An Experimental Trial Analyzing the Synergistic Effect of Imidacloprid and *Nosema ceranae* on Honeybee Mortality due to Starvation Induced Stress

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[student authors 1, 2, and 3]

The combined effects of the pesticide imidacloprid and the parasite *Nosema ceranae* greatly increase the rate of honeybee mortality, yet the specific mechanism of this synergy remains unknown. Using caged bees revealed a smaller number of *Nosema* spores on bees exposed to *Nosema* and imidacloprid simultaneously than in those exposed to *Nosema* alone, suggesting that imidacloprid suppressed *Nosema* proliferation. On the other hand, housing the honey bees in cages failed to account for natural environmental influences and may have put caged bees under unnecessary stress from the controlled environment, rendering results inconclusive. We plan to house honey bee hives in apiaries, providing natural conditions, and expose the hives to varying amounts of a food source, *Nosema ceranae,* and different doses of imidacloprid to observe their effects under natural environmental conditions. By exposing honeybees to the parasite and pesticide in different amounts of food, we can measure how starvation-induced stress influences susceptibility to disease and ultimately death, while determining the specific mechanism through which *Nosema ceranae* and imidacloprid synergistically affect mortality rate.

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**Introduction**

Harvard University and Princeton University request $313,140 from North American Pollinator Protection Campaign for the assessment of the interaction between the parasite *Nosema ceranae* and the neonicotoid (a nero-active insecticide) imidacloprid on the rate of mortality in honey bees. The money will be used in a four-part study that will evaluate how *Nosema* and imidacloprid affect honey bees placed under stress to find how this pathogen and parasite interact synergistically to amplify colony collapse in honey bees. Farmers currently use the pesticide imidacloprid to control numbers of sucking and chewing insects like termites and fleas, however, imidacloprid also affects organisms that are not targeted, like the honeybee (6). In a currently unknown interaction with imidacloprid, the pathogen *Nosema* infects the honeybee’s gut and kills it, causing major problems for the agriculture industry when entire colonies are infected.

The interaction between the pathogen and the parasite is causing many incidences of *colony collapse disorder* (CCD). Colony collapse disorder is defined as the sudden loss of worker bee populations within a colony, resulting in eventual colony extinction since worker bees are responsible for sustaining the colonies (9). CCD has a huge impact on agriculture all over the world. In the U.S., one-third of the average diet comes from agricultural crops that honey bees pollinate, valuing honey bees at a monetary amount of about $15 billion annually (7). Some of the crops honey bees pollinate include apples, nuts, citrus fruit, peaches, strawberries, melons, and even the alfalfa that cattle depend on (3). In fact, honey bees pollinate nearly the whole California almond industry. Without honey bees, we would not have these crops available for us to consume. Cattle would also not have the alfalfa they rely on to eat, meaning we would not have beef to consume either. Bee colony collapse reduces the amount of these vital pollinators. An estimate in 2006 found that between 651,000 and 875,000 of the nation’s estimated 2.4 million colonies died out, meaning 40% less bees were available to pollinate our crops (7). The loss of these key pollinators could completely change the American diet in a way that would not please most Americans.

Previous research that Alaux et al. performed found that the interaction between microspore parasites, like *Nosema*, and pesticides cause a higher rate of mortality among bee colonies (1). Pettis et al. later performed a similar experiment and determined that an interaction exists between the pesticide imidacloprid and *Nosema* but concluded that additional research needed to be performed to understand the underlying mechanism of this pesticide and pathogen interaction (10).

Our goal is to further analyze this synergistic interaction to find the mechanisms taking place between imidacloprid and *Nosema* that kills honey bee populations in the natural environment. We will base our experiment on previous studies done on the potato beetle and the pesticide imidacloprid since beetles are similar insects and would therefore yield similar conclusive results. The studies on potato beetles observed that starvation-induced stress reduced the beetle’s ability to deal with pathogens effectively (4). Besides the study on potato beetles, we’ve also constructed our experiment somewhat similar to Yang et al’s, which found that the pesticide imidacloprid affected the foraging behaviors of honey bees (11).

For the remainder of our proposal, we will devote our attention to a detailed analysis of the previous studies performed on *Nosema* and imidacloprid, give an in-depth description of our proposed research, introduce our key personnel and their qualifications, and finish with a justification of our requested budget.

**Background**

Contradicting results exist from various studies analyzing the relationship between the pesticide imidacloprid and the parasite *Nosema* on the rate of mortality of honey bees. Although both studies presented in this proposal observe the effects of imidacloprid and *Nosema* on honey bees, they used different methods in their experiments. Alaux et al. exposed caged honey bees to imidacloprid and *Nosema* and searched for differences in bees’ physiological processes to analyze the combined and individual effect of imidacloprid and *Nosema* on bees’ health (1). Conversely, Pettis et al. exposed honey bees to the same factors in *apiaries*, under natural environmental conditions (10). The conflict lies in how the combined effect of the pesticide imidacloprid and the parasite *Nosema* create a mechanism that ultimately kills honey bees, as well as determining if stress enhances this unknown mechanism.

Alaux et al. found smaller *Nosema* spore counts in bees exposed to both *Nosema* and imidacloprid simultaneously than in bees exposed to *Nosema* alone, suggesting that imidacloprid suppressed *Nosema* proliferation (1). However, their study failed to account for environmental influence on the relationship between imidacloprid and *Nosema*. In their experiment, honey bees were housed in cages and separated into four groups: a control group, groups infected with *Nosema* alone, groups chronically exposed to imidacloprid, and groups both infected with *Nosema* and chronically exposed to imidacloprid (1). Total hematocyte count (THC), phenoloxidase activity (PO), and glucose oxidase activity (GOX) were measured as well as the amount of *Nosema* spores found on bees (1). Unfortunately, their experiment was conducted on caged honey bees which might have experienced stress from the caged environment, rendering the results inconclusive.

On the other hand, Pettis et al. found that both treatment groups of honey bees exposed to imidacloprid, either at high or low levels, had a much higher *Nosema* spore count than the control group, indicating that imidacloprid enhanced *Nosema* spore production (10). In addition, Pettis et al. rendered more meaningful results due to their inclusion of natural environmental conditions: they conducted their experiments on several honey bee colonies that were housed in apiaries placed 0.5 km apart, allowing bees to experience environmental surroundings. Thirty honey bee colonies were used and divided into three treatment groups: control, groups exposed to low levels of imidacloprid, and groups exposed to high levels of imidacloprid (10). The imidacloprid groups were fed imidacloprid through a sucrose solution over several weeks. After five weeks, several honeycombs with brood were taken from each treatment group and placed in an incubator at 34º C overnight to hatch (10). Two days after the brood had hatched, some groups of newly emerged adult bees from selected colonies were removed and infected with *Nosema* in a 50:50 sucrose solution (10). Any bees that survived past 12 days were euthanized to analyze *Nosema* spore counts (10). Higher numbers of *Nosema* spore counts were found in bees that were exposed to imidacloprid, again suggesting that imidacloprid aids *Nosema* reproduction (10). Therefore, in a natural environment, imidacloprid kills bees more efficiently if paired with the *Nosema* parasite. However, Pettis et al. were unable to determine the specific mechanism through which imidacloprid and *Nosema* synergistically affect the rate of honey bee mortality.

Our research plans to focus on the specific mechanism created by the interaction between imidacloprid and *Nosema* that directly affects the physiological status of honey bees. Additionally, by exposing honey bees to starvation-induced stress, we hope to find how a weakened immune system makes bees more susceptible to the synergistic mechanism of imidacloprid and *Nosema*.

**Description of Proposed Research**

The objective of our study is to further explore the direct mechanism through which the parasite *Nosema ceranae* and the neonicotoid imidacloprid produce a synergistic effect on the rate of mortality in honeybees. Past studies showed that the interaction between the parasite and pesticide has an added effect on bees’ mortality, but were unable to determine the mechanism through which the interaction works. These past studies have failed to show how parasites and pesticides interact in the infection of bees, thus we plan to study how each works separately in the infection of bees. Using twelve new Langsthroth bee hives housed in apiaries, we plan to expose honeybees to varying amounts of a food source, the parasite *Nosema ceranae* alone, and to sublethal and lethal doses of the imidacloprid pesticide. By exposing honeybees to high, medium, and low levels of a food source, we can measure hormone levels in the bees’ brains to determine how stress brought on by possible starvation affects their physiological system. Exposing honeybees to the *Nosema ceranae* and imidacloprid individually in different amounts of food, we would be able to measure how starvation-induced stress influences susceptibility to disease and ultimately death. We hope that with the research we conduct we can become more definitive on how to reduce incidences of CCD.

*Animal Care*

To determine the mechanisms observed between imidacloprid and *Nosema*, we will be using honey bees as test subjects. Honey bees will be obtained from Rossman Apiaries Incorporated through an online order. We will follow the guidelines provided by the Honey Bee act of 1976 (8) as well as Harvard Animal Care and Committee guidelines to determine proper procedure and handling techniques of honey bees (2). All bees will be assumed to be sterile at the time of delivery.

*Set up of apiaries and colonies.*

Twelve new Langsthroth bee hives will be constructed and painted white externally, a standard beekeeping practice (8). Each hive will be six kilometers apart in the Carlson Orchards located in Harvard, MA. Bees will be provided with sources of sugar in accordance to their sample group. Each hive will be covered by tents in order to keep the bees from foraging and obtaining food from natural sources. Small feeders will be placed in the hives to allow them access to sugar sources that will be later provided.

*Control Groups*

We will have three control colonies with low, medium and high levels of food sources. The bees in the low food source will only have access to four flowering plants. The group with access to the medium source of food will have ten plants at their disposal and high food source group will have access to twenty plants. Subsequent groups will also be fed in similar fashion, but will be introduced to either imidacloprid or *Nosema*. 100 bees will be sampled for six months from each food group to measure stress levels associated with varying amounts of food. Stress levels of bees will be measured by removing the cerebral ganglia from bee heads in cold bee saline and placed in eppendorf tubes with perchloric acid centrifuged for 20 minutes. The octopamine, dopamine and serotonin levels will be measured through liquid chromatography and compared using the Biorad protein assay (5).

*Administration of Imidacloprid*

Imidacloprid will be administered in in two dosages. First we will use a high, medium, and low food group and expose them to a low sublethal dose of imidacloprid of 1/100th of LD50. We will use another high, medium and low food source groups and expose them a higher sublethal dose of imidacloprid of 1/50 of LD50. Stock solutions of imidacloprid will be made in advance and will be prepared in DMSO with 5.1g/L. for the sublethal dose we will dilute the stock solution to 1/100th and 1/50th of the lethal dose in cane sugar syrup. The solutions will be fed to their respective test groups via the feeders that will be placed in the hives. We will do daily monitoring of hormone levels by sacrificing 10 bees daily for six months and by processing them as previously mentioned.

*Infection of Nosema ceranae*

We will obtain the *Nosema* spores from previously infected bees by extracting the gut and grinding it in PBS solution. We will then centrifuge the solution and resuspended in sucrose at concentration of 125,000 spore/ml. The spore and sucrose solution will be given to the following treatment groups: (1) a group fed low amounts of food and exposed to 1/100th of LD50, (2) a group fed medium amounts of food and exposed to 1/50th of LD50, (3) a group fed just low amounts of food, (4) a group fed medium amounts of food and (5) a group that has been fed high amounts of food. Their hormone levels will be monitored daily by sacrificing 10 bees daily for six months and processing them as previously mentioned.

*Growth of Nosema in presence of imidacloprid*

We will culture *Nosema* spores that have been rehydrated in 50 ml of PBS with 10μl of our stock solution of DMSO and imidacloprid. Then, 50 μl of this new solution will be streaked onto Sabouraud Agar and incubated at 35º C for 24 hours. After the 24-hour time period, we will count the number of *Nosema* spores that were able to grow successfully and statistically analyze the data obtained. We will then conduct biochemical analysis on any compounds that may be produced when *Nosema* comes into contact with the pesticide.

|  |  |
| --- | --- |
| **Time Table** | **Milestone** |
| April 1, 2013 | Hives will be set up according to protocol. |
| April 9, 2013 | Bees will be placed in hives and given 1 month to grow into a functioning colony. |
| May 12, 2013 | Bees will begin their respective treatments. |
| May 19-October 2013 | Sampling of bees for hormone levels will begin and continue daily.  Nosema will be cultured in agar and exposed to imidacloprid. Biochemical analysis will b |
| October 2013- December 2013 | Statistical analysis will be done. |

**References**

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**Key Personnel**

Key personnel from Harvard University and Princeton University will be acquired to make sure that adequate and efficient staff are available throughout the duration of this experiment. In addition to the principal investigators, the staff will consist of pathologists, lab specialists, and undergraduates to fulfill the requirements of this experiment. The principal investigators will be in charge of ordering all needed equipment for the experiment and overseeing all of the other staff. The pathologist will be in charge of dissecting and analyzing the basal ganglia of the honey bees used in the stress testing phase of the experiment. The lab specialist will stay in the lab with the pathologists and help out with any needed analysis. The entomologist will stay on call at the site of the colonies and make sure all of the bees are being cared for properly and all procedures follow the Harvard Animal Care and Committee guidelines. Undergraduates will be responsible for performing data analysis and behavioral assessments.

**Key Personnel Chart**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Name** | **Degree** | **Organization** | **Role in Project** | **% Time Spent** |
| [student author 1] | Ph. D, M.D. | Harvard University | Principal Investigator | 50% |
| [student author 2] | Ph. D, D.V.M. | Cornell University | Principal Investigator | 50% |
| [student author 3] | M.S.,  D.V.M.. | Harvard University | Principal Investigator | 50% |
| John Parker | D.V.M. | University of California Davis | Pathologist | 20% |
| Mike Conners | B. S. | John Hopkins | Pathologist | 20% |
| Brenna Smith | V.M.V. | Harvard University | Lab Specialists | 15% |
| Paige Turner | D.V.M. | Cornell University College of Veterinary Medicine | Entomologist | 45% |
| Jennifer Williams | Undergrad | Harvard University | Data Analysts | 35% |
| Stacy Mahm | Undergrad | Princeton University | Data Analysts | 35% |
| Kendel Ram | Undergrad | Harvard University | Behavior Assessment | 35% |
| Tiffany Walters | Undergrad | Princeton University | Behavior Assessment | 35% |

**Biographical Sketch**

[student author 1] Ph.D., M.D Professor of Environmental Health

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Education

B.S. Animal Biology University of California-Davis 1997

M.D. Entomology University of California-Davis 2001

Ph. D. Environmental Health Harvard School of Public Health

2005

Teaching Experience

Lecturer of Environmental Health, Harvard School of Public Health

Lecturer of Environmental Exposure Biology, Harvard School of Public Health

Other Relevant Experience

Assisted in the development of cleaner beetles for the use in sewage sanitation

Professional Affiliations

Consultant for Bayer Bee Care Program

Consultant for U.S. Federal Drug Administration, Veterinary and Animal Division

Honors and Awards

Nobel Prize 2011 in Economic Sciences for advancing pollinator health and increasing  
pollination rates.

Selected Publications

Engel, E. Cayenne; Irwin Rebecca E. and [student author 1] *Linking pollinator visitation rate and pollen receipt.* American Journal of Botany 2003

Pankiw T, Waddington KD, Page RE Jr., [student author 1]. Modulation of sucrose response thresholds in honey bees (Apis mellifera L.): influence of genotype, feeding, and foraging experience. Journal of Comparative Physiology A, Volume 187, issue 4 (May, 2001), p. 293 - 301.

**Biographical Sketch**

[student author 2] Ph.D., D.V.M Professor of Entomology

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Ithaca, NY 14850

Education

B.S. Animal Biology University of California-Davis 1999

D.V.M Veterinary Medicine University of California-Davis 2004

Ph.D Entomology Cornell University

2009

Teaching Experience

Lecturer, Iowa State University, Department of Entomology

Lecturer, University of Maryland, Department of Entomology

Lecturer, Cornell University, Department of Entomology

Other Relevant Experience

Discovered 15 species of insects in the Amazon Rain Forest

Professional Affiliations

Entomological Society of America

BioOne

American Institute of Biological Sciences

American Holistic Veterinary Medical Association

Honors and Awards

Received the AVMA Lifetime Excellence in Research Award

Nobel Prize, 2011 for discovering the methods for tracking ants

Selected Publications

Pedersen, Stein, et al. "Automatic fault extraction using artificial ants." *Mathematical Methods and Modelling in Hydrocarbon Exploration and Production* (2005): 107-116.

Seeley, Thomas D.and [student author 2]. *Honeybee ecology: a study of adaptation in social life*. Princeton University Press, 1985.

**Biographical Sketch**

[student author 3] M.S., D.V.M. Professor of Animal Reproduction

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Education

B.S. Animal Science University of California-Davis 2000

M.S. Animal Behavior University of California-Davis 2003

D.V.M. Veterinary Medicine University of California-Davis 2008

Teaching Experience

Lecturer, University of California Davis, Department of Animal Science

Lecturer, California Polytechnic St. University San Luis Obispo, Department of Animal Science

Lecturer, Pennsylvania School of Veterinary Medicine

Other Relevant Experience

Researcher at University of California Davis School of Veterinary Medicine

Professional Affiliations

National Association of Animal Breeders

Association of Genetic Technologists

Honors and Awards

Nobel Prize for the development of miniature dairy cows that produced milk with lower

concentrations of lactose

Selected Publications

Bearden, Henry Joe, [student author 3] and John W. Fuquay. *Applied animal reproduction*. Reston Publishing Company, Inc., 1984.

Ginther, O. J. and [student author 3]. *Ultrasonic imaging and animal reproduction*. Equiservices Publishing, 1995.

**Budget Justification**

This study on how stress induced from varying food levels and pesticides hinder the ability for honey bees to fight off pathogens will in total cost approximately $521900. We expect approximately $313140 from the North American Pollinator Foundation (N.A.P.F.) as we have already been granted $208769 from the Preservation of Honey Bees. This estimated budget will cover the purchase of brand new Langstroth bee hives and accompanying materials as well as the new bee colonies that will provide this project with subjects. This budget will also cover the cost of Dr. Smith’s laboratory personnel and procedures as well as any equipment and supplies that they may use. Travel expenses from off site collaborators have also been included in our budget as various members of this project are from Cornell University, University of California Davis and Princeton University as well as the convention we will be attending in February. The most costly part of this study may be usage of land in order to provide enough space for our experiments as we will have multiple groups and they are required to be a minimum of 6 km apart. Field crews will be provided with several new beekeeper suits in order to prevent cross contamination between the colonies infected with *Nosema* as well as protect the crew members*.* Other expenses such as computers and statistical software have been previously purchased from other projects. But this is all justified as we believe that our study will provide much needed and critical information on how pesticide and parasite interactions lead to colony collapse disorder.

**Research Budget**

|  |  |  |  |
| --- | --- | --- | --- |
| **Personnel** | **Outside Agency** | **N.A.P.F.** | **Total** |
| [student author 1] 50%, 1 year @ 10,000/month | 24000 | 36000 | 60000 |
| [student author 2] 50%, 1 year @ 10,000/month | 24000 | 36000 | 60000 |
| [student author 3] 50%, 1 year @ 10,000/month | 24000 | 36000 | 60000 |
| Smith Loboratory of Entomology Research | 24000 | 36000 | 60000 |
| Veterinarian Staff | 6400 | 9600 | 16000 |
| Pathology Staff | 6400 | 9600 | 16000 |
| Undergraduate Student Assistants | 1200 | 1800 | 3000 |
|  |  |  |  |
| **Equipment and Consumables** |  |  |  |
| Medium Depth Hive with Accessories | 952 | 1428 | 2380 |
| BioLogic DuoFlow 10 System  @ 4000 from BioRad, will be used for Chromatography | 1600 | 2400 | 4000 |
| BioRad Protein Assay Kits (20 Kits) | 1200 | 1800 | 3000 |
| Premium Bee Suit with round Veil (24) | 768 | 1152 | 1920 |
| Laboratory Supplies and Media | 4000 | 6000 | 10000 |
| Northwest Territory Mountain Lodge 16-Person Tent - 16'x16' (14 tents) | 1120 | 1680 | 2800 |
| Monin Pure Cane Sugar Syrup  @ 9.00 for 750 ml | 4000 | 6000 | 10000 |
| Flowering plants (arobidopsis thailiana) | 800 | 1200 | 2000 |
|  |  |  |  |
| **Experimental Colonies** |  |  |  |
| Rossman Apiaries Inc- Queens (14) | 720 | 1080 | 1800 |
| Rossman Apiaries Inc-Packaged Worker Bees (14) | 1200 | 1800 | 3000 |
|  |  |  |  |
| **Experimental Field** |  |  |  |
| Carlson Orchards (12 Months) | 40000 | 60000 | 100000 |
|  |  |  |  |
| **Travel Expenses** |  |  |  |
| Collaborator's Travel | 40000 | 60000 | 100000 |
| The 4th International Congress on Insect Science , February 14-17, 2013 in Bangalore, India. | 2400 | 3600 | 6000 |
| **Total** | **$208,760** | **$313,140** | **$521,900** |
|  |  |  |  |