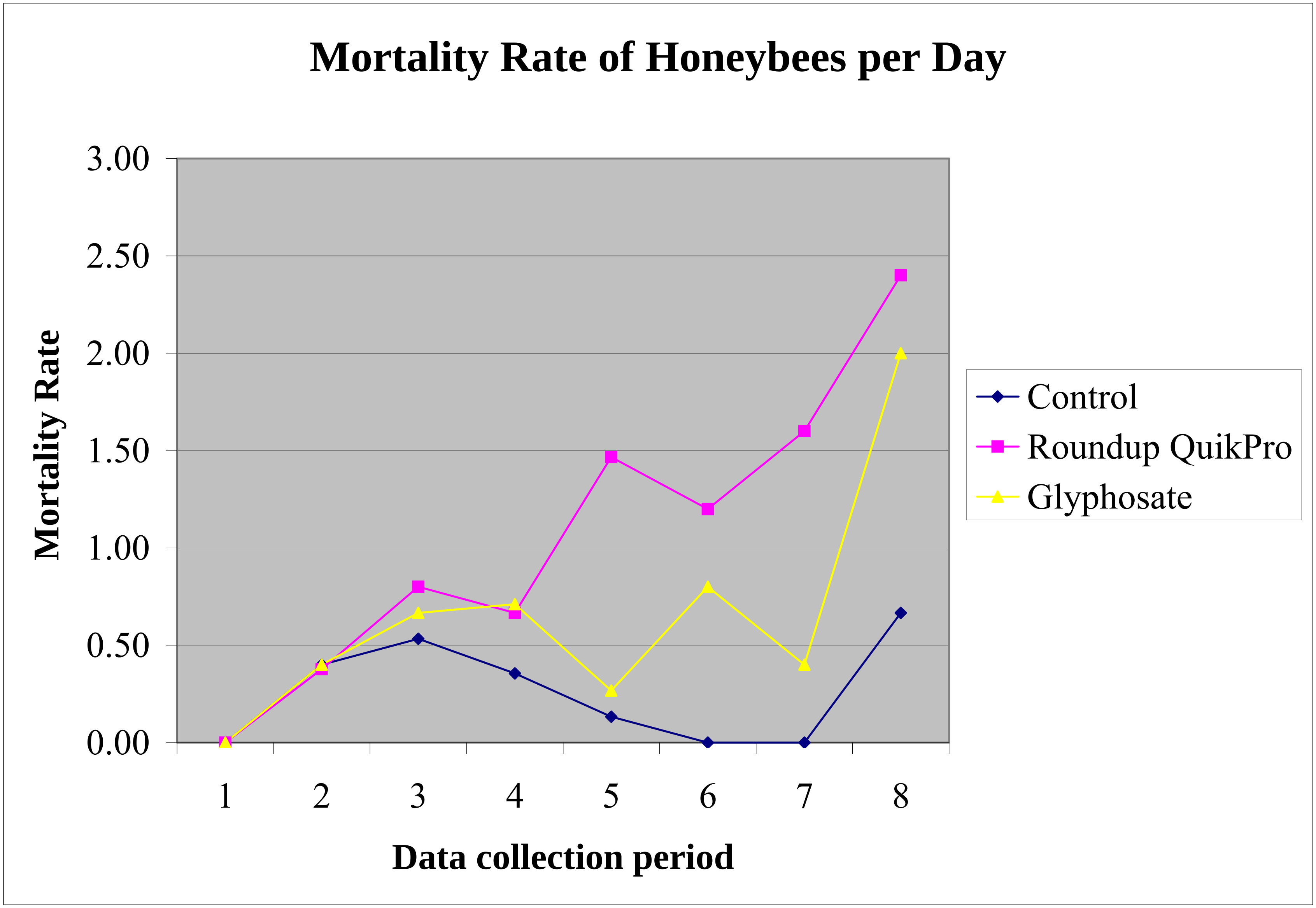
Figure 1



n=63

$p<0.0001$

Figure 2



n=63 $p<0.0001$

Attachment 1

Attachment 1: The hives during initial setup

Attachment 2

Attachment 2: Close-up of hives during initial setup

Attachment 3

Attachment 3: Close-up of a single hive during initial setup

Conclusions

Based on this research, the following conclusions can be drawn:

1. The initial hypothesis that hives with glyphosate-supplemented diets would exhibit a greater rate of mortality than hives with no supplementation was supported ($p < 0.001$).

2. The initial hypothesis that hives with Roundup QuikPro®-supplemented diets would exhibit a greater rate of mortality than hives with no supplementation was supported ($p < 0.001$).

3. The initial hypothesis that it would be possible to detect glyphosate in samples of honey and bees collected from hives with glyphosate-supplemented diets is as of yet unable to be determined.

4. The initial hypothesis that if glyphosate could be detected in samples of honey and bees from hives with glyphosate-supplemented diets, then there would be significantly greater levels of glyphosate in samples of such hives when compared to samples of hives with no supplementation is as of yet unable to be determined.

5. The initial hypothesis that if glyphosate could be detected in samples of honey and bees from hives with Roundup QuikPro®-supplemented diets, then there would be significantly greater levels of glyphosate in samples from such hives when compared to samples from hives with no supplementation is as of yet unable to be determined.

6. The initial hypothesis that if glyphosate could be detected in samples of honey and bees collected from hives with glyphosate-supplemented diets, then there would be significantly greater levels of glyphosate in samples from hives that have been affected by Colony Collapse Disorder when compared to samples from hives that have not been affected by Colony Collapse Disorder is as of yet unable to be determined.

Future Studies

The following studies could serve as an extension to this research:

1. Determine what level of exposure occurs to bees visiting flowers recently sprayed with glyphosate-containing herbicides.

2. Research the effects of other common herbicides, such as atrazine, on honeybee mortality.

3. Determine why Roundup QuikPro® increased mortality rates more than glyphosate alone.

Acknowledgements

Without the assistance from the following individuals this research would not have been possible:

Dr. Marion Ellis of University of Nebraska- Lincoln
-Provided insight about experimental design of the lab component
Dr. Mike Meyer of USGS Water Research Facility in Lawrence, Kansas
-Is in the process of developing a protocol to analyze honey, comb, and bee tissue samples
Dr. Nathan Leigh of University of Missouri- Columbia
-Assisted with the mass spectrometry aspect of the research
Mr. Chris Reeves of Camdenton High School
-Motivated me to begin *and* end this project and helped me every step of the way
Mrs. Jaimie Foulk of Camdenton High School
-Questioned my thoughts throughout the whole experiment and explained chemistry concepts
Mr. Lance Foulk of Camdenton High School
-Helped design and construct honeybee cages, transferred the honeybees into cages, and endured several stings
Dr. Brian Henry of Camdenton High School
- Allowed me to keep 2700 stinging insects in a closet in my high school
My IRB: **Dr. Marion Ellis, Mr. Jeff Whitney, Mrs. Jaimie Foulk, and Mrs. Kim Griggs**
Mr. Scott Moser
-Offered his advice and encouragement during experimental development
Mr. Larry Allman of Camdenton High School
-Allowed the use of his safe shop during cage construction
Mr. Ken Norman
-Allowed me to contact beekeepers at the MBA fall meeting
Mr. T.A. DeGraffenreid
-Showed me the beginnings of beekeeping when I first became a beekeeper
Camdenton R-III Schools Maintenance Department
-Provided me with Roundup QuikPro®
Missouri Beekeepers
-Provided samples of their personal hives
Miss Lori Schlatter
- Encouraged me at every research session, especially those that lasted until midnight
My family: Mom, Dad, and Courtney
-Showed undying support and shared my joys and trials throughout my research

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