

**The Effects of Pesticide-Contaminated Pollen on Larval  
Development of the Honey Bee, *Apis mellifera***

by

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## ABSTRACT

### **The Effects Of Pesticide-Contaminated Pollen On Larval Development Of The Honey Bee, *Apis mellifera***

Dorothy A Burlew

The Environmental Protection Agency (EPA) registers pesticides, in part, based on the median lethal dosages (LD<sub>50</sub>) for adult honey bees. However, recent evidence suggests that honey bee brood is sensitive to these chemicals at much lower levels of exposure than adult bees. Furthermore, rather than being protected from pesticides in the confines of the hive as was previously thought, the brood is fed large quantities of pollen that has been contaminated with pesticides in the field.

Until recently, pollen—the sole source of protein, lipids, vitamins and minerals for the rapidly developing larvae—was not considered a threat. But scientists are accumulating data that reveal most agricultural pollen, both here and abroad, is contaminated with multiple chemical residues that often display synergistic effects.

Using the primary literature, I studied the mechanisms by which larvae are exposed to toxins in pollen and how they are affected by them. I then compared the levels at which substantial adverse effects occur with the levels of pollen contamination found in agricultural areas. The analysis illuminated weakness in the EPA registration process due to changes that have occurred in pesticide manufacture and formulation, especially in the last 20 years. Pesticides are toxic in smaller doses than ever before, and they tend to be systemic—rather than surface—preparations. In order to protect honey bee health, we need to go beyond mortality testing of mature bees and establish protocols that are sufficient to protect honey bee larvae against sublethal injury.

Based on my review and synthesis, there is enough evidence to support 1) the restriction of systemic pesticide use on bee-pollinated plants, 2) the establishment of sublethal pesticide levels for larval pollinators, 3) the regulation of pesticide metabolites (breakdown products) as pesticides, 4) restrictions on the use of fungicides and herbicides during crop flowering, and 5) a prohibition on the use of pesticide combinations when detrimental synergistic reactions may occur.

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## **INTRODUCTION**

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Farmers throughout the world depend on animal pollinators to produce an estimated 35% of the global food supply (Klein et al. 2007). Pollinators come in many forms, including bats, butterflies, birds, beetles, flies, and wasps, but bees carry most of the load, accounting for about 75% of all crop pollination services worldwide (Buchmann and Nabhan 1996, Kremen 2002). In North America today more than 90 commercial crops are pollinated, at least in part, by bees (National Research Council 2007). In 2007 the U.S. almond crop alone was valued at upwards of \$1.5 billion—every last almond flower that produced a nut was pollinated by a bee, usually a honey bee (Schacker 2008). Forage crops such as clover, alfalfa, and lespedeza, which make their way into the human food chain via the mouths of livestock, are largely pollinated by bees, as well as most specialty crops such as herbs, nursery stock, and flowers. In 2007 the estimated value of all bee-pollinated crops in the U.S. was approximately 30% of the \$132 billion field crop total (USDA 2007).

But, for unexplained reasons, honey bee colonies are dying in record numbers throughout the world. The United States experienced its second worst loss on record in the winter of 2009/2010 when a total of 33.8% of all managed hives perished between October and April. (USDA-ARS 2010). Because honey bees are a vital part of American agriculture, researchers are in the midst of a massive hunt for the cause of dying bees. As of this writing no “smoking gun” has been found, but as results accumulate, pesticides are coming under close scrutiny because of their possible effects on honey bee brood. Brood,

as the term is used by beekeepers, comprises all the immature bees in a colony, including the eggs, larvae, and pupae.

As part of its registration process, the Environmental Protection Agency (EPA) requires testing of pesticides on adult honey bees to determine a median lethal dose (LD<sub>50</sub>). The companies desiring registration do their own testing under guidelines established by EPA. Once the LD<sub>50</sub> of adult bees and its confidence limits are established, the EPA uses the information to make decisions regarding usage and labeling if the chemical becomes registered, or to make requests for further tests if it does not.<sup>1</sup>

This system of testing was designed to work with the older classes of pesticides such as the carbamates, organophosphates, organochlorates, and pyrethroids. These pesticides, which are sprayed on the leaves of plants, have a relatively short active lifespan lasting just a few hours to a few days (Rortais et al. 2005). Unfortunately, the tests were not designed to measure the toxicity of the new systemic poisons that are applied to the soil or seed and move throughout the vascular system of the plant. These systemics turn the plant into a poison factory that emits toxins from its roots, leaves, stems, pollen, and nectar (Shah 2008).

Nevertheless, the EPA has approved the use of systemics even though their own recommendations for protecting bees—spraying at night when the bees are not present or spraying before the hives are brought to the field—does nothing to protect bees from the toxins in the pollen.

The EPA guidelines for testing pesticides on honey bees are outlined in three different documents: OPPTS 850.3020 *Honey Bee Acute Contact Toxicity*, OPPTS 850.3030 *Honey Bee Toxicity of Residues on Foliage*, and OPPTS 850.3040 *Field*

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<sup>1</sup> See appendix A for a description of pesticide regulatory authority.

*Testing for Pollinators*. The latter document may be used for any pollinator of “economic interest,” which includes the honey bee, the alfalfa leafcutting bee (*Megachile rotundata*), and the alkali bee (*Nomia melanderi*). These guidelines were posted in 1996 and, according to the EPA website, are still in draft form. No mention of bee brood, larvae, or pupae is made in the testing requirements, which deal exclusively with adult bees. In late 2009, in response to public concern about the fate of certain pesticides in the environment, EPA agreed to review the registrations of all pesticides on a 15-year cycle. However, as of this writing, the field testing guidelines remain unchanged (EPA, personal telephone communication).

The purpose of my paper is to survey the current literature on the effects of pesticides on developing honey bee brood and to synthesize an argument for a change in pesticide regulation—one that would require toxicity testing on honey bee brood in addition to testing on adult bees in the field—if it is warranted.

I tried to confine my research to 1) pesticides that enter the hive via pollen and 2) the effects of this pollen on the larval stage of development. I chose to examine the 6-day larval stage because it is a period of intense consumption and rapid growth sandwiched between the relatively stable egg and cocoon stages. I chose to look at pollen because it is the sole protein source for the developing larvae. However, due to the complex nature of a honey bee colony, it would be remiss to not discuss the other possible sources of pesticide contamination because the influences of the various contamination sources are difficult to separate.

The question of whether pesticides are causing the decline of honey bees and how the problem should be handled is a multidisciplinary problem with sweeping

implications. Answering the basic question of what is happening and why will take concerted effort by chemists, biologists, ecologists, and toxicologists to name just a few. As is demonstrated in this paper, the biological assessment has been hampered because of a lack of chemical detection, and chemical detection has been hampered by a lack of technology. The quantities of pesticide being measured are in the parts per billion range, and the animals being studied have a quirky life cycle that is not completely understood.

If we ultimately discover that pesticides are the culprit, then what? Can we ban the pesticides on which the world food supply depends? Will something less than a ban cause food prices to soar? Who gets hurt most by decreased pesticide use? Who gets hurt most by continued use? Do we protect the bees and preserve a large array of food choices? Or do we forsake the bees and have few choices beyond the grains? None of the answers will come easily, and policy changes will necessitate the involvement of politicians, lobbyists, and lawyers. But only by addressing the problem from a multidisciplinary perspective will we even begin to parse this complex, international conundrum that could affect every human being at the most basic level.

## **A SHORT HISTORY OF HONEY BEES IN AMERICA**

Honey bees were brought to North America by the English colonists (Horn 2005). Before the mid-1800s a honey bee colony could survive in the Americas, unattended, year after year (Ellis 2004). Because they were an introduced species, they had no natural enemies in the New World. Feral swarms that split from managed hives thrived in the forests and could be found living in recognized “bee trees” (Horn 2004). In *Notes on the State of Virginia* (1784), Thomas Jefferson commented, “The Indians . . . call them the white man’s fly, and consider their approach as indicating the approach of the settlements.” These feral colonies became significant pollinators of both native plants and cultivated crops (Delaplane and Mayer 2000), and both indigenous peoples and settlers collected honey from forest hives (Ellis 2004). When honey bee diseases began showing up in Europe, the United States enacted the Honey Bee Act of 1922, which prohibited the importation of honey bees from outside North America (NRC 2007).

In spite of Congress, the Honey Bee Act of 1922, and the subsequent Honey Bee Restriction Act of 1923—which decreed how honey bees could be housed—problems began to plague American honey bees. Feral hives began disappearing and beekeepers began losing vast numbers of managed hives (Kearns et al. 1998). The losses began just after World War II, commensurate with the rise in intensive agriculture (Horn 2005). In the United States alone, managed colonies dropped from over six million in 1944 (Schacker 2008) to about four million in the 1970s to about 2.4 million colonies in 2005 (USDA National Agricultural Statistics Service 1977, 2006). In the meantime feral hives almost completely disappeared: by 1994 over 98% of the feral colonies were eliminated

(Watanabe 1994). The shortage of bees, especially for almond pollination, became so severe that in 2005 the Honeybee Act of 1922 was altered to allow importation of bees from outside of North America (NRC 2007). Since that time, bees have routinely been shipped from Australia into the United States.

Honey bees suffered to a greater extent as agricultural ecosystems received more intensive management and monocultures became the norm (Jacobsen 2008). As agricultural intensity increased, so did the number of maladies affecting honey bees (Schacker 2008). Some of these maladies, although not new, began causing significant losses for the first time. Watanabe (2008) and others believe that modern management methods are weakening colonies to the point where they succumb to diseases that would otherwise have only minor impacts on colony health.

For example, in 2006 an unidentified malady now known as colony collapse disorder (CCD) appeared in North America. By spring of 2007, 25% of all managed hives in the United States were lost, and some individual beekeepers reported losses of 75-100% of their hives (James 2008). Several theories have arisen to explain CCD. Some scientists believe it is caused by one of the viruses carried by parasitic mites, some believe it is the result of the synergistic effects between pesticides, and others believe it is caused by sublethal doses of the agricultural insecticides imidacloprid or clothianidan, which can cause bees to become disoriented and lose their way home (Yang et al. 2008).

Whether or not agricultural chemicals cause CCD, many researchers believe that honey bees are losing vitality and disease resistance due to the accumulation of chemicals in the hive (Schacker 2008, Jacobsen 2008). Some of the chemicals are brought into the hives by the bees themselves in the form of contaminated pollen and nectar

(vanEngelsdorp 2009, Chauzat et al. 2006, Chauzat and Faucon 2007, Rortais 2005, Škerl et al. 2009). Other chemicals are introduced into the hive by the beekeeper in the form of drugs to combat the litany of diseases and parasites, which includes two types of foul brood, two types of *Nosema*, two types of mites, hive beetles, and wax moths (Watanabe 2008).<sup>2</sup>

While breeding resistance into plant or animal species is often a viable answer to health problems, a quirk in honey bee genetics makes this avenue extremely difficult. When the honey bee genome was first mapped in 2006, it was discovered that, in comparison to fruit flies (*Drosophila*) and mosquitoes (*Anopheles*), honey bees have only one tenth the number of genes involved in detoxification of environmental poisons (Schacker 2008). Thus, while fruit flies and mosquitoes rapidly develop resistance to industrial toxins such as pesticides, honey bees do not. Bee breeders, apparently hampered by this lack of genetic material, continue to be largely unsuccessful at developing resistant honey bee strains.

Pesticides have evolved over the years, but croplands were traditionally treated with insecticides before the honey bee colonies were trucked into the fields to pollinate the crops. Then, in the late 1980s, a new class of pesticides was approved for use in many nations of the world, including the United States. These new systemic pesticides were so powerful that an amount of product just large enough to treat the seed was enough to protect the plant for the entire growing season. Since honey bees do not eat plants, they

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<sup>2</sup> While many diseases and pathogens plague honey bees, those listed here are commonly treated with in-hive chemicals. American and European foul brood are bacterial diseases which kill brood, *Nosema apis* and *Nosema ceranae* are microsporidians that damage the honey bee gut, *Varroa* mites live off the hemolymph (blood) of the bee, tracheal mites affect breathing, and both hive beetles and wax moths destroy comb.



were thought to be safe from harm. It wasn't until new and powerful detection methods revealed pesticides in the pollen that toxicity concerns arose.

## **METHODS**

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In order to determine if the amount of pesticide present in pollen is enough to adversely affect the developing larvae, I scoured the primary literature for relevant research. I discovered that the first flurry of papers on the subject originated in Europe, with France leading the way. Subsequent papers came from Germany, The Netherlands, Spain, Great Britain, and Canada. Since then, the majority of the research has come from the United States. Indeed, a large number of projects are currently underway at major universities throughout the country.

Since the link between pesticides and brood development is a timely topic, new material was published frequently during the time I researched this paper. On a number of occasions I was able to query the authors and obtain additional or clarifying information.

After reading each of the papers, I grouped the research into categories based on the list below, and further divided them into pesticide types such as acaricides, fungicides, herbicides, insecticides, and insect growth regulators. I compared the amounts of pesticide measured in each paper with published data on lethal and sublethal levels to determine if those levels could be reached in accumulations of pollen.

The papers I read fell into the following categories:

- pesticides and how they work, especially the difference between systemic and contact poisons
- pesticide uptake in plants, especially what happens to the pesticide once it has been absorbed
- the kinds and amounts of pesticides being found in pollen

- lethal versus sublethal effects of pesticides in insects
- pollen collection by bees
- pollen consumption by bees
- growth and development of larval honey bees, especially the role of pollen in the diet
- synergistic effects among pesticides
- synergistic effects between pathogens and pesticides
- secondary sources of larval pesticide exposure, including wax combs, nectar, water, and propolis

I also discovered that there are two primary reasons why so little is known about this subject. The first is that, until very recently, there were no dependable and affordable ways to detect pesticides at the levels found in pollen. And second, there was no way to reliably detect the effect of low-dose pesticides on honey bee larvae. Since these issues are now being successfully addressed by researchers, I decided to add a short section on recent advances and how they may be used to further knowledge in the study of pesticide toxicity on honey bee brood.

However, the second issue—that of determining the effect of low dose pesticides on honey bee larvae—is still difficult to study, even with advanced detection techniques. The primary problem arises because of the way honey bee larvae are fed. A quick summary of the process reveals the problem:

1. The nurse bees consume large amounts of food, including pollen and nectar
2. The nurse bees use the products of this digested food to secrete royal jelly from their hypopharyngeal and mandibular glands
3. The royal jelly is fed to the larvae

This multi-step feeding route poses many questions, such as:

- Do the pesticides pass from the nurse bees to the larvae, or are they completely metabolized?
- Do the metabolites pass from the nurse bees to the larvae?
- If so, are the metabolites harmful to the larvae?
- If the larvae show signs of harm, is it the pesticides and their metabolites causing the harm or is it some exogenous variable such as poor diet, disease, stress, or environmental toxins unrelated to the pesticides?

Even if we can quantify the types and amounts of pesticides brought into the hives via pollen grains, and even if we can calculate the amount of pesticide the nurse bees eat, the amount of pesticide consumed by the brood and how it affects them remains nebulous. This is further complicated by the fact that the water they drink and the wax combs where they live can also be contaminated with pesticides.

In order to begin answering some of these questions, Aupinel et al. (2005) developed a technique for raising honey bee larvae *in vitro*. This technique has turned out to be one the most important advances in brood toxicity studies. However, the technology is new and, although many studies are now underway, few have been completed using the new system.

For the most part, the studies cited herein have been conducted using the best available science and, although limited in many respects, they have illustrated the need for continued research into brood toxicity, and proven the necessity of developing better techniques for detecting and measuring low levels of pesticides in plants, pollen, bee brood, and hive products.

A final note: Worldwide, a number of subspecies of *Apis mellifera* are used for pollination services. Beekeepers in the United States generally prefer either *A. m. ligustica* or *A. m. carnica*, while Europeans use *A. m. carnica* and *A. m. mellifera*. Whenever an author specified a particular race, I mentioned that in the text. However, I made no attempt to separate the races in the analyses of the data. Two major reasons account for this decision. First, many beekeepers stock more than one race in their beeyards, and since they freely cross, it is almost impossible to keep them separate in field conditions. Secondly, most authors don't specify the race they used in the first place, so rather than have a large class of "unknown" and smaller classes of specific races, I grouped all *Apis mellifera* together.

Several times in the paper I mention non-*Apis* bees as a point of comparison or interest. Since they all fall in the order Hymenoptera and have similar needs for pollen, I believe the papers I selected are useful and applicable to the problem of toxic pollen and bee brood.

## **BACKGROUND**

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To understand how contaminated pollen can affect larval development requires an understanding of both honey bee biology and basic pesticide chemistry. The next two sections provide an overview of how honey bees interact with their environment and a short summary of honey bee nutrition. The third section provides an overview of pesticides in the agricultural environment.

### **Honey Bee Biology**

In order to understand how honey bee larvae are exposed to pesticides, a basic understanding of the honey bee life cycle is necessary, along with an understanding of what materials bees collect in the field, and how these materials are used in the brood nest.

#### **The Honey Bee Life Cycle**

The reproductive cycle of a worker honey bee is approximately 21 days. Although the cycles are slightly different for drones (24 days) and queens (15 days), the vast majority of bees in any colony are female workers, so they are typically used as the standard.

The cycle begins when a fertile queen lays an egg in a wax cell that has been prepared by the house bees. After about three days the “shell” dissolves (Cobey, personal communication) and the larva emerges to float in a pool of royal jelly that has been secreted into the cell by the nurse bees. This royal jelly is produced by several glands in combination, but 60-80% of the royal jelly is secreted by the hypopharyngeal gland and about 20-40% is secreted by the mandibular gland (Sammataro and Avitabile 1998).

During a worker bee's stint as a nurse, she eats and digests large quantities of pollen and nectar. From these raw ingredients, her body produces the glandular secretions that nourish the next generation.

The larvae grow quickly during the next 6 days increasing their body weight 1500 to 1700 times (Kevan 2007, Oliver 2010a). As the larvae mature, the nurse bees gradually withhold the part of the diet secreted by the mandibular glands and increase the amount of bee bread<sup>3</sup> and nectar (Sammataro and Avitabile 1998). Towards the end of the 6-day period, the worker larvae even receive some whole pollen grains. Larvae defecate only once, right at the end of the larval stage. At this point each larva spins a cocoon within their wax cell, and the worker bees cover each cell with a wax coating. This is the beginning of the pupal or cocoon stage (Kevan 2007).

The pupal stage lasts 12 days. While in their cocoons, the pupae undergo complete metamorphosis, changing from the worm-like larval form into a recognizable adult bee. They do not eat during this period, but use the food they stored as larvae to form their new bodies. Soon after the young workers hatch, they go to work cleaning the cells in which they were born.

As the adult worker matures, she performs a number of other tasks. These tasks are performed sequentially, changing as she ages, although her tasks may also change with the colony's need. For example, she might spend the first day or two as a "house" bee, cleaning and polishing the brood cells, then spend the next 7 or 8 days as a "nurse," feeding and caring for the young. After that she may tend the queen, build comb, or become an "undertaker" bee that removes dead bees from the hive. Later she may become a "guard," monitoring bees as they come and go to assure that "foreign" bees are

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<sup>3</sup> Bee bread, also known as ambrosia, is a combination of pollen, nectar, and bee-secreted enzymes.

not admitted. Guard bees also attack intruders, such as skunks, dogs, mice, and beekeepers.

The last stage of a worker's life is that of a forager. A forager may collect water, pollen, nectar, or propolis, depending on the colony's need. Foragers work until they wear themselves out. Devoid of energy and tattered of wing, they usually die in the field. The entire life span of an adult bee in summer is about 4 to 6 weeks.

The 6-day larval stage is the portion of the life cycle when bees are most susceptible to environmental toxins. They are not protected by a shell, cocoon, or wax covering. Their bodies are exposed to any contaminants in the brood comb, water, or nectar, as well as any that may be adhering to the bodies of the nurse bees. In addition, the incredible rate of growth rate mandates that all the essential nutrients be present in the diet. If toxins are present, they are most likely to disrupt the bee biology at this incredibly rapid and vulnerable stage of development.

## **The Colony Reproductive Cycle**

While individual bees are born and die on a relatively short cycle, the colony as a whole operates on a much different calendar. In the spring, when a queen begins laying in earnest, the hive population increases rapidly along with the availability of nectar and pollen. The queen can lay about 2000 eggs per day, and the colony can increase from a few thousand to tens of thousands of bees in several weeks (Tautz 2008).

A summer colony may contain 40,000-70,000 members, of which 40-45% are brood and 55-60% are adult workers. The rest of the bees—perhaps as many as 15%—are male drones (Sammataro and Avitabile 1998). Drones have no duties except to mate with young queens from other hives. They perform no hive chores, nor do they collect



provisions or build comb. They are devoid of stingers and therefore cannot defend the hive.

At the peak of spring build-up, the colony may split into two parts, thus beginning a new colony. This phenomenon, called swarming, occurs when over-crowded worker bees produce a second queen. When this queen is nearly ready to hatch, the old queen along with about 40-70% of the workforce leave the hive to take up residence in a new location, and the new queen reigns over the old hive. Thus, in honey bees, whole-colony reproduction occurs as well as individual bee reproduction.

As the summer wanes the number of colony members decreases. As fall approaches any remaining drones are ousted from the hive, and the workers begin preparations for the long winter. Egg-laying is greatly reduced, but collection and storage of supplies continues unabated as long as the weather permits. By winter, the colony may be down to about 10,000 members. These bees are the ones who will tend the queen and sustain the colony during the winter months. Bees do not hibernate but actively work to keep the colony warm and the queen healthy. Unlike summer bees, winter bees can survive long periods—perhaps as long as 320 days (Sammataro and Avitabile 1998) [Table 1].

	Egg	Larva	Pupa	Total	Adult Life Span	Weight
Queen	3 days	4.6 days	7.5 days	15-17 days	2-5 years	178-292 mg
Worker	3 days	6.0 days	12.0 days	19-22 days	15-38 days summer 140-320 days winter	81-151 mg
Drone	3 days	6.3 days	14.5 days	24-25 days	4-8 weeks	196-225 mg

**Table 1. Average Development Time and Lifespan of European Honey Bees. Adapted from Sammataro and Avitabile (1998).**

## **Collection of Materials by Foragers**

Foraging bees collect four main items from the environment: pollen, nectar, water, and propolis (plant resins). A fifth item—honeydew—a sweet substance secreted by certain insects and collected like nectar, is included with the nectar supply.

### **Nectar Collection**

Nectar is usually secreted from glands called floral nectaries that are found in various places in a flower depending on the species. They are usually found at the base, but may also be on the sepals, petals, or stamens. While foraging bees climb deep inside the flower looking for the sweet liquid, pollen sticks to the bee's body. On any given foraging trip a honey bee tends to visit only one species of flower. As she travels from bloom to bloom, pollen grains are inadvertently transferred from one flower to the next. Quite accidentally—at least from the bee's perspective—cross pollination has occurred.

The fossil record tells us that flowering plants co-evolved with bees (Hu et al. 2008). The bee pollinated plants that survived were the ones that produced the sweetest, most attractive nectar. Since the bees flocked to these plants, they were the ones most likely to get pollinated and produce the next generation. This phenomenon is known as a plant-pollinator mutualism: the plant benefits from the bee and the bee benefits from the plant and they both evolve to work ever-more-closely together. While most bee species collect both nectar and pollen, honey bee colonies have a division of labor that allows some individuals to carry only nectar, and some to carry only pollen—although a few manage to carry both (Tautz 2008).

Over the course of about 80 million years (Raven 2005), flowers have developed other specialized ways to attract bees including colorful petals, distinctive patterns called

“honey guides” that lead the bee to the nectar, and landing platforms—widened or fused lower petals that make foraging easier for the bee. Many of the patterns are ultraviolet—unseen by humans but extremely attractive to the pollinators. The bees, in turn, developed tube-like mouthparts that can reach deep into a flower like a straw, brushy bodies that collect pollen, and bristly legs that can be used like combs to remove pollen from their abdomens (Raven et al. 2005).

The nectar is swallowed into an organ known as the “honey stomach,” a part of the esophagus that expands as it fills. Once the honey stomach is full the bee returns to the hive where the payload is transferred to a waiting worker in a process called trophallaxis—the direct transfer of water or food from one bee to another. A colony may collect about 220 pounds of nectar per year.

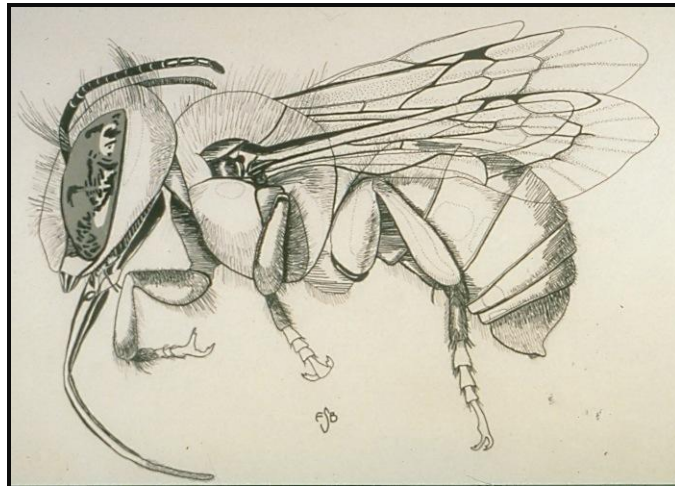
### **Pollen Collection**

While we normally think of honey bees collecting nectar, an average-size colony may collect and utilize 100 pounds of pollen in a single season (Standifer 1967, Sammataro and Avitabile 1998). Pollen is an essential part of the honey bee diet (Kevan 2007), providing a wide range of nutrients including protein, carbohydrates, lipids, vitamins, and minerals (vanEngelsdorp et al. 2009).

Pollen is collected in the field by foraging bees and stuffed into hairy receptacles on their hind legs called corbiculae. Additionally, some pollen sticks to the hairy surface of the bee, and it is this pollen that rubs against the stigmas of subsequent flowers resulting in cross pollination [Figure 1]. A single bee can carry about half her own body weight in pollen.

Once the forager arrives back at the hive, she pushes the pollen pellets from the corbiculae and drops them into a prepared wax cell. Unlike nectar-carrying bees, pollen-carrying bees have to off-load by themselves. In addition to depositing the pellets from their legs, they may also groom away any pollen that is stuck to their bodies. The pollen is stored in cells at the perimeter of the brood nest, forming a ring between the nest and the honey storage area.

In-house bees sometimes known as “food handlers” preserve the pollen by adding enzymes and honey, then packing it tightly into the cells. The resultant mixture is called “bee bread.” Cells filled with bee bread are not capped with wax but remain visible as a mosaic of multi-colored hexagons (vanEngelsdorp et al. 2009). During the brood rearing season, the pollen is stored for only a few days. During the winter it is stored for much longer.



**Figure 1. Drawing showing the fine hairs on the bee's body which capture pollen and aid in the cross-pollination of flowering plants. Pen and ink illustration by Frederica Bowcutt. Used with permission.**

## **Water Collection**

Water has several uses in a honey bee hive. During certain times of the year foragers find a source of water, fill their crops, and ferry it home. The number of bees foraging for water depends on the needs of the colony. If the in-hive workers accept the water quickly, the foragers sense that the need is still high, and they will go back for another load. If the in-hive workers are slow about “unloading” the water, the foragers sense that the need for water has lessened and fewer bees will return for more.

Bees find water in a number of places including damp rocks, branches, muddy puddles, pond edges, and drops adhering to vegetation. They swallow the water and store it in their crops before flying home. The water is transferred to the waiting in-hive workers through the process of trophallaxis.

Bees rarely store water, but bring it in as needed. In the heat of summer it is used for evaporative cooling. The water is spread in a thin film atop sealed brood or on the rims of cells containing larvae and eggs. The in-hive workers then fan their wings vigorously, setting up air currents which evaporate the water and cool the interior of the hive. The process is the same as the human-designed swamp cooler

Nurse bees, who feed the developing larvae, also have a high demand for water. The nurses consume large amounts of pollen, nectar, and water so that their hypopharyngeal glands can produce the jelly that is used to feed the larvae, and to a lesser extent, other bees in the hive.

A third use for water occurs in the winter. Stored honey—especially honey high in glucose—tends to crystallize as it dries. Bees need water to dilute the crystals back into liquid before they can eat it. The same occurs if a beekeeper feeds crystalline sugar to

bees as a winter supplement: the bees need to dissolve the crystals before they can eat the sugar. In very cold periods, the bees may use moisture that has condensed on the inside of the hive.

### **Propolis Collection**

Bees collect propolis from plants. It is a sap-like substance exuded from flower and leaf buds as a defensive coating. It protects those delicate plant parts from pathogens, fungi, and insects. Foraging honey bees scrape it off plants and carry it in the pollen sacks on their hind legs. It often looks like a load of pollen except that it glistens in the sunlight and is usually a rich chestnut brown. The color however varies with the source. It may be a whitish gray, tan, a variety of browns, or nearly black.

The composition of propolis also varies considerably, and so do its antibacterial and antifungal properties. As a general rule it averages about 50% balsams (a general term for sticky, aromatic resins), 30% waxes, 10% essential oils, and 5% pollen. The rest is a vast composite of amino acids, vitamins, and minerals.

Bees use propolis for a number of purposes. They seal small cracks with it, they use it as a polish on the inside of brood cells, and they wrap dead organisms with it to prevent putrefaction in the hive. Dead mice and snakes—which are too big for the bees to remove—have been found completely sealed in propolis, much like mummies. They also smear it over rough places in the hive to reduce wear and tear on their delicate wings, and sometimes will use it to reduce the size of their entrance to protect the hive from invaders or to reduce drafts.

Since propolis is annoying for beekeepers to handle and can stain the wax comb, some breeders have selected against propolis collection and have produced genetic lines

that collect considerably less than others. As often occurs after human interference, there is now some question about whether these bees can adequately defend their hives against pathogens (Watanabe 2008).

## **Glandular Secretions of Nurse Bees**

Honey bees have several pairs of exocrine glands in the head segment, including mandibular, hypopharyngeal, and salivary glands [Figure 2]. The mandibular glands are attached to each of the mandibles and, when the workers are young, the glands produce a lipid-rich white secretion. This is mixed with a protein-rich secretion from the hypopharyngeal glands. Together, these secretions form a substance known as worker jelly or royal jelly. The jelly flows from the glands to the mouth through a long duct (Huang 1990).



**Figure 2: Honey bee salivary glands are shown in upper left. The large mass to the right is the hypopharyngeal gland. Photo by Zachary Huang. Used with permission.**

The nurses consume large quantities of pollen in order to stimulate these glands and provide the raw materials for the jelly (Frazier 2009, Rortais et al. 2005). Drops of the royal jelly are released from openings on the inner sides of the mandibles and placed in the cells containing larvae (Huang 1990). After about 6 days, the larvae are sealed

under wax coverings, cocoons are spun, and the developing pupae begin their metamorphosis.

Although nurses can produce some amount of jelly from birth, production peaks at 6-12 days and may continue at a reduced level until day 20 (Feng et al. 2009). Usually a nurse bee stays in service 8 to 10 days before she moves on to some other job within the hive. As a worker ages past the nurse bee stage, the glands produce other substances and the size of the duct decreases (Tautz 2008).

From egg-hatch to about three days, the larvae receive straight jelly. Afterwards, the jelly is mixed with bee bread—a mixture of whole pollen, honey, and enzymes—and fed to both worker and drone larvae until they spin their cocoons. The queens receive a steady diet of royal jelly throughout their development.

Each larva must eat enough in its 6 days of development to increase its weight by as much as 1700 times (Oliver 2010a) [Table 2]. The larvae lay directly in a pool of jelly. Because the jelly is purified, sterilized, and highly digestible with little bulk, the larvae do not produce feces during this critical growth stage. Consequently, their food does not become contaminated with fecal material and the nurse bees are not burdened with excessive cleaning (Webster and Peng 1988).

During extremely active periods, even drones and foragers may be fed some amount of royal jelly to keep them working at their maximum (Schmickl and Crailsheim 2004). In his series of articles called, “The Economy of the Hive,” Randy Oliver of *ScientificBeekeeping.com* describes this conversion of pollen into protein-rich jelly the “essence of bee economics.” He writes, in part, that “jelly is the true currency of colony



wealth” (2010a, 162). He stresses that nurse bees must remain healthy and hungry in order to fulfill the many protein demands of the hive.

Days	Developmental state	Weight	Length	Food source
1	egg	0.132 mg	1.2mm	yolk
2	egg	not listed		yolk
3	egg	0.09 mg		yolk
4	larva	not listed		Royal jelly
5	larva	3.4 mg		Royal jelly
6	larva	33.3 mg		Royal jelly/honey and pollen (bee bread)
7	larva	100.1 mg		honey and pollen (bee bread)
8	larva	134.5 mg		honey and pollen (bee bread)
9	larva	155.2 mg		honey and pollen (bee bread)

**Table 2.** During the first three days, the egg decreases in weight as it loses moisture. Then, from the end of the egg stage until the end of the larval stage (six days), a typical larva can increase its weight over 1700 times. From Stone (2006). Used with permission.

### **The Production of Bee Bread**

Bee bread is different from pure pollen collected in the field. It is often seen in the comb as a matrix of colorful hexagons—a testament to the many varieties of pollen collected and stored by the colony [Figure 3]. To make bee bread, the honey bees mix the pollen with nectar to which has already been added a number of antifungal and antibacterial peptides and enzymes (Tautz 2008). These enzymes, secreted by the third pair of exocrine glands in the head segment, allow both the nectar and pollen to be stored for long periods without becoming moldy. In spite of this, research by Gilliam et al. (1989) has shown that bee bread contains an average of 107 molds, 81 yeasts, and 29 bacteria.

It has long been suspected that a fermentation process takes place in the bee bread which helps preserve its nutritious properties, but the mechanism was not understood.

Recently, however, the source of the fermentation bacteria was discovered in the honey stomachs of bees. The fermentation bacteria was found to be a collection of several types of lactic-acid producing bacteria (Vásquez and Olofsson 2009).

In addition to fermenting the bee bread, the lactic acid bacteria produce antimicrobial substances, such as organic acids, hydrogen peroxide, and antimicrobial peptides, so eating the fermented product is probably a colony-wide defense against disease (Forsgren et al. 2009). The uniformity of lactic acid-producing bacterial colonies—which were similar in all the populations studied—helps to explain the similarity of microflora in bee bread collected from colonies that forage on diverse pollen sources (Vásquez and Olofsson 2009).

Since young nurse bees produce the vast majority of the glandular secretions needed for colony development, the health of the entire colony is dependent on the health of the nurse bees. And because the young nurse bees eat the vast majority of pollen in the form of bee bread, that substance must be wholesome and free of contaminants.



**Figure 3. Pollen stored in brood nest. Flickr photo by Maja Dumat. Used with permission.**

# **Pollen and Honey Bee Health**

## **The Composition of Pollen Grains**

Pollen is a collective term for the microspores that contain the male gametes or sperm of flowering plants [Figure 4]. Depending on the species of flowering plant, pollen is produced by the anther and must be transported to a stigma of a flower on the same plant or another individual of the same plant species in order for sexual reproduction to occur. Individual pollen grains are covered by a tough outer wall or exine that protects them from environmental hazards such as desiccation on their journey from flower to flower.

The outer surface of the exine is coated with a sticky substance called pollen coat or pollenkitt that is often scented, pigmented, and enzyme rich (Raven et al. 2005). The pollenkitt is especially attractive to bees, and its stickiness allows it to adhere to the bee's body and pack easily into the corbiculae (Pacini and Hesse 2005). Pollen grains vary in size from less than 20  $\mu\text{m}$  to more than 250  $\mu\text{m}$  in diameter, and vary in shape and composition as well (Raven et al. 2005).

The content of pollen varies widely from species to species. For example, crude protein may be 2.5-61% of the total dry weight (Roulson and Cane 2000), but the individual amino acids vary even more (Bell et al. 1983). Although 18 amino acids are commonly found in pollen, a particular plant species may be entirely lacking in one or more (Standifer 1967).

Pollen also contains a range of carbohydrates (about 55%), crude fiber (4-18%), lipids (1-20%), vitamins, and minerals. Calcium, chlorine, copper, iron, magnesium, phosphorous, potassium, silicon, sodium, sulfur, and zinc are all found in pollen, along

with ascorbic acid, biotin, vitamin D, vitamin E, folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine (Bell et al. 1983, Standifer 1967). The high proportion of crude fiber is probably due to the structure of the cell walls (Bell et al. 1983). These tough coatings protect the pollen from environmental stressors such as pathogens and parasites, and drying due to sun and wind (Kearns and Inouye 1993).

Honey bee enzymes capable of digesting protein are secreted by the midgut and hypopharyngeal glands and reside in the alimentary canal. The enzymes split the pollen grains apart at a weak point—usually the soft germinal pore area—and digest the interior portions only (Standifer 1967). The cell walls or “husks” pass through the honey bee gut without being digested (Babendreier et al. 2004).



**Figure 4: Red pollen on flower anthers. Flickr photo by Kris Gabbard. Used with permission.**

## **The Relative Quality of Pollen Sources**

Although colonies bring in vast amounts of pollen every year (Standifer 1967, Sammataro and Avitabile 1998), honey bees cannot distinguish high-quality from low-quality sources. When protein quality is low, honey bees respond by simply bringing a

greater volume of pollen into the hive regardless of its composition (Pernal and Currie 2001).

Like honey bee larvae, sweat bee larvae (*Lasioglossum zephyrum*) were found to grow larger when fed pollen with higher protein content. Unlike honey bees, however, adult foragers did not adjust the provision size based on the type of pollen collected (Roulston and Cane 2002).

So while honey bees adjusted the quantity of pollen but not the quality, sweat bees adjusted neither. The researchers in both studies concluded that bees are unable to make floral choices based on the quality of pollen. Although the honey bees reacted to the low quality pollen, they didn't react in a way that alleviated the problem. Because of this inability to determine protein quality, bees do best when they are able to forage on a large number of flowering species and return with a mixture of pollen types.

Tasei and Aupinel (2008) tested a number of different pollens on the development of the bumble bee, *Bombus terrestris*. They used six single-source pollens and nine commercially-available pollen mixes which are commonly purchased by bumble bee producers in Europe. The six single-source pollens ranged widely in their crude protein content, and produced significantly different developmental results. The protein content of these pure pollens is shown in Table 3.

Pollen type (100% purity )	Nitrogen content (% dry weight $\pm$ sd)	Protein content (% dry weight)
<i>Actinidia</i>	2.91 $\pm$ 0.15 (a)	18.1
<i>Cistus</i>	2.31 $\pm$ 0.03 (b)	14.4
<i>Papaver</i>	3.98 $\pm$ 0.07 (c)	24.9
<i>Helianthus</i>	2.30 $\pm$ 0.26 (b)	14.4
<i>Castanea</i>	3.25 $\pm$ 0.04 (c)	20.3
<i>Rubus</i>	3.08 $\pm$ 0.07 (a)	19.2

**Table 3: Protein content of single-source pollen samples. From Tasei and Aupinel (2008).**

The mean individual weight of larvae was highest when fed *Castanea* (0.15 g), and lowest when fed *Helianthus* (0.02 g), followed by *Cistus* (0.05 g) and *Actinidia* (0.09 g). The highest larval rejection rates (larvae expelled by worker bees) were in colonies fed *Papaver* (27%) and *Helianthus* (22.4%), and the lowest rejection rates were found in colonies fed *Castanea* (4.5%) and *Rubus* (6%). The highest worker mortality was found in colonies fed *Helianthus* (25.6%) and the lowest was found in colonies fed *Actinidea* (2.6%). In contrast, the colonies fed pollen mixtures were more consistent in their growth and development. The results suggest that protein composition—not merely the amount—is necessary for proper bee development (Genissel et al. 2002).

Similar results were obtained in several studies of *Taraxacum* and *Helianthus*—two members of the family Asteraceae (Compositae) known for their low pollen protein levels. A steady diet of *Taraxacum* pollen was found to hinder larval development in *Osmia lignaria* (Levin and Haydak 1957), prevent brood production in *Apis mellifera* (Loper and Berdel 1980), and cause 100% larval rejection in *Bombus* spp. (Genissel et al. 2002). A diet of pure *Helianthus* reduced adult longevity and stunted development of the hypopharyngeal glands in honey bees (Schmidt et al. 1995, Pernal and Currie 2000). Loper and Cohen (1987) believe these pollens have inadequate ratios of at least four amino acids—arginine, isoleucine, leucine and valine.

## **Why and How Bees Consume Pollen**

Besides being the sole source of protein in the hive, pollen is a source of lipids, vitamins, minerals, and carbohydrates (vanEngelsdorp et al. 2009). The mass build-up of bee populations in the spring is dependent on an adequate and nutritious pollen supply.

Without the mass build-up of individual bees, there can be no reproduction at the colony level (Schmickl and Crailsheim 2004).

Larvae need a constant supply of nutrients (Schmickl and Crailsheim 2004). Although very young larvae are fed royal jelly exclusively, older larvae also receive whole pollen and nectar. But Babendreier et al. (2004) found that the direct feeding of whole pollen to larvae constituted only about 5% of protein-containing brood feedings. During this 6-day period, larvae increase their body-mass from about 0.11 mg to about 159 mg (Wang 1965), so an impressive amount of jelly is needed.

### **Most Larval Protein Comes From Nurse Bees**

Using the fact that honey bee larvae do not defecate until larval development is nearly complete<sup>4</sup>, Babendreier et al. (2004) designed an experiment to determine exactly how much whole, undigested pollen is fed directly to larvae. They placed hives of honey bees in field enclosures where maize was the only source of pollen available to foragers. When the larvae reached maturity, the researchers split the larval guts of approximately 30 bees from each of five colonies and counted the number of pollen grains that had accumulated over the development period. They found pollen in three distinct stages: undigested pollen, shrunken pollen (partially digested), and completely digested pollen (recognized by an empty exine) [Figure 5].

The larval guts yielded a range of 1720 to 2310 maize pollen grains, of which 2.2% were undigested, 23.3% were partially digested, and 74.5% were completely digested. Based on the mean weight of a single grain of maize pollen, Babendreier et al.

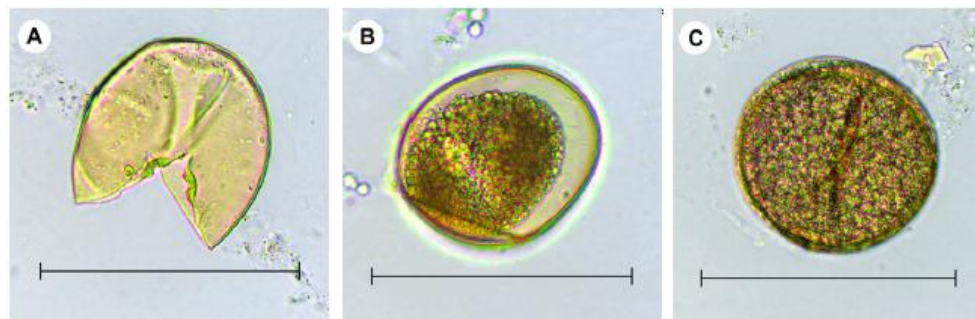
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<sup>4</sup> According to Dr. Marion Ellis at the University of Nebraska, honey bee larvae defecate just one time just prior to spinning their cocoon. They defecate on the cell wall, but then spin a cocoon that isolates them from their waste. When the fully developed adult bee emerges, she cleans the cell and removes the silk and fecal material (personal communication.)

calculated that a typical worker bee larva ate between 1.5 and 2 mg of whole pollen during the 6-day development period ( $1.52 \pm 0.108$  to  $2.04 \pm 0.104$  mg).

However, data show that a single honey bee pupa, after defecation, contains 1.85 to 1.87 mg of nitrogen (Imdorf et al. 1998). Using a conversion factor of nitrogen to protein of 6.25 (Maynard and Loosli 1969) and a protein content of 16.7% for the maize variety Monumental, and subtracting 20% for loss of nitrogen through defecation, Babendreier calculated that 86 mg of maize pollen is necessary to rear a single honey bee larva.

These calculations indicate that the 1.5 to 2 mg of pollen eaten “whole” during the larval period satisfies only about 5% of the total amount of protein needed to reach the pupal stage. The rest of the protein comes indirectly from the hypopharyngeal secretions of the nurse bees.



**Figure 5. Varying degrees of maize pollen digestion in the honey bee gut. (A) fully digested (B) partially digested and (C) undigested. From Brabendreier et al. (2004).**

Since 95% of the larval protein is coming through the hypopharyngeal glands, the proper development of nurse bees is essential for colony survival. A system for measuring the amount of pollen consumed by nurse bees is currently being developed by Maryann Frazier at the University of Georgia (Frazier 2009). She is measuring the pollen consumption of adult bees during their first 8 days of adult life by feeding them



controlled amounts of pollen in an observation chamber and calculating average pollen consumption per bee. Her research will likely provide a better understanding of the flow of protein from pollen to larvae via the nurse bees.

## **Pollen Fed to Larvae is Selected by Foragers**

Pollen is both selected and collected by the foraging bees. When their corbiculae are full the foragers return to the hive and deposit the pollen pellets into cells by themselves. The foragers do not eat any of the pollen, but simply release the pellets and return to the field. The younger nurse bees then examine the pollen to determine its suitability as feed<sup>5</sup>. If it passes their inspection, the nurses consume the pollen in preparation for feeding the larvae (Schmidt et al. 1987). If the pollen is not preferred, it may remain stored in the comb for long periods of time. Most pollen is stored very near—or even within—the brood nest so that it is readily available for the nurses who are busy feeding the larvae (Schmickl and Crailsheim 2004).

Although there are usually vast stores of honey in a hive, pollen collection is tailored closely to use. As the demand increases, pollen collection must increase. If collection cannot keep up with demand, the demand must be lowered by producing less brood and distributing the remaining pollen in a different fashion. In a normally functioning hive, about 5% of the individuals are eggs, 10% are larvae, and 21% are pupae. The other 64% are adult bees. When pollen supplies are short, the workers may expel larvae or eat eggs in order to change the proportions and lower the number of necessary feedings (Schmickl and Crailsheim 2004).

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<sup>5</sup> For example, nurse bees require phagostimulants (eating stimulants) for optimal feeding (Schmidt 1984). The authors speculate that sesame pollen—a material that has very little taste or odor for humans—may not be phagostimulatory to bees, and so they ignore it in favor of other pollens.

# **Pesticides in the Environment**

## **A Short History of Pesticides**

Although the very first application of pesticide is unknown, there is some evidence that elemental sulfur was used as a crop dust in Mesopotamia about 4,500 years ago (Miller 2002). Later, up through about the 15<sup>th</sup> century BP, a number of naturally-occurring elemental toxins were in use, including arsenic, lead, and mercury. In the latter part of the 19<sup>th</sup> century, compounds became more sophisticated (Hodgson 1991). Naturally-occurring organic compounds were extracted from some plants and used as pesticides on others. Notable among these were pyrethrum, derived from chrysanthemums, rotenone, derived from a number of tropical genera including *Lonchocarpus* or *Derris* (Ambrose and Haag 1936) and nicotine extracted from tobacco. Nevertheless, the arsenic-based compounds remained the pesticides of choice all the way through the 1950s (Perkins 1982).

The technologies related to the production of explosives during WWI and WWII led directly to the manufacture of modern chemical pesticides. One of the first, paradichlorobenzene (PDB), was a by-product of the production of picric acid. When a USDA entomologist, E. B. Blakeslee, found it to be highly effective against the peach tree borer, the synthetic pesticide industry was born (Perkins 1982). Many of the early breakthroughs in pesticide science remained war-related. Armies needed ways to protect woolen uniforms from moths and to rid soldiers of body lice. Ultimately, a Swiss researcher, Paul Herman Mueller, isolated a chemical that would not only perform these duties but also control flies and Colorado potato beetles. It was dichlorodiphenyltrichloroethane, DDT (Perkins 1982).

From the inception of commercial pesticides until the mid-1970s, almost all pesticides were sprayed on the leaves of plants to kill insects or weeds, or were applied to the ground to kill soil-borne organisms (Klingman and Ashton 1975). These chemicals were contact poisons, designed to kill the organism on contact or upon ingestion.

In agriculture, contact poisons were often sprayed on crops to kill harmful insects before the honey bees were brought into the field to pollinate. The poisons disintegrated quickly and most bees survived (Jacobsen 2008). However, as with any system managed by humans, mistakes were made. Crops were sprayed while the bees were working, decimating entire colonies. Winds carried poisons onto non-target plants. Insect pests attacked the fields while the bees were working and nothing could be done to save the crop—it would either be consumed by the predator or not pollinated at all—a loss either way.

As scientists recognized the need to prevent waste, spray drift, and collateral damage, new types of pesticides were developed. Systemic pesticides—poisons that are absorbed by the plant and circulated throughout by way of the vascular system—first came onto the market in the 1960s. They turned out to be much more effective than previous pesticides because they persisted in the plant for long periods, and when an insect ate the plant or sucked on the juices, it was poisoned and died. At first systemics were used only for ornamental plants, and never for plants that were intended for human consumption. Although the older pesticides were also poisonous to humans, they could be washed from the plant, leaving very little residue (Jacobsen 2008).

Honey bees were believed to be safe from the systemic insecticides because bees do not eat the leaves of plants or suck their juices. For the most part, bees were observed

collecting nectar and pollen with no visible effect. The new chemicals seemed to be a panacea: they killed the bad insects and spared the pollinators.

By the late 1990s, however, agronomists in France were reporting colony deaths in bees that had worked in systemically-poisoned crops the previous year (Kirchner 1999). A number of papers appeared in the French scientific literature questioning whether nectar and pollen could harbor enough poison to kill a colony of bees (Rortais 2005). Aside from the French research, little attention was given to the pesticide-in-pollen question, and systemic pesticides continued to evolve.

In 1991 Bayer CropScience released the first neonicotinoid pesticide called imidacloprid. The neonicotinoids were generally lethal in much smaller quantities and persisted for longer periods (Jacobsen 2008). Growers liked them because they could reduce the frequency of pesticide applications. Although the compounds were known to be toxic to bees on contact, the applications were made before the honey bees were brought into the fields to pollinate, just as they had done in the past.

A further refinement of this system appeared when Bayer modified the neonicotinoids so they could be painted on the seed before planting, rather than being sprayed on the plant itself. These applications were effective in minute quantities and so saved money and reduced damage to the environment. Because the seed was sown directly into the ground, wind-blown contamination didn't have a chance to occur. Imidacloprid soon became one of the best-selling pesticides in the world, used on plants to control herbivory and on animals to control biting and sucking pests.

The systemic neonicotinoids were generally accepted as safe by beekeepers until the appearance of Colony Collapse Disorder in 2006 (Shah 2010). Although bees had

been in decline for years, colony losses suddenly accelerated. Something was killing bees by the millions, but no single biological agent could be found in all the dead hives (Schacker 2008). Researchers then turned their attention to the materials the bees were bringing back into the hive: nectar, pollen, propolis and water.

### **The Amount of Pesticides in Use**

By 2000 world pesticide use exceeded 5 billion pounds per year. Of that the United States accounted for 20% of the total. By category the U.S. accounted for 25% of the herbicide use, 10% of the insecticide use, 15% of fungicide use, and 30% of all other pesticide use. The “all other” includes nematicides, fumigants, rodenticides, molluscicides, piscicides, and other chemicals used as pesticides including sulfur and petroleum (EPA 2009).

## **DATA SECTION**

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Before beginning an in-depth study of the effects of contaminated pollen on honey bees, I wanted to know how often chemical contamination is actually detected in pollen samples collected from the agricultural environment. If contamination were seldom detected in pollen, then that mechanism of honey bee harm would likely not be a significant factor in the demise of millions of honey bee colonies. I believed that the *frequency* of pollen contamination would be critical to assessing the harm of agricultural pesticides to larvae in particular.

I was able to find four studies that addressed this question, three of which were published during the time I was researching this paper. This first data section is an overview of the four studies. I have not attempted to quantify all the chemicals found in each study, or to compare concentrations and detection methods. I'm simply using the information to illustrate the frequency with which pesticide-contaminated pollen may be encountered and collected by bees. Subsequent sections will detail specific interactions between bees and chemical pesticides.

### **How Frequently are Pesticides Found in Pollen?**

In 2002 Chauzat et al. initiated the first field survey to monitor pesticide residues in pollen loads carried back to the hive by honey bees. Although they were not able to determine a significant correlation between pesticide residues and colony mortality, a number of their findings suggested that more study was in order (Chauzat et al. 2006, 2009).

## **Pollen Loads in France**

In their three-year field study conducted from 2002 to 2005, Chauzat et al. (2006, 2009) examined hives that produced various types of honey, but mostly sunflower, canola, chestnut, and local mixed-flower honey. The apiaries were run by both professional and hobby beekeepers, and ranged in size from 15 to 1500 hives. The beekeepers were asked to continue with their normal management regimes, except that hives selected for monitoring were not to be moved for the duration of the study. From this pool of apiaries, 125 colonies were selected at random. Pollen loads sampled were taken from pollen traps only—and not from bee bread—to eliminate contamination from wax combs, nectar, and honey bee digestive enzymes.

Each pollen sample was tested for 41 pesticides and some of the more common metabolites (pesticide breakdown products), such as those from imidacloprid and fipronil. All the chemicals tested were legally registered for agriculture in France, with the exception of lindane, and they were chosen based on either their frequency of use and/or their high toxicity to honey bees. Thirty of the pesticides were registered as insecticides or acaricides, and 11 were registered as fungicides.

Although Chauzat et al. grouped their findings to include all detections in honey, wax comb, and pollen they found that the number of pesticides in any one sample ranged from 0 to 9 with an average of 2. Imidacloprid and 6-chloronicotinic acid (an imidacloprid metabolite) were the most frequently detected compounds. In pollen, 57.3% of the samples contained imidacloprid or its metabolites, 16% contained one or more of 6 fungicides, 13.5% contained carbaryl, 12.4% contained fipronil or its metabolites, 7.6%



contained endosulfan, and 15% contained other miscellaneous pesticides. Only nine of the pollen samples contained no pesticides; the remainder contained from one to five.

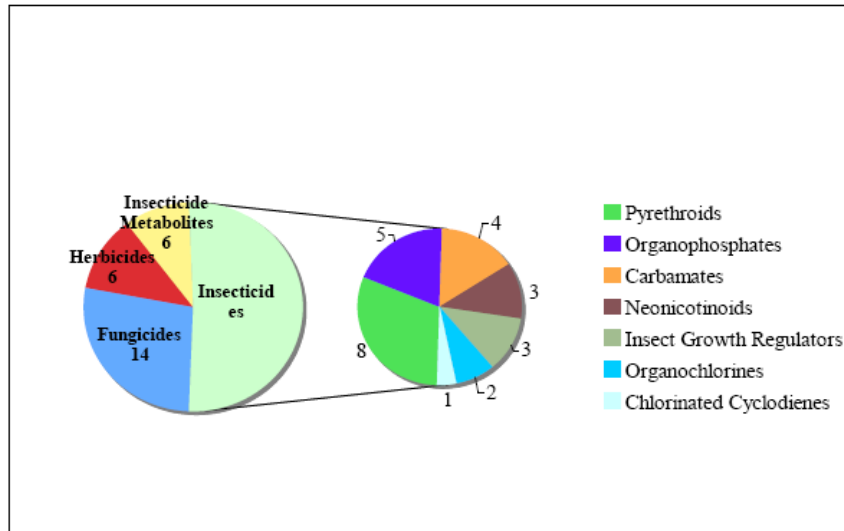
Even though Chauzat et al. found no pesticides at acute toxicity levels, they found that colonies are chronically exposed to multiple foreign substances and that these were stored for future use by the colony. Since little is known about the interactions between these chemicals, and little is known about the fate of these chemicals during in-hive storage, it was impossible for them to draw conclusions about the ultimate effect these chemicals would have on colony health.

However, since it has been shown that doses of imidacloprid between 60 to 6000 times lower than the LD<sub>50</sub> can produce sublethal effects in honey bees (Suchail et al. 2001), Chauzat et al. concluded that LD<sub>50</sub> values are not sufficient to assess the effects of pesticides on colony health. In addition, they expressed the need for more study on the long-term health of colonies exposed to sublethal doses of pesticide, the need to study honey bee susceptibility to pesticides in an environment of restricted nutrition (monocultures), and the need for more study on the possible synergistic effects between pesticides, and between pesticides and pathogens.

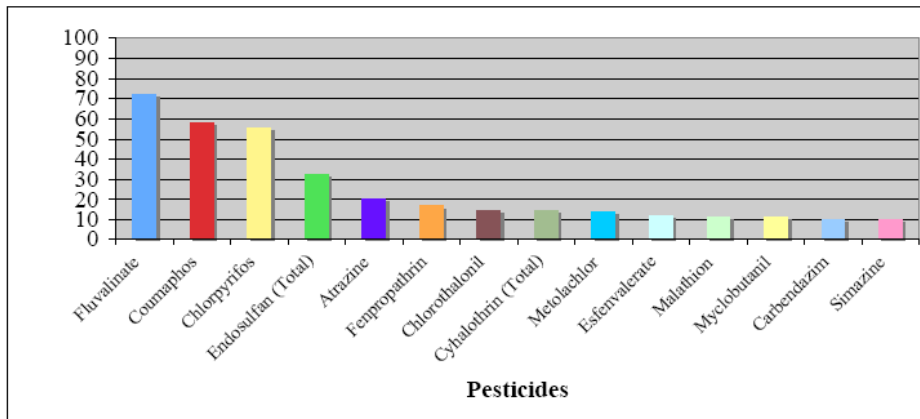
### **Pollen Loads in Pennsylvania**

In 2007 the Department of Entomology at Penn State University (Frazier et al. 2008) analyzed pollen samples from 108 Pennsylvania hives, looking for the most common pesticides as well as many of their metabolites. Altogether, the samples were analyzed for 171 different chemicals. The researchers were able to identify 46 pesticides and six metabolites [Figures 6 and 7] with up to 17 different pesticides in a single sample. On average, samples contained five pesticides, and only three samples had no pesticides.

According to the report, “Some samples had multiple pesticide residues including insecticides from several chemical classes in combination with fungicides and less commonly with herbicides.” Such findings raise questions about acute and chronic exposures of individual chemicals as well as synergistic effects among the chemicals.



**Figure 6. Pesticide classes and types of compounds detected in 108 pollen samples from Pennsylvania in 2007. (Frazier et al. 2008).**



**Figure 7. Most frequently detected pesticides in bee bread trapped at hive entrances (Frazier et al. 2008).**

## **Pollen Loads in Connecticut**

As part of a USDA-CAP study (Coordinated Agricultural Project), a number of researchers are currently looking at the “medical records” of 420 colonies of honey bees in seven states. Brian Eitzer of the Connecticut Agricultural Experiment Station is responsible for analyzing the pollen collected by the other researchers. His protocol, which uses high performance liquid chromatography/mass spectrometry, allows him to test for 140 different pesticides in the parts per billion range. So far he has analyzed 29 pollen samples from stationery (non-migratory) hives and has detected 32 different pesticides or metabolites, which included 14 insecticides, 1 pesticide metabolite, 9 fungicides, and 8 herbicides. The pollen samples contained an average of 4.1 pesticides each—usually in the <1 to 30 ppb range, although some were much higher (Eitzer et al. 2010, Spivak 2010). Only one sample was found to contain an acute lethal dose (LD<sub>50</sub>) of any pesticide. That sample contained imidacloprid at 123% of the LD<sub>50</sub> for an adult bee (Stoner and Eitzer, 2010).

## **Pollen Loads Across North America**

Mullin et al. (2010) analyzed 350 pollen samples collected from 23 states and one Canadian province in 2007-2008. Samples were tested for a total of 200 pesticides and metabolites using a variety of chemical analysis techniques that could detect chemical presence in the parts per billion (ppb) range. Collectively the 350 pollen samples tested positive for 98 different pesticides and metabolites, with a maximum of 31 different pesticides in one sample. The samples averaged 7.1 pesticides each.

Levels of chlorothalonil the authors described as “unprecedented” reached as high as 99 parts per million (ppm). Other ppm levels were found for aldicarb, captan, carbaryl,

myclobutanil, pendimethalin, fluvalinate, and coumaphos. The acaricide fluvalinate was found in 88.3% of the samples, and coumaphos was found in 75.1%. Nearly 92% of all samples contained multiple pesticide residues.

### **Summary: Contamination Frequency**

There is no dispute that pesticides are found in virtually all pollen samples taken from agricultural areas in the U.S. and France. In the four studies cited (Chauzat et al. 2006, Frazier 2009, Eitzer 2009, and Mullin et al. 2010) the respective authors found a range of 2 to 7 pesticides per pollen sample with multiple chemical residues in 56-92% of the samples. The chemicals detected included insecticides, acaricides, fungicides, nematicides, insect growth regulators and various metabolites. Frazier et al. (2009) found 17 pesticides in once sample, while Mullin et al. (2010) found 31 in a single sample.

Although only one of the pollen samples contained a lethal dose of any one chemical for adult bees ( $LD_{50}$ ), the possibility for sublethal chronic effects, and lethal or sublethal synergistic effects, exists in nearly all the samples. In Mullin et al. (2010), for example, 10 different pesticides were found in pollen at greater than 1/10 the  $LD_{50}$  for each one. Even though the average number of pesticides was only 7 in the Mullin study, they state, “As pollen is the main protein source for developing brood . . . surviving on pollen with an average of 7 different pesticides seems likely to have consequences” (2010, p. 15).

### **How Different Types of Pesticides Affect Larvae**

There is an overwhelming consensus that honey bee brood is affected, not just by insecticides but also by a host of other pesticides, including acaricides, herbicides, fungicides, and insect growth regulators. In the past, honey bees were generally

considered safe from many of these chemicals, especially the fungicides and herbicides. But the research shows that not only are these chemicals harmful by themselves, but due to synergistic effects, they may be particularly potent when used together.

## **Pathways of Pesticide Exposure**

Honey bee larvae are exposed to harmful chemicals via two major pathways—in-hive acaricides used by beekeepers to control mites and out-of-hive agricultural pesticides used by growers to control insects, fungus, and weeds (Chauzat et al. 2009). The acaricides are placed directly in or above the brood chambers in honey bee hives. The agricultural pesticides—if they do not kill or disorient the foraging bees immediately—are brought back to the hive in contaminated nectar, water, and propolis, but most significantly, pollen.

Because pollen grains are relatively high in lipids, ranging from 1-10% by weight (Bogdonov 2004), they become excellent storage vehicles for pesticides which are often lipophilic. In addition, pesticides are often mixed with adjuvants such as solvents, emulsifiers, stickers, and spreaders, which may be separately toxic in their own right (Oliver 2009). How much, if any, of the adjuvants make it into the lipids of the pollen is unclear, but the pesticides themselves appear in most modern pollen samples in agricultural North America (Mullin et al. 2010).

Pollen can transmit pesticides to bees by both contact (dermal) and oral exposure. Besides stuffing large amounts of pollen into their corbiculae, honey bees often become heavily coated with the sticky grains [Figure 8]. Any residue on the pollen has the potential to enter the bee trans-dermally. Multiple exposures can build up as the foragers take one trip after another into treated fields and orchards. Oral exposure occurs when the

nurse bees consume the pollen themselves, and feed bee bread to other member of the colony.



**Figure 8. Bee covered in pollen. Flickr photo by Louse Docker. Used with permission.**

### **Pesticides Used in the Hive**

Since *Varroa* mites first came to U.S shores in 1986, a variety of miticides have been used inside the hive to control them. This is a tricky process since too much acaricide is usually toxic to insects as well as arachnids (Jacobsen 2008).

One of the surprises shared by researchers was the vast amount of beekeeper-introduced acaricides in the hives. While this is not introduced via pollen, it is virtually impossible to separate its influence on the honey bee brood because, once pollen is introduced into the hive and stored as bee bread, it absorbs the acaricides from the surrounding comb (Lodesani et al. 2003). Ultimately the pollen becomes the delivery route for acaricides reaching the bee brood.

**Commercial Acaricides:** The two most common acaricides—fluvalinate and coumaphos—are impregnated into plastic strips that the beekeeper places between the frames of the hive. The formulations are designed to kill the mites without killing the bees. However, during the short history of their use, several problems developed. Mite

resistance to fluvalinate was first noticed in 1998 and spread quickly across the country (Sammataro and Avitabile 2005). Its successor, coumaphos, produced resistant mites just three years after it received an emergency registration from the EPA (Pettis and Jadczyk 2005). Left without an effective way of controlling mites, beekeepers began using off-label products, often killing their bees in the process (Oliver 2009).

Fluvalinate and coumaphos have been found to persist in the hive—especially in wax combs—for long periods of time (Chauzat and Faucon 2007). Fluvalinate, for example, has a half-life of about five years (check Bogdanov 2004). If a beekeeper applies new strips of fluvalinate every year without replacing his comb, the bees are soon exposed to unacceptably high doses.

Recent research conducted in Georgia and South Carolina compared the viability of hives treated with the common in-hive acaricides fluvalinate, coumaphos, and amitraz with untreated hives. The untreated hives had higher brood viability, greater homing ability of adult bees, greater foraging rates, and a lower incidence of queen supersedure<sup>6</sup>. The number of mites in the untreated hives was not significantly different from the treated hives (Alaux and Berry 2010).

Although the incidence of acaricide detection in bee bread was not surprising to the pollen researchers, the amounts detected were “startling”. The Pennsylvania study found fluvalinate in 70% of the pollen samples (Frazier et al. 2008). Mullin et al. (2010)

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<sup>6</sup> Supersedure is a process in which the worker bees in a colony raise a new queen to replace the current queen. It often occurs when the bees sense that something is wrong with the queen, such as when she has a physical injury, illness, or a depressed laying rate. However supersedure may also occur when something is wrong with the colony that is not caused by the queen, but is perceived to be caused by her. Examples can be excessive disruptions to the hive (such as beekeeper interference) or elevated levels of some chemical toxins.

found fluvalinate in 88.3% of the samples, and coumaphos in 75.1%. Concentrations in the latter study reached 2670 ppb for fluvalinate and 5828 ppb for coumaphos.

Since these mite treatments are applied inside the hive, the researchers believe acaricides are moving from contaminated comb into the pollen. Unfortunately, these “unprecedented” (p.4) levels are being found in hives where the chemicals were used according to manufacturer’s directions. Mite treatments are applied one or two times annually. But until recently no one knew the chemicals were accumulating in the comb, or migrating into fresh bee bread. Many of the pesticides—including the acaricides—are relatively stable in the dark confines of the hive. With new material being added every six months and the old material persisting, the ambient levels continue to rise.

**“Natural” Acaricides:** As an alternative to commercial acaricides, a number of naturally-occurring organic acids have also been used as mite controls, but even they have been found to produce toxic effects. Gregorc et al. (2004) tested for cell death in larvae treated with both formic and oxalic acids, two of the most commonly used organic acids for the control of *Varroa* mites. In both 3-day old and 5-day old larvae, cell death occurred in 25% of the mid-gut epithelial cells after five hours of treatment with oxalic acid. After 21 hours, cell death increased to 70%, with the 5-day old larvae showing more damage than the younger ones. Increased cell death eventually led to the death of the entire organism. Although these treatments are usually used at the end of the summer when little brood is present, their use nevertheless places a severe stress on the colony, often killing any remaining larvae.



## **Pesticides Used Outside the Hive**

Honey bees are exposed to a tremendous variety of pesticides, both from agricultural crops and from residential applications. The formulations include insecticides, herbicides, fungicides, insect growth regulators, and transgenic plants.

**Insecticides:** It should not be surprising that insecticides are toxic to honey bees at very low levels of exposure. Honey bees, after all, are insects. It is unreasonable to assume that we can kill all the unwanted insects and preserve the beneficial ones by slight manipulations of dose and timing. Many researchers have discovered that honey bee brood exposed to low doses of insecticide exhibit a variety of sublethal symptoms that may affect colony survival (De Wail et al 1995, Kadar and Faucon 2006, Morandin 2005, Ping-Li Dai 2010, Tasei et al. 1988, Tesoriero et al. 2003).

Morphogenic effects such as wing malformations, small size, crippled legs, and stunted bodies have been shown in adult honey bees that were exposed to realistic doses of dimethoate, malathion, carbaryl, and captan (fungicide) during the larval stages of their development (Atkins and Kellum 1986). Although not “lethal,” these handicaps so severely affect the performance of adults that the survival of the colony is called into question (Thompson 2003).

Research on related Hymenoptera larvae has also shown adverse response to pesticides. Tesoriero et al. (2003) tested three pesticides on larval instars of *Osmia cornuta*, an efficient pollinator of pears. They tested various concentrations of three preparations that are commonly used in pear orchards in Italy. Two are fungicides, kresoxim-methyl and copper oxychloride, and the third is an insecticide certified for use by organic farmers. The insecticide, quassin, is an extract of a small tropical tree, *Quassia*

*amara* L. (Simaroubaceae) commonly known as bitterwood. It is used against the larvae of *Hoplocampa* spp., the pear-fruit sawfly. Both *Osmia* and *Hoplocampa* are in the order Hymenoptera.

Not surprisingly, the insecticide quassin resulted in a mortality rate in *Osmia cornuta* of 82.8% ( $p < 0.05$ ). The copper oxychloride yielded 44.8% mortality, and the kresoxim-methyl yielded 13.2%. The untreated control showed 10% mortality. Although their results are preliminary, the authors recommended that treatment of pear trees should be limited to the kresoxim-methyl fungicide in those orchards where the long-term establishment of *Osmia* populations is desirable. Quassin is unsuitable for use in the presence of *Osmia* (Tesoriero et al. 2003).

A laboratory study by Tasei et al. (2000) using small bumble bee colonies showed that a diet that included imidacloprid significantly reduced brood production at 10  $\mu\text{g}/\text{kg}$  active ingredient in syrup and 6  $\mu\text{g}/\text{kg}$  active ingredient in pollen.

**Herbicides:** Although herbicides are often considered harmless to honey bees, research shows otherwise. For example, Papaefthimiou et al. (2002) found cell death in the isolated atria of the honey bee heart (*Apis mellifera macedonica*) after exposure to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). Only 1  $\mu\text{M}$  (micro mol) of 2,4-D was required to reduce the force and frequency of heart contractions by 70% in 20 minutes. The honey bee is much more sensitive to this chemical than other insects tested, including the beetle *Tenebrio molitor*, which required more than 1000  $\mu\text{M}$  of 2,4-D to produce the same result.

In an earlier study, ten different herbicides were fed to honey bees in a 60% sucrose solution to determine their effects on brood production. Picloram, 2,3,6-TBA,

and dicamba had no adverse effects at 1000 ppm, but chloramben and dalapon caused a reduction in brood development, and 2,4-D, 2,4,5-T, silvex, 2,4-DB, and EPTC severely reduced or eliminated brood production (Morton and Moffett 1972).

**Fungicides:** As mentioned by Frazier et al. (2008), pesticides don't have to target insects to be lethal. Fungicides, which are often freely applied to flowering crops, are a case in point. Ruben Alarcon of the Tucson Bee Lab reported that "research has shown that feeding larval honeybees pollen contaminated with fungicides can lead to increased mortality. Exposure to pollen containing captan, ziram, or iprodione led to 100 percent mortality of larvae."

Alarcon explained that "high levels of fungicides in stored pollen might also inhibit the growth of certain strains of fungus that are necessary to convert pollen into bee bread. The loss of the fungus could reduce the nutritional value of the pollen to bees" (Oliver 2010b). In addition, fungicides may disrupt the lactic acid bacteria. Without the lactic acid the beneficial microbes produce, other microorganisms may decompose the bee bread such that it becomes useless or harmful to the bees (Vasquez and Olofsson 2009).

**Reversible Conjugation of Fungicides Makes Them Invisible:** Back in the 1990s, long before much attention was paid to the question of pesticides in pollen, Kubik et al. (1999) made a prescient recommendation:

. . . great attention must be paid to the determination of residues of pesticides in pollen. Pesticide residues may be present in conjugated form difficult to detect with standard procedures, which may result in a gross underestimation of contamination (530).

In that paper, the authors searched for residues of fungicides (vinclozolin, iprodione, and methyl tiophanate) in bee products collected from treated cherry trees. Fungicides are generally considered safe for bees and are often sprayed on the trees during bloom. Using high-resolution liquid chromatography (HRLC) and gas liquid chromatography (GLC), the authors determined the levels of residue in nectar, pollen, and bee bread for each of the fungicides. They found contamination of honey at 0.0589, 0.107, and 0.0231 mg/kg for methyl tiophanate, vinclozolin and iprodione, respectively. Contamination of pollen was found at 0.25, 0.12, and 0.009 mg/kg. But, surprisingly, contamination of bee bread was 1.929, 23.6, 3.055 mg/kg—levels that are 200 times that of plain cherry pollen for vinclozolin and 300 times plain cherry pollen for iprodione.

To explain this anomaly, Kubik et al. suggest that some pesticide residues may become covalently conjugated within the pollen grain. A conjugate of this type could not be extracted using the standard preparation procedures or, even if it were extracted, it would have different chromatographic properties and not look like a pesticide. Hence, readings for the residue would be low. However, during the fermentation of bee bread, the conjugate could be broken down and the pesticide released into the bee bread. This would account for the very large amounts of residue found in bee bread. Corroborating evidence for this theory was discovered when the authors measured the vinclozolin residues in stored honey. Vinclozolin concentrations increased steadily during the first six months of storage and then dropped. Since the honey was no longer exposed to pesticides, reversible conjugation seems to be a reasonable explanation for this phenomenon.

**Entombed Pollen:** A completely new pollen phenomenon was discovered during the summer of 2008 by researchers looking into the causes of Colony Collapse Disorder (VanEngelsdorp et al. 2009). The new syndrome, named “entombed pollen,” is highly associated with colony mortality, although no causal relationship between entombed pollen and colony mortality has been found.

Entombed pollen takes the form of capped and sunken cells of pollen (bee bread) scattered amidst “normal” uncapped pollen cells within a hive, and the pollen in these cells is often a deep, brick-red color. In a healthy hive, brood cells in the pupal stage and honey are the only things that are covered with wax cappings—not bee bread. And whereas normal pollen will fluoresce under ultraviolet light, entombed pollen will not (VanEngelsdorp et al. 2009).

The brick red color of entombed pollen is sometimes found throughout the cell contents, while at other times it is found only in the top layers. A third type of cell, referred to as “capped pollen” contained normally-colored pollen that was capped. VanEngelsdorp et al. conducted melting point tests on the cappings and found that the cappings of entombed (red) pollen were most often composed of propolis, but sometimes contained beeswax as well. The cappings on normally-colored capped pollen was formed of propolis only.

A variety of diagnostic tests were performed on the entombed pollen, including pesticide levels. Although thirty different pesticides and metabolites were found in the samples, the most striking finding was that the fungicide chlorothalonil was found in 100 percent of the entombed pollen samples, but in only 45.5 percent of the normal pollen

samples. However, the amounts of chlorothalonil were approximately 40-times higher in the entombed pollen than the normal pollen.

VanEngelsdorp et al. then fed the entombed pollen to larval honey bees to see if it caused adverse effects on their health and development. To conduct the test, a mixture of royal jelly, fructose, glucose and either entombed pollen or normal pollen was fed to larvae that had been reared *in vitro*. Although the survival rate of those eating entombed pollen was less (13/24), it did not differ significantly from those fed a normal diet (35/48). A one-tailed Fisher's exact test resulted in  $P = 0.09$ .

Although this was the first and only research to date on entombed pollen, it is clearly a situation that requires further study. In a healthy hive, it is not uncommon to find a hive invader (such as a mouse or snake) killed and covered in beeswax and propolis. These waxy coatings contain antibacterial phytochemicals that protect hive members from the toxins of decomposition. It is possible that the bees, sensing the high-level of chlorothalonil in the pollen, try to cover it so it doesn't poison the hive. Why they would collect it in the first place is unclear, unless the fungicide metabolizes into a product more poisonous to the bees than the original product. Based on work performed by Chaves et al. (2008) that explained the behavior of chlorothalonil in soil and water, VanEngelsdorp et al. speculate that the highly reactive quality of the metabolites of chlorothalonil may cause the brick-red color in the pollen.

**Fungicides are the Major Contaminant of Pollen:** Similarly, fungicides were found in all the pollen studies at very high levels. In fact, fungicides made up the bulk (by weight) of all pesticides found in pollen. This is not at all surprising because fungicides are considered safe for bees by the EPA, and are even allowed to be sprayed during

flowering. One fungicide in particular, chlorothalonil, was found in 53% of all the samples (Mullin et al. 2010).

The high frequency of fungicides is particularly troubling because, when tested in vitro, some fungicides have been shown to have a lethal effect on larvae (Frazier et al. 2008), some have synergistic effects with certain insecticides (Pilling & Jepson 1993), and some are suspected of having synergistic effects with other categories of pesticides (Thompson & Wilkins 2003).

**Insect Growth Regulators:** Insect growth regulators (IGRs) have been used as pesticides for about 35 years. They differ from other pesticides in that they are not poisons but synthetic compounds that mimic actual insect hormones. Used in the right quantities at the right stages of growth, they can interfere with various developmental processes such as molting or cuticle formation (Tasei 2001). Although IGRs are generally considered safe for adult bees, both *Apis* and non-*Apis*, the brood is highly susceptible. IGRs are delivered to the larvae by adult bees via contaminated pollen and nectar (Davis 1989; Tasei et al. 1988).

**IGRs and Bumble Bees:** DeWael et al. (1995) tested several IGRs on the brood of bumble bees. The IGRs in question—fenoxycarb, pyriproxifen, and teflubenzuron—are important in the control of greenhouse pests, including the white fly. Since bumble bees (*Bombus terrestris*) are raised specifically to pollinate greenhouse crops, the toxicity of IGRs to bumble bees is an important consideration. Wael et al. used photographs, taken every day for six weeks, to examine egg development and larval mortality inside bumble bee nests where the adults had been fed sugar solutions containing IGRs at various concentrations.

The authors found no change in brood development except in the case of teflubenzuron, which was previously known to be toxic to bumble bees. All the brood raised by adults receiving the fenoxycarb and pyriproxifen developed normally according to the photographs. The authors concluded that “During 28 days after administration of an overdose of pyriproxifen and fenoxycarb no effect on bumble bee brood development was detected, so that the products seem safe for bumble bee colonies used for pollination in greenhouses. If no effect is observed, the substance is considered to be safe for bumble bee brood.” In this study, however, there is no mention of sublethal effects and no follow-up on the next generation of bumble bees to see if they function normally or if they reproduce. Since “detection” was limited to observations from the photographs, the authors could not consider behavioral, reproductive, or other sublethal impacts.

**Different Genera React in Different Ways to IGRs:** Fenoxycarb, a juvenile hormone mimic, fed at a rate of 100mg/L caused the death of nearly all honey bee larvae, and those that survived were deformed (Van der Steen and de Ruijter 1990). On the other hand, fenoxycarb had no significant effect on bumble bee brood (Tasei 2001). Diflubenzuron, a chitin inhibitor, had the opposite effect, damaging bumble bee brood at standard application rates but sparing honey bee brood.

In both species of insect, the growth regulators caused abnormalities in eggs, larvae, and/or pupae, depending on the compound used. In honey bee colonies, dead or malformed individuals were typically removed from the hive by adult workers. Some products produced typical malformations. For example, fenoxycarb produced pupae with atrophied wings, flat or shortened abdomens, and white or red rims around the eyes (Van



der Steen and de Ruijter 1990). Diflubenzuron reduced the amount of capped and uncapped brood, even though it increased the number of eggs (Chandel and Gupta 1992).

Tasei (2001) concluded that the apparent safety of IGRs to pollinating insects is deceiving, because although they have no effect on the foraging population, they have severe effects on the brood. Furthermore, since honey bees and bumble bees reacted in opposite ways to specific IGRs, risk assessment for one species cannot be transferred to another species, even in the same family.

**Transgenic Plants:** Transgenic crops were first introduced into the United States in 1996 and have become a major component of American agriculture (Johnson et al. 2010). In a transgenic organism (also known as a genetically modified organism) some genes from one species are spliced into the chromosomes of another species. This is quite different from traditional plant or animal breeding in which individuals with desirable characteristics are crossed with other individuals having desirable characteristics.

By 2007 three transgenic crops—soybeans, cotton, and corn—were planted on 113 million acres worldwide, mostly in the United States (Ellis 2010). In Canada, most of the 16.6 million acres of oilseed rape is transgenic and the percentage is increasing every year (Clay 2010). Many of these plants are registered as pesticides with the EPA.

There are two major types of genetic modification, both of which have implications for honey bees. One type of transgenic crop is resistant to certain herbicides, and one type is resistant to insects. Some crops, such as cotton, have been modified to resist both (Ellis 2010). Honey bees are regular pollinators of oilseed rape, frequently visit cotton and corn, and occasionally visit soybeans.

**Insect-Resistant Crops:** The insect-resistant genes have been transferred from *Bacillus thuringensis* (Bt), a bacterium that lives in the soil. The introduced genes produce an endotoxin throughout the plant that causes damage to the walls of the gut in susceptible insects. The damaged walls leak their contents into the lumen (interior space) of the gut, causing death of the insect. Researchers fear that Bt toxin in the pollen could damage the adult nurse bee gut or the larval honey bee gut.

Many researchers have looked at the possible effects of Bt plant pollen on bees but, so far, there is no evidence of injury (Babendreier 2005, Apraia 1996). However, because the toxins in the plants are able to kill the larval stages of other insects such as the lepidoptera (moths and butterflies) and coleoptera (beetles, weevils and fireflies), many believe that trouble may lie ahead as other plants—which may produce slightly different toxins or different quantities of toxin—become available. Currently only corn, cotton, potatoes, and tomatoes are commercially available with Bt genes. But in 2008 pre-release field trials were conducted on 30 additional crops, including apples, cranberries, grapes, peanuts, rice, soybeans, poplar, sunflowers, and walnuts (Ellis 2010).

Malone et al. (2004) concluded that the current commercial varieties of genetically modified plants are not adversely affecting honey bees. Their research has measured adult and larval development, survival, food consumption, digestive activity, flight activity, olfactory learning responses, and foraging behavior. They caution, however, that future transgenic traits may affect bees differently.

Embrey et al. (2004) also found no detrimental effects from transgenic sweet corn pollen on honey bees in laboratory tests, but warn that the tests may not be appropriate since pollen in the hive is predigested by nurse bees and fed as secretions to larvae, while

the transgenic pollen in their experiment was fed directly to the larvae and did not go through the nurse bees first. They caution that their experiment did not fully replicate field conditions, and so may have achieved skewed results.

**Herbicide-Resistant Crops:** The herbicide-resistant crops have a gene that resists glyphosate (Round-Up). This gene, too, was isolated from a bacterium. While the gene itself seems to have no adverse effect on insects, the application of glyphosate eliminates all the plant life except the resistant crops. Flowering weeds within the crops, as well as those in ditches, borders, paths, and irrigation canals are all killed, leaving the bees a very poor diet of only one flowering species (Ellis 2010). Many believe that these monoculture diets are a major factor in honey bee decline (Eischen and Graham 2008).

## **Toxicity of Pesticide-Contaminated Pollen to Brood**

### **Contact vs. Systemic Pesticides**

A major change in agricultural chemicals has been the recent shift to the neonicotinoid and phenylpyrazole pesticides, both of which are systemic poisons that move through all parts of the plant. They can be used as seed, soil, or foliage treatments, depending on the formulation. Currently, they are applied to a wide variety of crops in the United States, including grains, vegetables, turf, and ornamental species.

**Imidacloprid as a Contact Poison:** In 2004 Iwasa et al. working at North Carolina State University, determined the LD<sub>50</sub> concentrations for several insecticides applied topically to adult honey bees in the laboratory. Of seven neonicotinoids tested, they found that imidacloprid was most toxic at 17.9 ng/bee. Clothianidan and thiamethoxam were close behind at 21.8 and 29.9 ng/bee, respectively. These were

followed by dinotefuran (75.0 ng/bee) and nitenpyram (138 ng/bee). Acetamiprid and thiacloprid, which have slightly different chemical structures, were much less toxic to bees.

**Imidacloprid as a Systemic Poison:** In a study using maize plants treated with Gaucho (a commercial formulation of imidacloprid), Bonmatin et al. (2005) analyzed the leaves, panicles, and pollen for levels of the chemical. Of the 47 pollen samples taken directly from plants in the field, 38% of the samples had concentrations between 0.3 and 1 µg/kg, 45% had concentrations between 1 and 10 µg/kg, and 4% had concentrations above 10 µg/kg, with a maximum of 18 µg/kg. The imidacloprid in the study was present at an average level of 2.1 µg/kg with a standard deviation of 2.7 (reflecting the heterogeneity of the samples.)

Bee-collected pollen samples were also analyzed for contamination. Using standard pollen traps, pollen pellets were collected from two hives placed at the perimeter of the treated fields. Of the 11 samples 45.5% contained less than 0.3 µg/kg imidacloprid, 18% ranged from 0.3 to 1 µg/kg, and 36% contained between 1 and 10 µg/kg. Of the total number of samples, 54% contained significant amounts of chemical, and the average level was 0.6 µg/kg. The bee-collected pollen comprised many pollen types—not just maize—accounting for the 3-fold lower concentrations of imidacloprid.

The concentrations found in this study were comparable to those found in other crops. The field-collected pollen of sunflowers treated with Gaucho has been found to contain 3 µg/kg imidacloprid (Bonmatin et al. 2003), and the pollen of seed-treated rape was found to contain 4.4-7.6 µg/kg (Scott-Dupree and Spivak 2001).

## **Lethal (acute) vs. Sublethal (chronic) Effects**

Traditionally, the measurement of pesticide toxicity to arthropods has been determined by measuring the median lethal dose (LD<sub>50</sub>) or median lethal concentration (LC<sub>50</sub>) for an adult organism. In the last few years, however, more and more attention has focused on sublethal toxicity. Desneux et al. (2007) define a sublethal effect as “an effect—either physiological or behavioral—on individuals that survive an exposure to a pesticide” (p. 82).

Sublethal effects can be temporary or permanent. Furthermore, they may affect multiple stages of the life cycle, not just the adults. Over 20 years ago Haynes (1988) pointed out that, “The assumption that a colony of honey bees is healthy simply because no increase in mortality is noted immediately after exposure to an insecticide may not be valid” (p. 150).

This broader view of toxicity is vital for several reasons. A sublethal dose, while not killing the organism outright, can have deleterious effects on both the physiology and behavior of individuals that can show up immediately or much later. In the case of honey bees and other social arthropods, these changes not only affect the individual, but can potentially compromise the entire colony. Adult honey bees exposed to sublethal effects of imidacloprid, for example, often appear intoxicated and have difficulty returning to their hive.

As a case in point (Bonmatin et al. 2005) demonstrated that although acute levels of imidacloprid are seldom carried back to the hive in pollen, the chance for chronic and sublethal exposure to brood is significant—even when contaminated pollen is mixed with clean pollen. They write, “At this point, it appears that imidacloprid levels measured in

maize pollen is one of the major factors contributing to the weakening of bee colonies” (p. 5339). This conclusion derived from their calculations with no consideration of the imidacloprid that may be accumulating from other sources, such as contaminated nectar and water, nor does it consider contamination from other pesticides which may be found in the pollen, nectar, or water. In addition, synergistic effects from multiple pesticides are not considered.

According to Rortais et al. (2005), “Such impacts might affect honeybees by disrupting their cognitive capacities (i.e. the learning and orientation abilities) and behaviors (i.e. the collection of food). In such condition, a forager might not be able to return to the hive and, as it relies on the colony for its survival, might die within a few hours. Therefore, the initial sublethal effect might eventually become lethal to honey bees.” Furthermore, pesticides at sublethal levels have been shown to suppress the honey bee immune system (Frazier et al. 2008). And, according to Peters et al. (2010), minute amounts of pesticide in the parts per billion range can cause morphological changes, immune deficiencies, heart deformities, and reproductive abnormalities.

**Sublethal Effects of Imidacloprid:** The insecticide imidacloprid is extremely toxic and it is often regarded as one of the main threats to honey bee populations. It is effective against a broad range of insects and can be applied in ways that reduce wind-borne cross-contamination, such as soil treatment and seed dressing. It is frequently used in rice, maize, sunflowers, rape, potatoes, sugar beets, and many vegetables and fruits (Bonmatin et al. 2005). It is registered for use on 140 crops in 100 nations, making it one of the best-selling pesticides in the world (Jacobsen 2008).

Beginning in 1995 beekeepers in France noticed increased mortality of bees working in fields of maize, rape, and sunflowers (Comité Scientifique et Technique 2003), a phenomenon that spurred in-depth research into the effect of imidacloprid on honey bees. Since then, a number of nations have placed restrictions on its use or have banned it altogether in certain crops (Suchail et al. 2003).

Several types of imidacloprid toxicity have been described. *Acute* toxicity (LD<sub>50</sub>) has been measured at concentrations from 3.7 to 40 µg/kg. Mortality of 50% can also be achieved by *chronic* exposure to imidacloprid at 0.1 to 10 µg/kg for 10 days. *Sublethal* toxicity has been observed beginning at 1 µg/kg in an adult bee. The ranges are due to variations in treatment and measurement protocols and natural variability in honey bee populations (Bonmatin et al. 2005).

For example, imidacloprid is used as a foliar spray or a seed treatment on a variety of crops that includes corn, canola, blueberries, and sunflowers. Usage labels require the bees to be protected from the foliar spray, even though it has been found to be more toxic to honey bees when ingested than when exposed by physical contact (Suchail et al. 2000). A similar product, clothianidan, is licensed in Canada for use as a seed treatment for these same crops (Abbott et al. 2008). Both of these products persist well in the soil and move freely through it, which means plants readily absorb them into the root system. These products are in the pollen of corn, canola, and sunflowers at about 8 ppb. And although some experiments show that adult honey bees can tolerate 10-20 ppb, sublethal effects in larvae at much lower levels (Rortais et al. 2005).

**Sublethal Effects of Carbofuran and Dimethoate:** Davis et al. (1988) found that the systemic insecticides carbofuran and dimethoate affected larval development at

concentrations that were sublethal to adults. In their experiments, pre-measured concentrations of the insecticides were mixed with royal jelly and fed to larvae at various life stages. They found that although adults appeared to be unaffected by carbofuran at 1.25 µg/g royal jelly, dosages as low as 0.625 µg/g caused mature larval weights to be significantly lower than the controls. At 1.25 µg/g the number of potentially viable pupae was also lower than the controls.

Honey bee larvae exposed at an early age (44 hours) to low doses of dimethoate showed a reduced number of viable pupae at concentration as low as 0.313 µg/g. Larvae fed 1.25 µg/g dimethoate often matured sooner and larger than the controls, but many of these failed to spin a cocoon. Some lost their characteristic C-shape in the cell and, instead, were found stretched out straight. The failure to spin cocoons led to speculation that dimethoate may interfere with normal metabolism.

Based on their finding for these two insecticides, the authors concluded that “long-term exposure of honeybee larvae to insecticide-contaminated diets at concentrations not immediately lethal to worker adults may cause significant hidden damage to colonies” (146).

**Sublethal Effects Occur Even at Prescribed Application Rates:** Using colonies of carniolan honey bees in Slovenia, Škerl et al. (2009) measured residues of pesticide in both bee bread and pollen loads. The hives were located in apple orchards treated with two insecticides (diazinon and thiacloprid) and one fungicide (difenoconazole) *in accordance with government-specified application rates and times*. The pollen, taken from inside the hives, contained residues of all three treatments in both the pollen loads and the bee bread at from 1 to 18 days after treatment. Although the detected levels were



below the LD<sub>50</sub> for adult honey bees, and no dead bees were seen at the hive entrance, the chemicals were present at levels that could produce sublethal effects on brood and colony development.

Škerl et al. concluded that, even when orchardists use these crop protection products in full accordance with government regulation, contamination of pollen occurs. And even though the contamination rates are below the specified LD<sub>50</sub>s for adult bees, the tainted pollen is stored in the hive and used by nurse bees to feed the brood. They suggest that studies be conducted to determine the effects these sublethal doses may have on honey bee larval tissue.

In a more recent study, Ping-Li Dai et al. (2010) examined the sublethal effects of two pyrethroids—bifenthrin and deltamethrin—on the growth and development of *Apis mellifera ligustica*, the most common subspecies of honey bee in the United States. The pyrethroids are synthetic forms of pyrethrin, the insecticide derived from certain chrysanthemums. They are potent neurotoxins which typically cause paralysis in the target organisms. Pyrethroids are problematic because they are widely available in both commercial and consumer formulations, and because they are often considered safe and natural alternatives to the organophosphates.

Ping-Li Dai and his colleagues used sublethal doses in their study. *The dosages were at or below the concentrations honey bees would typically encounter when pollinating crops treated with these chemicals.* Nevertheless, they found that queens in the exposed colonies didn't lay as many eggs, the number of eggs that hatched was far fewer, and the number of hatchlings that made it to adulthood was even fewer. As an example, queens in the control group laid 1,000 eggs per day, while queens exposed to

bifenthrin laid about 900 eggs per day, and queens exposed to deltamethrin laid only 600 eggs per day. Of the exposed eggs, fewer metamorphosed into viable adults.

Several studies indicated that the neonicotinoid insecticides are found in pollen at levels that affect learning and cognition in bees (Chauzat et al. 2006, Halm et al. 2006, Desneux et al. 2007). Since these sublethal levels are substantially below the regulatory adult LD<sub>50</sub>s for these chemicals, spraying at these levels is not prohibited by the EPA.

**Sublethal Effects on Different Categories of Honey Bees:** Rortais et al. (2005) studied honey bee exposure to systemic insecticides based on the different categories of bees found in the hive. Since the different categories of bees eat differing amounts of pollen and nectar, they wanted to see if some bees were more vulnerable to toxins than others. They divided bees into categories based on high levels of pollen consumption (larvae and nurses) and high levels of nectar consumption (wax-producers, brood attenders, winter bees, and foraging bees.) They chose imidacloprid because much data is available about the concentrations found in seed-treated sunflower and maize, and because its lethal, sublethal, acute, and chronic toxicities to bees have been well documented.

Using a survey of published literature, Rortais et al. (2005) first determined the amount of pollen and nectar consumed by each category of bee. For example, a worker larva will consume a total of 5.4 mg of pollen and 59.4 mg of nectar within the first five days, and nurse bees consume an average of 65 mg of pollen over the course of ten days.

Next they used published data to determine how much imidacloprid is found in the pollen of sunflower and maize plants that have been seed-treated with the chemical, and found that it averaged 3.4 µg/k of pollen. They also looked at the amount of

sunflower and maize pollen in the loads brought back to the hive by the bees and found that during the flowering period of 1 to 1.5 months the pollen from sunflower and maize together made up 80-90% of the total weight of all pollen types collected by the bees.

Using the above data Rortais et al. were able to calculate that worker larvae could consume about 0.3 ng of imidacloprid in the first five days of development, and nurse bees could consume up to 0.2 ng in 10 days. The lethal dose of imidacloprid ranges from a few pg to about 3.7 ng, depending on the age and condition of the bee (Suchail et al. 2001, Schmuck et al. 2001), but sublethal effects are suspected of occurring at much lower dosages.

Rortais et al. concluded that in agricultural areas where bees are feeding heavily on sunflower and/or maize pollen, bees of different categories are potentially subjected to both lethal and sublethal doses of systemic imidacloprid through contaminated nectar and pollen.

**Sublethal Effects in Other Genera of Bees:** Various studies have shown similar effects on other genera of agriculturally important bee species.

**Sublethal Effects in Bumble Bees:** At Simon Fraser University in British Columbia, Morandin et al. (2005) tested for lethal and sublethal effects of spinosad on bumble bees. Spinosad, a microbial biopesticide, is listed by the EPA as a “reduced risk” application because, although it is active against Diptera, Lepidoptera, Hymenoptera, Thysanoptera, and Siphonaptera, it has little or no effect on other insects, mammals, or other wildlife. Although the EPA states spinosad is highly toxic to honey bees with an LD<sub>50</sub> of 0.0029 µg/bee (Exttoxnet 2009), no information exists about wild pollinators.

Bumble bees have a life cycle that is very similar to honey bees, except that the colony is quite small and the queen is the only bee that overwinters. In the spring the queen lays her eggs on a provision of pollen and nectar and allows them to develop for about five days. Once the eggs hatch, the newly emerged larvae are fed large amounts of pollen and nectar by the queen for about nine days before they enter the pupal stage. Ten days later the adult bees emerge from their cocoons (Morandin et al. 2005).

Morandin et al. fed bumble bees a mixture of spinosad and pollen at four different treatment levels: 0.0 (control), 0.2, 0.8 and 8.0 mg/kg. The lower dosages (0.2 – 0.8 mg/kg) are levels that would normally be found in treated crops. The 8.0 mg/kg dose was used as a “worst-case scenario” such as might be encountered if application guidelines were not followed. Colonies were fed the treated pollen for four weeks during the ten-week study in order to replicate what they might experience in the field.

They found that pollen treated at the 8.0 mg/kg level resulted in colony death. Although the queen continued to lay eggs, most larvae failed to reach the pupal stage. No immediate detrimental consequences to the colonies were noted at the normal treatment levels. However at 0.8 mg/kg worker bees that had been larvae during the treatments were below average weight, and when these bees began to forage they displayed impaired foraging ability. The impaired bees would land on the lip of a flower, tremble slightly, and fall back before entering the flower tube.

#### **Sublethal Effects of Imidacloprid on *Osmia lignaria* and *Megachile***

***rotundata*:** Abbott et al. (2008) tested imidacloprid for lethal and sublethal effects on larval development in the orchard mason bee, *Osmia lignaria*, and clothianidin in the alfalfa leafcutting bee, *Megachile rotundata*. Larvae were exposed to control (0 ppb), low

(3 or 6 ppb), intermediate (30 ppb), and high (300 ppb) doses of both the insecticides. The doses were injected into bee-collected and pre-mixed pollen provisions, and the larvae were monitored for development, emergence, weight, and mortality.

No acute lethal effects were found in either species at any dosage level, and no sublethal effects of clothianidin were found in *M. rotundata* at any dosage level. However, imidacloprid produced statistically significant sublethal effects on the larval development times of *O. lignaria* at the 30 and 300 ppb dosage levels. The total time to reach the last larval stage was greater for both males and females, the time to complete cocoon spinning was greater for males, and both males and females took longer to finish darkening their cocoons. The authors concluded that although these effects are minor, they should be studied further to determine if the increased development times may have further impacts on the adult bees<sup>7</sup>.

#### **A Method for Assessing Environmental Exposure to Systemic Pesticides**

**Shows “Alarming” Risks:** In a 2006 paper, Halm et al. tried adapting a measurement system that is currently used to protect ecosystems in France. Since honey bees—unlike most other species—live in colonies where they depend on each other for survival, Halm et al. compared them to an ecosystem, wherein a number of species are interdependent on one another. They argue that “the functioning of a [beehive] is similar to that of an ecosystem in the sense that each unit (temporal castes in a colony and species in an ecosystem) is essential to sustain the system as a whole” (2448).

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<sup>7</sup> According to Heather Higo at Simon Fraser University (personal communication), she and her colleagues did not test the control pollen for ambient levels of pesticide. It is possible that the “minor” differences they recorded between the controls and the test groups was influenced by unmeasured contaminants in the test pollen. Although their test pollen was not collected from an agricultural area, it could have nevertheless been exposed to pesticide contamination from residential gardens.

The measurement system used a ratio of predicted environmental concentration divided by the predicted no effect concentration (PEC/PNEC). To test this system, Halm et al. used imidacloprid-treated sunflowers and maize. To find the PEC, they collected and analyzed samples directly from flowers in the field. Contaminated sunflower pollen was found to average 3.3 µg/kg imidacloprid, and contaminated maize averaged 3.5 µg/kg imidacloprid. Additionally, contaminated sunflower nectar averaged 1.9 µg/kg. Using previously published data (Rortais et al. 2005), they determined how much pollen and nectar is consumed by different categories of bees. From these data points they were able to establish a PEC (the predicted amount of pesticide that each category of bee was likely to be exposed to.)

For the next step—establishing a PNEC—Halm et al. used existing data from a number of studies. However, since there were no existing data on chronic and sublethal toxicity in larvae, the authors had to derive data obtained from adult workers. They acknowledged that larvae may have more or less susceptibility to these chemicals, and applied assessment factors to help correct for the unknowns. Nevertheless their calculations indicated that all categories of bees have an “alarming” (2451) risk from imidacloprid used at field-recommended levels.

### **Pesticide Metabolites**

The surprising thing about metabolites is they can be many times more toxic than the original formulation, and they are routinely found in pollen (Kadar and Faucon 2006, Nauen et al. 1999, Suchaie et al. 2001).

Suchail et al. (2003) studied the metabolic breakdown of imidacloprid in adult honey bees. They found that although honey bees show almost immediate intoxication

from the ingestion of imidacloprid, mortality is delayed and is correlated with the increase of the imidacloprid metabolites, 5-hydroxyimidacloprid and olefin.

The two metabolites are very similar in molecular structure to imidacloprid, however 5-hydroxyimidacloprid is slightly less toxic and olefin is slightly more toxic than the parent compound. Other researchers (Nauen et al. 1999) have found that olefin is about ten times more toxic than imidacloprid to the cotton whitefly.

In an earlier study, Suchail et al. (2001) found that both the metabolites were equally toxic to honey bees during 10-day chronic toxicity experiments, but the dose needed to produce 50 percent mortality ( $LD_{50}$ ) was 3000 to 100,000 times less than the dose needed to produce an acute  $LD_{50}$ . This could have a significant effect on colony survival because, as the chronically exposed bees continue to function in the period before death, they could easily pass the dosages required for chronic toxicity to other individuals in the hive as well as to the larvae.

Although the half-life of imidacloprid after ingestion is very short—between 4.5 hours and 5 hours—the metabolite presence peaks at about four hours. Honeybee acute mortality begins at about four hours after ingestion and continues to about 96 hours. Chronic toxicity extends for at least 10 days.

Other research, however, has shown that honey bee colonies fed dosages of imidacloprid and other systemic insecticides comparable to the levels found in contaminated hives showed no increase in colony mortality (Nguyen et al. 2009). Further research is needed to answer questions such as whether the metabolites of the insecticides that appear after long-term storage in the hive are more toxic than the raw product, or if

there are synergistic effects between chemicals or between chemicals and other hive products that may be causing honey bee declines.

**Metabolite Summary:** Because of the way larvae are fed, it is difficult to ascertain how much of each pesticide is metabolized by the nurse bees and how much is passed onto the larvae. It is believed that some pesticides, such as imidacloprid, are metabolized quickly by the nurse bees, some kill the nurse bees before they can pass it onto the brood (Oliver, personal communication), and some is channeled to the larvae by way of the jelly. In addition, pesticides can breakdown into metabolites that are just as harmful—or more so—than the original product (Kadar and Faucon 2006, Nauen et al. 1999, Suchaie et al. 2001). If these metabolites are passed on to the brood they have the potential for causing harm. If the chemical in its original configuration is passed on, it may cause harm. Or, if the nurse bees are killed or rendered unable to properly feed the brood, the hive may be lost.

### **Synergistic Effects Between Pesticides**

Unfortunately, multiple pesticides are the rule rather than the exception in modern commercial agriculture. It is not unusual for a crop to be sprayed with one to several of each class of chemical during one growing season. Some are sprayed much more. “Stacking” is a common practice in which farmers mix multiple chemicals into one sprayer to maximize efficiency and reduce costs.

There exists a small but growing body of data that suggests pesticides and their metabolites sometimes have a synergistic effect that make them even more toxic to bees than when they act alone. At this time, very little is known about these interactions and under what conditions they occur. Most of the research cited in this paper looks at single-



pesticide toxicity. That is, the research assumes that one pesticide acting alone is causing the symptoms. It should be noted, however, that the general feeling among researchers is that synergistic effects are real and dangerous and should not be overlooked when making final decisions about the safety of pesticides in the environment (Mullin et al. 2010).

In a study of how in-hive acaricides kill mites but spare honey bees, researchers found that bees have the ability to detoxify three of the commonly used acaricides. When used singly, bees are able to detoxify coumaphos, fenpyroximate, and tau-fluvalinate. But when exposed to a combination of these poisons—as often occurs with re-used comb—bees were far more susceptible to the poisons (Johnson et al. 2010). For example, bees treated with a sublethal dose of coumaphos were 14 times more susceptible to tau-fluvalinate. And bees treated with sublethal doses of fenpyroximate were 5-7 times more susceptible to the other two.

Johnson et al. (2010) also found that when bees were exposed to prochloraz—a common agricultural fungicide—bees were 72 times more susceptible to coumaphos, 23 times more susceptible to fenpyroximate, and 1,118 times more susceptible to tau-fluvalinate. They believe that the class of fungicides that includes prochloraz is able to inhibit the detoxifying enzyme that normally protects the bees from the acaricides.

Although studies on brood are lacking, much research has been done on the synergistic effects of pesticides on adult bees. For example, the physiology of adult bees has been assessed by measuring enzyme activity during and after pesticide exposure (Desneux et al 2007). In one experiment, enzyme disruption by the insecticide deltamethrin caused cardiac contractions. A fungicide, prochloraz, produced a similar but

stronger reaction. Worse, when the two compounds were used together, a synergistic interaction occurred (hypothermia) that did not occur at significant levels with either compound alone (Desneux et al. 2007).

Other synergistic interactions have been recorded, especially between pesticides and fungicides. While fungicides are not normally considered toxic to honey bees, these synergies can cause lethal conditions. One theory is that the fungicides decrease metabolism, detoxification, and excretion—possibly due to the hypothermic effects—which causes the pesticide to become more harmful (Desneux et al. 2007).

These synergistic responses need more investigation, especially in light of the many chemicals and chemical combinations that honey bees routinely encounter. Indeed, the practice of stacking needs to be re-evaluated in the face of the existing studies.

### **Synergistic Effects between Pesticides and Pathogens**

Other studies (Cox-Foster et al. 2007) have demonstrated increased susceptibility to pesticides that exposure to parasites and pathogens can produce.

**Nosema and Pesticides:** Two species of fungi classified as microsporidia, *Nosema apis* and *Nosema ceranae*, are frequently found in honey bee colonies. The organisms live in the honey bee gut and can damage the digestive tract, which makes the bee more susceptible to infection by bacteria and viruses (Watanabe 2008).

However, Alaux et al. (2010) found that the *Nosema* microsporidians and the insecticide imidacloprid caused greater mortality when acting together than either did alone. When they looked at the mechanism of action they found that glucose oxidase activity was not reduced by either agent alone, but in combination the activity was significantly decreased. Glucose oxidase activity enables bees to secrete antiseptics into

both honey and brood food. When those antiseptics are missing, the colonies can succumb to various ailments.

**Varroa destructor and Pesticides:** According to the Dyce Laboratory for Honey Bee Studies at Cornell University, A female *Varroa* mite enters a brood cell 1 to 2 days before it is capped. About 60 hours later, she starts to lay her eggs at a rate of one egg every 24 to 30 hours. While the honey bee is in the pupal stage, the mite eggs hatch and the immature mites bite into the pupa and feed on the hemolymph. Not only do mites weaken the bee directly, they are also vectors for some of the worst honey bee viruses, including acute Israeli paralysis virus and deformed wing virus.

However, a researcher at Washington State University recently found that pesticide contamination in the hive caused a delay in honey bee larval development. Because of the delay, the parasitic *Varroa* mite had time to produce an extra mite in each infested cell (Sorensen 2010). Repeated in thousands of brood cells, this simple delay can cause the *Varroa* population to soar in a very short time.

### **Summary: Larval Sensitivity to Pesticides**

Except for the one study funded by Bayer CropScience (Schmuck et al 2001), all the research reviewed for this paper found that honey bees in agricultural service are routinely exposed to toxic levels of pesticide. Since this paper seeks to discover how contaminated pollen affects the health of the brood, I tried to separate those factors from others—such as contamination from soil, water, and drifting air. However, the complexity of the honey bee life cycle makes this extremely difficult.

The problem of controlled experimentation with honey bee larvae and pollen is mentioned many times in the literature, and, in fact, many of the papers dealt with ways of improving the methods used for studying both the bees and the pollen.

But as difficult as the process is, most of the researchers—each in his or her own way—have come to similar conclusions about the danger of pesticide-contaminated pollen to honey bee brood, and hence to the future practice of bee-pollinated agriculture. Because the methods, the pesticides, the crops, and the bee species differed in each study, it would be meaningless to compare them mathematically. Nevertheless, each of the researchers represented here were able to produce statistically significant results, or results that clearly demand more study (vanEngelsdorp 2009). Some interesting trends revealed themselves throughout this literature review.

Honey bee larvae are particularly sensitive to low levels of pesticide for a number of reasons.

- Since honey bee larvae do not defecate until the end of the larval period, food and metabolic waste must be retained within the organism during this rapid stage of growth. As such, larvae are in physical contact with any insecticides or metabolites in the waste for an extended period of time—much longer than most other bees<sup>8</sup>.
- The extreme rapidity of larval growth requires the ingestion of an average of 70 mg/day of pollen for each 100 mg body weight, while adult bees eat 5 mg/day of pollen for each 100 mg body weight. This means larval exposure to xenobiotics is 35 times greater than adult exposure (Villa et al. 2000).

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<sup>8</sup> An exception is overwintering adults who may hold their feces for weeks if the weather is too cold to permit them to fly.

- The extreme rapidity of larval growth means that harmful mutations are quickly telegraphed throughout the organism and may prove fatal.
- The larval stage is fed from the secretions of nurse bees. Pesticides digested by the nurse bees often form metabolites many times more toxic than the original chemical.
- The larval stage is soft-bodied and has little protection from environmental toxins that may be stored in the wax comb or the bee bread. Toxins may be absorbed transdermally as well as orally.
- Many pesticides are rapidly decomposed by sunlight, but in the dark recesses of the hive, pesticides can persist much longer, perhaps years.

The importance of these findings cannot be overstated. For years the agricultural community has determined lethal levels based on adult bees and treated their crops in accordance with EPA “safe for honey bee” regulatory standards. During this same period, the number of honey bee colonies dropped precipitously. While most researchers do not believe pesticides are the sole cause of colony loss, most think it is an important factor. The published research overwhelmingly supports their conclusions of significant pesticide causation.

Because larval development is essentially invisible to the beekeeper, it is possible that they have been suffering from pesticide contamination for years without it being recognized. But now with increased awareness of the possible problems, and with improved detection techniques, honey bee larvae are getting long-overdue scientific scrutiny.

## **EXOGENOUS VARIABLES**

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When studying honey bee larval development, there are many variables that make determining cause and effect especially difficult. Not only is the agricultural environment complex, but honey bee colonies themselves have been compared to “superorganisms” wherein separate individuals act as if they were merely parts of a single living being. As such, each bee performs in a way that affects the colony—rather than the individual (Tautz 2008). In a contaminated environment, for example, the forager’s selfless act of gathering food for the young may end up poisoning the entire colony—not just the individual. Also, because so many potentially contaminated items are brought into the hive, it is often difficult to determine exactly where the toxics originated. The sections below outline some of the confounding variables that complicate the study of larval development in relation to the pollen supply.

### **Diet**

Honey bees are naturally polylectic which means they forage from many different species. In the natural environment this trait assures that the colony as a whole will be fed a variety of nutrients. However, one of the hallmarks of modern farming is large expanses of monoculture. The placement of hives amid a single crop limits honey bee choice in the selection of pollen and increase the probability of collecting large doses of a contaminant over a short period of time. In addition, some crops do not produce pollen that sufficiently meets the needs of the bees. Since pollen is the only source of protein and lipids in the bee diet, it has to be of high quality in order to sustain life (Chauzat et al. 2009).

## **Pollen Must Contain all the Necessary Nutrients**

In a study conducted at the Carl Hayden Bee Research Center in Tucson, Schmidt et al. (1987) measured the life span of honey bees fed four types of blended pollen and 25 different types of pure pollen. The bees fed a five-pollen mix lived an average of 41 days longer ( $40.6 \pm 0.6$ ) than the control group fed only sugar syrup. Overall, bees fed one of the four blends lived an average of 29 days longer than the control group.

Of the pure pollens, all but three increased the life span of bees over the control group, but none were as effective as the five-pollen mix. Overall, lifespan increased only 19.5 days over the control group. The increase ranged from  $5.4 \pm 0.3$  days for *Haplopappus* to  $38.0 \pm 0.9$  days for *Populus*. It is interesting to note that four of the pollens actually decreased the lifespan of bees compared to the syrup-fed controls. Bees fed *Ambrosia*, *Typha*, and *Kallstroemea* lived from 3.9 to 2.4 days *less* than those receiving no pollen at all.

Schmidt et al. believe that to be of nutritional value to bees, pollen must be collected, have a proper texture and consistency, and be consumed. Once consumed, it must contain the nutrients necessary for bee health, and be non-toxic, digestible, and properly absorbed into the hemolymph. Because an individual pollen source may be more-or-less lacking in any of these areas, these findings suggest that pollen from diverse sources is necessary for good health and longevity of the individual as well as the entire colony.

## **Polyfloral Pollen Boosts the Honey Bee Immune System**

Researchers at the French National Institute for Agricultural Research recently found a correlation between diet diversity (polyfloral pollen) and a healthy immune system in honey bees. In laboratory experiments, the researchers found that bees fed five different types of pollen had higher levels of glucose oxidase than bees that were fed only one type of pollen, even if that one type had higher protein content. As mentioned earlier, glucose oxidase is used by the bees to preserve honey and protect the hive against invading pathogens (Alaux et al. 2010).

Seven types of fresh pollen were collected from local species of *Acer*, *Castanea*, *Cistus*, *Erica*, *Quercus*, *Salix*, and *Taraxacum* and tested for both nitrogen and protein content. Groups of bees were fed one of the pollens or a blend of five of the pollens.

The effects of the different diets were assessed by measuring hemocyte concentration and fat body content. Hemocytes are necessary for the phagocytosis and encapsulation of parasites, while fat bodies provide antimicrobial peptide synthesis. They also analyzed glucose oxidase, which is produced by the hypopharyngeal glands and catalyzes the oxidation of beta-D-glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide is secreted into the larval food and helps with sterilization and preservation (Alaux et al. 2010).

While hemocyte activity did not seem affected by the pollen source, both fat body and glucose oxidase activity were significantly enhanced. The authors concluded that a varied diet yielded more of the amino acids necessary to increase antiseptic protection of the hive (Alaux et al. 2010). Similar results were achieved by Tasei and Aupinel (2008) who were able to grow heavier bumble bees with larvae fed a polyfloral diet than a



monofloral diet. This research is consistent with earlier reports that pollens vary considerably in their composition, and some are deficient in the essential amino acids (Bell et al. 1983).

### **Bees that Consume Polyfloral Pollen Live Longer**

In a similar experiment, Schmidt et al. (2000) fed honey bees either a mixture of 15 pollens from spring-blooming Sonoran desert plants or a monoculture diet of rape, sesame, or sunflower pollen. Control bees were fed only sucrose syrup with no pollen. The 15-pollen mixture was collected from the corbiculae of worker bees during a period of profuse flowering. Since the bees had many options, it was assumed that the pollens in this mixture were the most attractive to bees. The pollens were fed to newly-hatched nurse bees because it is the nurse bees who consume the largest amounts of pollen in the hive.

Compared with the bees fed the 15-pollen mixture, the rape-fed bees ate 73% ( $73.4 \pm 1.3$ ) more pollen and they lived 2.5 times as long as the sucrose-fed controls. The sunflower-fed bees ate 58% ( $58.5 \pm 6.7$ ) as much pollen as the bees eating the 15-pollen mixture but lived only 1.6 times as long as the controls. The bees given sesame pollen ate only 34% ( $34.2 \pm 5.0$ ) as much as the bees eating the 15-pollen mixture and lived 1.7 times longer than the controls.

In a related study Sagili and Breece (2010) measured the protein composition of nurse bee hypopharyngeal glands that were fed either single source pollen or multi-source pollen. They also measured the lipid content and emergence weights of newly hatched bees. They found both hypopharyngeal protein content and colony growth to be significantly lower in the single-source pollen consumers.

Although worker honey bees collect a large variety of pollens and store them in the hive, the nurse bees have definite preferences about what they eat. For example, although *Kallstroemia grandiflora* and *Baccharis sarothroides* are frequently collected, they are seldom consumed (Schmidt et al. 1987). On the other hand, certain species such as *Taraxacum officinale* are readily collected and eaten but, if they are the sole food source, result in poor survival (Schmidt et al. 1987). By having a large variety of pollen on hand, bees increase the probability of achieving a balance of vitamins, minerals, and proteins, and decrease the chances of toxic-pollen poisoning.

### **Monoculture Diets May Be Excessively High in Pesticides**

The French have reported huge winter losses of honey bee colonies that pollinated seed-treated maize and sunflowers during the previous year. Maize does not produce nectar but is a popular pollen plant for honey bees. Sunflowers, which produce both pollen and nectar, are also a favorite of honey bees and usually yield a high-quality honey. In recent years, however, the French report rapidly declining honey crops from sunflowers in addition to subsequent losses of colonies.

Some speculate that poor diet is the culprit. As we have seen, bees raised on monocultures do not collect the variety of pollen that is needed to provide all the proteins necessary for the proper growth of bees. Others believe that it is the bioaccumulation of insecticide in the bodies of larval bees that is causing the problem. At this point there is no conclusive evidence either way, but monocultures exacerbate both of these problems.

In polleniferous crops such as maize and sunflowers, a hive can collect 20 or more kg of pollen from each of these crops in a month, a sum that may represent 80-90% of the total weight of a year's collection. When these crops are treated with one of the

neonicotinoids, such as clothianidan or imidacloprid, a large amount of contamination enters the hive. Rortais et al. (2005) say “When comparing the known toxicity doses for imidacloprid to the estimated amounts of imidacloprid consumed . . . we find out that honey bees are potentially exposed to lethal (acute) and sublethal (chronic) doses.”

## **Guttation**

Guttation is a natural process seen in many vascular plants whereby drops of xylem sap exude from hydathodes at the leaf margins or tips. Honey bees are known to drink this exudate, especially in the early spring before large numbers of nectar-processing flowers are available to foragers.

A problem with this type of water collection occurs in agricultural areas where plants are treated with systemic insecticides. Bees collecting guttation drops can be poisoned by systemic pesticides flowing through the xylem. Worse, sublethal, but potentially harmful, doses of pesticide can be carried back to the hive and fed to the developing larvae by way of the nurses. Researchers are currently trying to determine the type and frequency of damage this may cause to honey bee colonies.

Girolami et al. (2009) performed tests in Italy to see if the guttation drops of corn treated with seed dressings of neonicotinoid pesticides contained enough pesticide to damage bees. The authors used corn seed treated with three different systemic neonicotinoids (imidacloprid, thiamethoxam, and clothianidan) and one systemic phenylpyrazole pesticide (fipronil). The seeds were purchased from the manufacturer with the pesticide already applied and ready for commercial distribution.

Guttation drops were collected from emergence through the first three weeks of growth, after which the guttation rate dropped substantially. For each individual seedling,

the drops were collected and held at 2°C. After 2-3 days half the liquid was sent for analysis and half was used for the bee experiments.

Bees held in captivity were chosen at random and caged with the drops. The bees were monitored constantly and timed from the beginning of drinking until they began to show symptoms. The two symptoms monitored were arching of the abdomen, and paralysis of the wings. Because previous research has shown that wing paralysis is not reversible, the time until death was not recorded.

Their results showed that there was easily enough of all three of the neonicotinoid pesticides in the drops to cause wing paralysis. The mean concentration of imidacloprid was  $47 \pm 9.96$  mg/l. Clothianidan was measured at  $23.3 \pm 4.2$  mg/l, and thiamethoxam was  $11.9 \pm 3.32$ mg/l. Fipronil was not found in the guttation drops (Girolami et al. 2009). Previous work by Yang et al. (2008) has shown that honey bees in the field fail to return home after ingesting imidacloprid at  $\geq 3$  mg/liter.

## **Pesticides in Beeswax**

Another difficulty in measuring the in-hive effects of contaminated pollen is beeswax is a repository for chemical pesticides. Chauzat and Faucon (2007) monitored 125 honey bee colonies in France for the presence of pesticides in wax combs. They searched for 18 specific compounds (16 insecticides/acaricides and 2 fungicides) chosen for their high toxicity to honey bees and their frequent use in field crops. During the two-year study they found 14 of the compounds in wax combs. Tau-fluvalinate was found in 61.9% of the samples, coumaphos in 52.2%, and endosulfan in 23.4%. The other 11 pesticides were found in 2.1 to 21.9% of the samples. The two most commonly found—

tau-fluvalinate and coumaphos—are both acaricides used to control *Varroa* mites in the hive; the third, endosulfan, is an organochlorine insecticide.

A similar study in Canada found tau-fluvalinate in 91.7% of wax samples from brood chambers (Chauzat and Faucon 2007), and a German study found it in 62.5% of commercial foundation wax samples<sup>9</sup> produced in that country (Wallner 1999). Other studies have found high levels of coumaphos in foundation wax (Lodesani et al. 2003, Masr and Wallner 2003).

Because beeswax is a lipid-based substance (Bogdanov et al. 1997; Wallner 1999, Mullin et al. 2010), lipophilic pesticides are easily dissolved into the material. In fact lipophilic pesticides in honey have been found to migrate from the honey into the wax, especially when the water content of the honey is high. Thus, under the right conditions, wax combs act like a sink for hydrophobic compounds such as coumaphos (Tremolada et al. 2004). Furthermore, the findings by Kubik et al. (1999) that bee bread had higher vinclozin residues than pollen could be explained by its close contact with contaminated wax comb. If comb were high in vinclozin, it is possible that the compound moved from one matrix into the other (Chauzat and Faucon 2007).

Honey bees are in physical contact with the comb throughout their lifetimes. Although honey bees don't eat comb, adult bees constantly reshape it as the needs of the colony change (Chauzat and Faucon 2007). This reshaping requires chewing by the bees, and thus further increases their exposure to the toxins contained therein. Honey bee brood is in close contact with the wax combs from egg deposition through the larval and pupal

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<sup>9</sup> Foundation wax is beeswax that is pressed into sheets and embossed with a hexagon design of the size used by bees. The sheets are slotted into frames by beekeepers to speed the process of comb building. Wax for foundation is collected from many hives, melted together, formed, pressed, and sold. Since the wax is collected in the field, it often contains pesticides—especially the acaricides used to control *Varroa* mites.

stages—a period of about 21 days. Although the egg casing and the pupal cocoon probably provide some protection against pesticides, the larval stage is particularly vulnerable because of its direct physical contact with wax comb. Tremolada et al. (2004) have already documented acaricides residues in larvae.

## **Spray Drift**

Vincenzo Girolami, an entomologist at the University of Padua, reported in 2010 (unpublished manuscript) that the seed-planting machines commonly used to sow imidacloprid-treated seeds kick up a cloud of soil dust that can reach 20-50 meters wide. The fine dust—contaminated with pesticide—can drift and float above the field for long periods of time. Girolami has measured the concentrations of imidacloprid in the clouds directly above the machines and found them to be as much as 1,000 times the LD<sub>50</sub> for adult bees. According to Girolami, “Bees that cross the fields, making a trip every ten minutes, have a high probability of encountering this cloud. If they make a trip every five minutes, it is certain that they will encounter this cloud.” He believes that 90% of bee die-offs in areas using the seeding machines may be the result of the direct poisoning of adult bees (Shah 2008).

## **Stress**

During the annual migration to California’s Central Valley, over 80 *billion* bees are trucked from all over North America to pollinate the almond crop. Afterward, many of these bees are moved into other states as far away as Maine to pollinate blueberries, Massachusetts for cranberries, and Washington for apples. Others go to Texas for cotton, North Dakota for clover, and Florida for citrus (Mares 2005).

Bees do not react well to migratory beekeeping. In preparation for their journey, they are often fed large quantities of high fructose corn syrup<sup>10</sup> and dosed with antibiotics and acaricides. Stacked by the hundreds on the back of flat bed trailers, the hives are bounced around on the nation's freeways, parked on hot tarmac, and assailed by loud noise. The bees are unable to remove their dead, collect water or pollen, or ventilate the hives. They often arrive at their destination queenless, apparently having "blamed" the queen for their bad fortune (Mares 2005).

Once they arrive at their destination, they are plunked down in large fields or orchards of monoculture crops along with thousands of other hives. There they must compete for scarce resources of an often protein-limited pollen source. The dense populations of bees easily share parasites and pathogens while they are assailed by the agricultural chemicals applied to the crops (Jacobsen 2008).

Many researchers believe the stress of migratory beekeeping alone is a major factor in the collapse of honey bee colonies (Schacker 2008, Jacobsen 2008, Mares 2005).

## **Pathogens and Parasites**

Other research is focusing on whether pesticides are causing a decline in overall health and immune function that is allowing normally-occurring parasites and pathogens an unprecedented advantage over the honey bee.

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<sup>10</sup> Beekeepers often supplement a colony's diet with high-fructose corn syrup, especially in the early spring when they want to build large populations in time for the pollination season. Recently, researchers have found that when high-fructose corn syrup is heated, even slightly, it produces a compound called hydroxymethylfurfural (HMF) that causes ulceration of the honey bee gut. The more high-fructose corn syrup is heated, the more HMF is formed, and the production increases considerably at around 120°F (LeBlanc et al. 2009).

For example, Wu et al. (2010) studied the growth and development of honey bee brood in pesticide-contaminated comb compared to brood raised in “clean” comb. They found that those raised in the contaminated environment lived an average of four days less than their counterparts. In a separate study they found that the brood raised in the contaminated comb was infected with the microsporidian *Nosema ceranae* at a younger age and at higher infection levels, suggesting that brood exposed to pesticide residues may succumb to more easily to pathogens or parasites in the environment.



## **DISCUSSION OF NEW TECHNOLOGY**

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One of the major problems surrounding the testing of larval bees has been the lack of a viable method. It is difficult or impossible to track individuals in the brood nest— brood cells number in the thousands and they are attended by tens of thousands of workers. In addition, the response to a particular contaminant is difficult to assess because a wide variety of contaminants pre-exist in the wax comb, the honey, the pollen, the water, and the workers who attend to them. Furthermore, previous methods of weighing or measuring an individual larva involved moving it from its cell, a procedure that often damaged or killed the larva or caused it to be expelled from the hive. For these and other reasons, controlled experiments in the field are nearly impossible.

### **Raising Bee Larvae *in Vitro***

Recently, researchers have begun an aggressive search for new and better tools for studying developing larvae. Aupinel et al. (2005) recently developed a method of raising bee brood *in vitro* by grafting first instar larvae into individual plastic cell cups of the type commonly used by queen breeders. Unlike previous methods, larvae can be weighed and monitored without disturbing them from their position. The cell cups can be tared before grafting, manipulated, and monitored all without touching the organism after the initial graft. In addition, Aupinel et al. discovered they could feed the larvae just once per day with a pipette. This schedule produced larvae equivalent in size and health to those raised by worker bees without the interference of the workers. Because worker bees often provide food of unknown origin with unknown levels of contamination, elimination of the workers provides better experimental control.

In their experiments, Aupinel et al. prepared a diet of 50% royal jelly mixed with yeast extract, D-glucose, D-fructose and purified water. The royal jelly was chemically analyzed for 28 common insecticides and 9 fungicides before being mixed into the brood food. This assurance of purity is essential for any experimentation dealing with chemical contaminants in the bee food supply. Lethal and sublethal effects can be assessed by weighing and monitoring individuals into the adult stages. The adults can then be tested for both morphological and behavioral abnormalities.

The *in vitro* method of rearing can be used not only to monitor sublethal doses of pesticide, but to chart the LD<sub>50</sub> levels that are unique to larvae. These additional measurements could better determine whole-colony toxicity than the currently used LD<sub>50</sub> of adult bees. The *in vitro* method can also be used to study the synergistic action that may occur between pesticides, and between pesticides and pathogens. This unique method of rearing is a significant step forward in the study of bee ecotoxicology.

### **Current Studies Using the *in Vitro* Method**

Jamie Ellis at the University of Florida is currently researching the effects of pesticides on honey bee larvae using the *in vitro* method. The pesticides being tested—all routinely found in beehives—include coumaphos and fluvalinate (miticides), chlorothalonil and mycobutanil (fungicides), simazine and glyphosate (herbicides), and chlorpyrifos, imidacloprid, and amitraz (insecticides). Ellis is testing at contamination levels that are commonly found in pollen and beeswax, and assessing individual chemicals as well as combinations (Flottum 2009).

To isolate the larvae, Ellis is transferring them into special containers where they are fed a prescribed diet mixed with varying amounts of pesticides singly or in

combination (University of Florida Institute of Food and Agricultural Sciences 2009).

Although results have not yet been published, preliminary data indicate that many of the chemicals are indeed toxic to the test larvae at levels normally found in beehives (Flottum 2009). When the research is complete Ellis hopes to examine how pesticide exposure at the larval stage affects adult bees, and how those bees react to common bee stressors such as mites and pathogens (University of Florida Institute of Food and Agricultural Sciences 2009).

### **Advanced Detection of Trace Chemicals**

Other scientific developments have been equally important. While many of the lipophilic compounds can be monitored with conventional gas chromatography-mass spectrometry (GC-MS), the more recently developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) is necessary for measuring the systemic pesticides that are only found in the parts-per-billion range in pollen samples. The detection of many of the water-soluble (polar) pesticides and their metabolites also could not be accomplished without the advent of LC-MS/MS (Mullin et al 2010).

In the past, the inability to detect trace amounts of pesticides and their metabolites has limited scientific knowledge of their actions at low concentrations. Fipronil, for example, is a pesticide that has long been suspected of harming bees at sublethal levels in the  $\mu\text{g}/\text{kg}$  range, inhibiting both foraging activity and learning performance (Kadar and Faucon 2006). However, until recently, techniques for measuring this contaminant were not sufficiently sensitive for detecting the trace quantities found in pollen.

In 2006 Kadar and Faucon developed a method for detecting fipronil and its metabolites—fipronil sulfone, fipronil desulfinyl, and fipronil sulfide—down to 0.1

$\mu\text{g}/\text{kg}$  using a pollen test sample of only 5 grams. Although the three-step technique is complex, utilizing liquid chromatography with electrospray ionization-tandem mass spectrometry, it can be adapted to analyzing honey bee adults and larvae, as well as vegetation samples. This is just one of many new developments in chemical analysis that is allowing the study of low-dose concentrations of pesticides on plant and animal life.

## CONCLUSIONS

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Several important conclusions can be drawn from the current primary literature regarding honey bee brood, pesticides in pollen, and the future of American agriculture.

- Honey bees at all stages of development are exposed to multiple chemical residues of pesticides and their metabolites through the collection and consumption of pollen.
- Honey bees at all stages of development—particularly the larvae—experience adverse effects at doses far below the median lethal doses (LD<sub>50</sub>s) established by the EPA.
- Many of the modern pesticides persist in the environment by design. The systemic pesticides in particular are meant to protect a plant throughout the entire growing season. Persistence, especially in the soil, increases the chance of contamination of non-target species.
- Pesticides used in combination can produce synergistic effects which increase their toxicity to honey bees.

The studies have shown that low-level pesticide exposure has detrimental effects, not only on the brood of honey bees, but of bumble bees, mason bees, and leafcutter bees as well. The thresholds for damage vary with the species and the pesticide used but, with one exception (Schmuck et al. 2001),<sup>11</sup> the papers cited revealed unsettling effects to developing larvae of all domestic and wild bees tested.

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<sup>11</sup> This study funded by Bayer CropScience, the manufacturer of imidacloprid, is the only one that found no adverse effects on honey bee colonies from imidacloprid-treated seeds.

Bee pollination is necessary if we are to maintain diversity in the human diet. Our caloric requirements can be met with sufficient quantities of the wind-pollinated cereal grains. However, we also need a regular supply of fruits and vegetables in our diet in order to meet our daily requirement for vitamins, minerals, and phytochemicals. Since many of these fruits and vegetable crops are pollinated by honey bees or various wild pollinators, human health is linked to the health of these organisms.

But while the USDA recommends that every American eat a colorful variety of fruits and vegetables every day, another segment of our government, the EPA, declines to require sufficient testing on the bees which will pollinate them. These policies are at odds with each other: one promotes the proliferation of flowering plant foods while the other may be hastening their demise.

It is true that we cannot simply ban pesticides in a society that has become completely dependent on them. However, by not mandating complete testing on the organisms that pollinate the crops, we are operating our agricultural industry with our hands tied. How can we best make decisions about agricultural practices if we don't have access to all the available information?

Growers are working under the assumption that the pesticides are safe for bees if applied according to the manufacturer's label. But, as many of the above studies show, there is compelling evidence that the assumption is simply not true.

The growers and the agricultural industry as a whole could make better choices if they had more information at their disposal. For example, plant breeders in California are developing a new type of almond that is self-fertile (USDS-ARS 2010a). A tree of this type could greatly reduce the almond industry's dependence on honey bees, which every

year causes the greatest migration of bees anywhere on the planet. Other answers, too, could probably be found for other crops. But without a full disclosure of what the pesticides are doing to the bees, we are delaying the research into possible pesticide alternatives.

The USDA's sole reliance on a pesticide's LD<sub>50</sub> for adult bees is unconscionable now that methods for controlled rearing of larvae and testing of pesticides in the parts per billion range have become available. There is no longer a valid reason to forego these tests, and every reason to require them. Nor is there any reason to allow pesticides to be used in combination without first studying possible synergistic reactions between them.

The public, at least, is becoming more sophisticated about pesticide-pollinator issues and more aware of the processes by which these poisons are released into the environment. As a case in point, Bayer Crop Science was recently forced to withdraw its pesticide Movento (spirotetramat) from the market after a federal district court found the EPA had granted registration without holding the public comment period (*Natural Resource Defense Council & The Xerces Society v. USEPA & Bayer CropScience* 2009). While the breach was one of procedure only, it is interesting to note that the plaintiffs were motivated to act because the chemical was labeled as being dangerous to honey bee brood. Because of the potential of further harming an at-risk resource, the plaintiffs wanted to assure that the product did not slip through the regulatory cracks but met every letter of the law—even though that law is not sufficient.

For years, groups like these have pointed to regulatory gaffs that have perpetuated the use of pesticides that have not been fully tested. Bayer's imidacloprid, for example, has had 163 emergency exemptions since 1980 (EPA 2010). It has never been fully tested

in most of the crops in which it is used. Bayer CropScience merely applies for—and receives—one “emergency exemption” after another, without ever completing the registration process for those particular crops (EPA 2010). Conservation groups, like the above plaintiffs, believe this is an abuse of the system by both the EPA and the pesticide manufacturers. If the EPA is unable to handle pesticide regulatory matters, we need to find an alternative.

From a regulatory point of view we have done a disservice not only to honey bees, but to wild pollinators, beekeepers, and the food-consuming public. The establishment of spray tolerances based on the median lethal dose to adult bees is useless for assessing pollinator risk in typical foraging conditions. The problem is even worse when you factor in the possible synergistic effects between pesticides or between pesticides and pathogens and the known increased toxicity of various pesticide metabolites.

This paper illustrates the wide gap between our knowledge of what pesticides are doing to the brood of honey bees, and the criteria being used to approve those chemicals for use on the crops that bees pollinate. And while pesticides are not the sole reason for the decline in bee populations or the appearance of Colony Collapse Disorder, many researchers believe it is a major factor in both. When the health or immune system of a colony is compromised by poison, it is not in a condition to effectively resist diseases, pathogens, or the stresses of migratory beekeeping and monoculture diets. Each has a part to play in the complex life cycle of the honey bee.

In the words of Jeff Pettis, lead researcher at the USDA-ARS Bee Research lab, “The general feeling is that we need to move beyond mortality testing to sublethal testing



that looks at the shortening of life span, disorientation, and reduced vigor” of the honey bee (Sunshine 2009).

## **RECOMMENDATIONS FOR REGULATORY CHANGE**

Based on the above findings it would be prudent to change the regulatory processes for the registration and labeling of pesticides for agricultural use. Even if pesticides cannot be eliminated from modern agriculture, we need to test and label according to the best available science—not the science of past decades. Specifically, I recommend:

- *The restriction or prohibition of systemic pesticides on bee-pollinated crops.* From the studies above, it appears that any pesticide that enters the vascular system of the plant will ultimately find its way to the pollen and injure the pollinators.
- *The establishment of sublethal pesticide levels for larval pollinators.* We must have detailed information on how much pesticide can be used before sublethal toxicity begins harming the pollinator larvae, whether this pesticide arrives in the hive via pollen, water, propolis or nectar.
- *The regulation of primary pesticide metabolites as pesticides.* Since the metabolites can be many times more toxic than the original formulation, the metabolites should be regulated separately based on their larval toxicity. As an alternative, the original formulation could be regulated based on the most toxic of its metabolites.
- *The prohibition of fungicide and herbicide use immediately prior or during crop flowering.* Fungicides and herbicides cannot be assumed to be

non-toxic to bee larvae and should not be sprayed at any time that will allow the toxins to enter the larval food supply.

- *The prohibition of pesticide stacking when synergistic reactions are known or suspected.* The EPA needs to compile a list of pesticides that are safe for use in combination. All other combinations should be disallowed.

As illustrated in this paper, even when agricultural chemicals are used legally and responsibly according to EPA regulations, they can cause serious harm to bee larvae (Dai et al. 2010, Morandin et al. 2005). Until EPA regulations catch up with modern pesticide technology, honey bees, other pollinators, and the fate of the food supply will continue to be at risk from the detrimental effects of pesticide-contaminated pollen on beneficial insect larvae.

## **APPENDIX**

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### **Laws: Federal Statutes, Regulations and Guidelines**

An understanding of pesticide regulation in the United States requires a basic knowledge of federal law. The basic, essential and underlying approval of pesticides is under control of the federal government by statute, Congress' authority for enactment arising under the U.S. Constitution's commerce clause. Federal control of pesticides, in terms of the role of statutes, regulations, and administrative guidelines, is typical in many areas of federal law. The variations include the subject, the regulatory agency, and the extent of regulation.

President Truman signed FIFRA, the Federal Insecticide, Fungicide and Rodenticide Act, in 1947. Subsequent laws, including the Federal Environmental Pesticide Control Act (FEPCA) enacted in 1972, have changed the law and given authority to the Environmental Protection Agency to establish regulations under the act. Those regulations are established after drafting, publication, comment periods and administrative hearings pursuant to the Administrative Procedures Act (APA) of 1946.

The general framework of pesticide regulation, then, is:

(1) Congress passed statutes that established the policy goals and any specific restrictions within their authority, and granted regulatory authority to the EPA. The authority granted to the EPA cannot exceed the authority Congress is granted by the U.S. Constitution, and the EPA's regulations cannot exceed the authority granted by Congress.

(2) The EPA established regulations, codified in the Code of Federal Regulations (CFR), such as 40 CFR 159.165, that provide detailed regulations, including methods for approval for pesticides.

(3) The EPA published guidelines. Even the level of detail found in the CFR is insufficient to establish all needed guidance for interested parties. This occurs, for example, with standards associated with the testing of pesticides on honey bees, which are not covered in the Code of Federal Regulations at all. The solution is guidelines, such as OPPTS 850.3020 and OPPTS 850.3030, written by the Office of Prevention, Pesticides and Toxic Substances, an office within the EPA.

Guidelines can be included in the CFR, but an executive agency may choose not to do so if the guidelines are not controversial, help the general users, and correspond well with the intent of the existing regulations and statutes. One reason to use guidelines is they can be changed by the department anytime, when new information is available.

Although state regulation is not fully preempted by federal law in the field of pesticides, pesticide regulation as applicable to possible adverse affects on honey bees is not more restrictive, or substantially so, by the states than under federal regulation. Therefore, an understanding of state regulatory law is not relevant to this thesis.

## **EPA Pesticide Registration Review: An Update**

A new procedure called “registration review” will be replacing the old “pesticide re-registration and tolerance reassessment programs.” The program will operate continuously and encompass all registered pesticides.

In the future, the EPA will review each registered pesticide to see if it still meets all FIFRA standards for registration. According to EPA “the scope and depth of the Agency’s reviews are tailored to the circumstances, so registration reviews are commensurate with the complexity of issues currently associated with each pesticide (EPA 2009).

By law, the Agency must complete its first round of reviews by October 1, 2022.



## GLOSSARY

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**abdomen:** The posterior segment of the bee containing the honey stomach, stomach, intestines, reproductive organs, and stinger.

**acaricide:** a chemical designed to kill arachnids such as mites

**ambrosia:** see bee bread

**amino acid:** an organic compound composed of an amine group and a carboxyl group. The amino acids are the “building blocks” of proteins.

**anemophilous:** pollinated by the wind

**anther:** a sac-like component of a flower where pollen grains are produced; part of the stamen

**apiary:** a place where bee hives are kept and managed

**bee bread:** pollen mixed with nectar and bee secretions and stored in the comb for later use as brood food

**beeswax:** a substance secreted by four pairs of ventral glands in the bee abdomen that is molded to form combs and cappings

**biodiversity:** the relative abundance and variety of plant and animal species and ecosystems within a particular habitat

**brood comb:** any comb in the hive in which brood is found

**brood food:** glandular secretions of nurse bees that are used to feed larvae and, to a lesser extent, to feed the queen, drones, and foragers

**brood nest:** the area in a hive devoted to brood rearing

**brood:** all immature bees in a hive, including the eggs, larvae, and pupae

**capped brood:** pupae

**cappings:** wax coverings used by the bees to seal either pupae or honey

**cell:** a hexagonal compartment in a honey bee comb used for rearing of brood and storage of pollen and honey

**chorion:** the membrane covering a bee egg



**cluster:** a group of bees clinging together to maintain temperatures inside the hive; the cluster expands as the seasonal temperature increases

**cocoon:** the protective covering around the pupae

**coleoptera:** an order of insects that includes the beetles, weevils and fireflies

**colony:** a community of bees composed of one queen and many workers. In the spring and summer it also includes drones. See hive.

**comb:** an interconnected group of wax cells

**complete metamorphosis:** the four stage development process of an insect that includes egg, larva, pupa, and adult

**corbiculae:** a widened portion of the rear legs of female honey bees covered by curved spines where pollen is stored for transport, also known as pollen baskets

**cross pollination:** fertilization by transfer of pollen from the anthers of one flower to the stigma of another

**cuticle:** the waxy outer layer of an insect

**dearth:** a lack of availability usually referring to nectar or pollen

**diploid:** having two set of homologous chromosomes

**drone:** a male haploid bee that develops from an unfertilized egg

**endotoxin:** a toxin secreted by certain bacteria that is released into the surrounding environment only when it dies

**enzyme:** a protein with specific characteristics that allow it to aid certain chemical reactions

**exine:** the outer covering of pollen grains, often containing sporopollenin

**exocrine:** a gland that secretes externally through a duct

**flow:** the presence of large amounts of nectar or pollen, usually used in reference to a particular plant species, as in “a good maple flow”

**foraging:** the collection by bees of water, nectar, pollen, and propolis from their environment

**foundation:** a commercial product made from beeswax that is used as a starter substrate for bees to build comb; its use results in evenly-spaced and parallel combs

**frame:** a rectangular structure, with or without foundation, in which bees build comb. Frames allow combs to be removed for inspection or harvest without damaging the colony

**fructose:** a monosaccharide (simple sugar) frequently found in honey

**fungicide:** a chemical designed to kill fungus or mold

**guttation:** The exudation of water from leaves as a result of root pressure.

**haploid:** having only one set of chromosomes

**hemolymph:** The circulatory fluid of invertebrate animals that is comparable to blood

**herbicide:** a chemical designed to kill plants

**high-fructose corn syrup:** corn syrup that has undergone enzymatic processing to convert its glucose into fructose and then has been mixed with pure glucose to produce a desired level of sweetness

**hive:** usually refers to a manmade structure that houses bees, but may also be a synonym for colony

**honey stomach:** an enlargement of the esophagus that is used to collect and transport nectar

**honey:** nectar that has been dehydrated by the bees so that it contains no more than 17-18% water

**honeydew:** a sweet liquid excreted by aphids, leafhoppers, and some scale insects that is collected by bees, especially in the absence of a good source of nectar.

**hydathode:** a specialized leaf structure through which water is discharged from the interior of the leaf to its surface

**hydroxymethylfurfural (HMF):** an organic compound derived from the dehydration of sugars, HMF can form in both honey and high-fructose corn syrup (HFCS) when heated, and is toxic to bees

**hymenoptera:** an order of insects that includes sawflies, wasps, bees, termites, and ants

**hypopharyngeal:** in honey bees, a pair of exocrine glands in the head segment which secrete a protein-rich substance used to feed certain categories of bees, including larvae

**IGR:** an insecticide that works by disrupting the growth or development of an insect by mimicking natural hormones (insect growth regulator)

**insecticide:** a chemical designed to kill insects

**instar:** a stage of larval development between two molts; the first instar occurs after the first molt

**larvae:** an immature bee, grub-like, bee intermediate between egg and pupal stages

**lepidoptera:** an order of insects that includes moths and butterflies

**lumen:** the interior space of a tubular structure

**mandibles:** the jaws of an insect

**metabolite:** a substance that is the product of biological changes to another chemical, such as those from pesticides

**monoculture:** the agricultural practice of growing one single crop over a wide area

**monolectic:** a pollinator that visits only one species of plant

**nectar:** a sweet solution secreted by the glands of plants

**nematicide:** a chemical designed to kill roundworms

**neonicotinoid:** a class of insecticides which act on the central nervous system of insects and are chemically similar to nicotine

***Nosema apis:*** a microsporidian parasite of honey bees that lives in the intestines and destroys the epithelial cells of the midgut. It affects honey bee nutrition and shortens the life of worker bees.

**nurse bee:** a young worker bee that produces brood food and feeds the larvae

**oligolectic:** a pollinator that visits only a small number of plant species

**over-wintering:** the process of survival during the winter months, during which the bees live on stores collected during the spring and summer. Bees do not hibernate but actively maintain colony temperatures by forming a cluster.

**panicle:** a type of inflorescence with a pyramidal, loosely branched flower cluster

**parthenogenesis:** development from unfertilized eggs. In honey bees the drones (males) result from parthenogenesis

**pellet:** the contents of a pollen basket (corbicula)

**pesticides:** a chemical designed to kill a pest

**pheromone:** a chemical substance released by an animal to induce a response in another animal of the same species

**phytochemical:** chemical compounds that occur naturally in plants, such as beta-carotene, lycopene, and resveratrol. The term usually refers to those chemicals that may affect health but are not established as essential nutrients.

**pistil:** the female ovule-bearing part of a flower composed of stigma, style, and ovary

**pollen trap:** a device for removing pollen pellets from the corbiculae of incoming bees

**pollen:** a powder-like substance produced by the anthers of flowering plants and containing the male gametes

**pollenkitt:** a sticky substance adhering to the outside surface (exine) of a pollen grain, which aids bees in the collection of pollen

**pollination:** the movement of pollen from the anthers of one flower to the stigma of a compatible flower

**pollinator:** an agent that transfers pollen from one flower to another

**polyfloral:** made from many different flower types, as polyfloral pollen

**polylectic:** pollinators that visit many different plant species

**prepupa:** a stage between the last larval instar and the true pupal stage

**proboscis:** the “tongue” of a bee used to suck nectar and water

**propolis:** plant resins that are collected by bees and used to seal cracks and soften rough edges in the hive. Also called “bee glue,” propolis is high in antimicrobial substances

**protein:** an organic compound made of amino acids arranged in a linear chain in an order specified by a gene's DNA sequence

**pupa:** the stage of development immediately preceding the adult stage. A pupa is sealed under a wax capping where it spins a cocoon and completes development

**queen:** a fully developed female honey bee. Once mated, the queen stores sperm for as long as three or four years and lays eggs at varying rates throughout the year. Normally, a hive has only one queen.

**queenless:** a colony without a mated queen

**queenright:** a colony with a fully functioning mated queen

**re-queen:** a process in which a beekeeper removes the queen from a colony and replace her with a different one

**royal jelly:** a glandular secretion originating in the head segments of nurse bees and used to feed the larvae

**spermatheca:** an organ in the queen abdomen in which sperm is stored

**sporopollenin:** a cyclic alcohol that is chemically stable and highly resistant to decay, often found in the outer walls of spores and pollen grains

**stamen:** the male (pollen-bearing) part of the flower consisting of the anther and the filament

**sublethal dose/concentration:** a dose or concentration that induces no statistically significant mortality in the experimental population

**sublethal effect:** a physiological or behavioral change found in individuals that survive an exposure to a pesticide

**supersedure:** a process in which a colony replaces its queen with a different one

**swarm:** the reproduction of an entire colony that occurs when a colony splits into two parts. The old part is left with a new queen, and the part that splits off takes the old queen

**synergistic:** co-operative, working together, interacting, mutually stimulating. Synergistic toxicity occurs when two pesticides acting together are more toxic than the sum of the toxicological effects of each.

**systemic pesticide:** a pesticide that is absorbed and circulated by a plant or animal so that the plant or animal is toxic to pests that feed on it.

**thorax:** the middle segment of a bee body that supports the wings and legs

**tracheal mite** (*Acarapis woodi*): parasites that live in the trachea

**trachea**: a breathing apparatus consisting of branching tubes that conduct oxygen to the inner tissues of the bee

**transgenic**: an organism that has had genes from another organism inserted into its chromosomes

**trophallaxis**: direct food transfer between bees

**uncapped brood**: eggs and larvae not covered by wax cappings

**Varroa mites** (*Varroa destructor*): parasites that feed on the hemolymph of bees and reproduce on the pupae

**worker**: an infertile, diploid female bee adapted to perform a variety of functions in the colony depending on her age and the colony's needs

**xenobiotic**: chemical substances that are foreign to a biological system

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