Pesticides and Honey Bees: State of the Science

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Pesticide Action Network North America

Pesticide Action Network North America (PAN North America) works to replace the use of hazardous pesticides with ecologically sound and socially just alternatives.

As one of five PAN Regional Centers worldwide, we link local and international consumer, labor, health, environment and agriculture groups into an international citizens' action network. This network challenges the global proliferation of pesticides, defends basic rights to health and environmental quality, and works to ensure the transition to a just and viable society.

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I - Introduction

Honey bees and other pollinators are dying off at unprecedented rates around the world. First in France, then in the U.S. and elsewhere, colonies have been mysteriously collapsing with adult bees abandoning their hives. In 2006, two years after this phenomenon hit the U.S., it was named "Colony Collapse Disorder," or CCD. Each year since, U.S. beekeepers have reported annual hive losses of 29% - 36%. Commercial beekeepers tell us that their industry, which is the care and cultivation of an indicator species, is on the verge of collapse.

While claims of imminent food system collapse are not supported by pollination biologists, we do know that honey bees pollinate 71 of the 100 most common crops that account for 90% of the world's food supply, making managed honey bees the most economically important pollinator.[i] In the U.S. alone, the U.S. Department of Agriculture (USDA) indicates this industry is worth more than $15 billion per year.[ii] Without pollination, agriculture quickly becomes less efficient—requiring more land and water to grow the same amount of food—and our diets lose nutritionally important variety. For instance, most fruits and many nuts rely on the pollination services of bees, including almonds, blueberries, apples and melons. Altogether, more than one in every three bites of food depends on honey bees for pollination.

While few contest that the recent, dramatic decline of honey bee populations presents serious challenges to an already-stressed food system, the public debate over what lies behind CCD is at this point so polarized and confusing that concerned citizens find it difficult to know how or where to intervene. Indeed, the debate over the causes of CCD has become a case study in public, scientific controversy[iii]. As with other recent matters of public and environmental health, such as the link between tobacco and cancer and anthropogenic climate change, this issue has become characterized by policymaker inaction in the face of irreducibly complex science.

Two increasingly intractable sides have emerged in this controversy: beekeepers and environmental health advocates vs. pesticide companies and the scientists supported by them. While PANNA's position in this line-up is clear enough, we have sought to hew to a commitment to non-partisan, scientifically literate public discourse. We believe that engaged forms of scientific citizenship are a vital part of democratic civic life, and a needed force in environmental decision-making. In cases such as this where the debate has become intractable and position-driven, as is reported by participants and scholars alike, the conversation between experts has clearly broken down. Historically, these kinds of logjams are broken either through concerted public demand, or through a catastrophic focusing or "triggering" event that compels policymaker action—or both in conjunction. With one-third of our bees dying off each winter and wild pollinators facing similarly catastrophic declines, it would seem that we have before us the focusing event. Needed now is public demand for policy action.

Thus, the purpose of this document is to inform public debate and build national will for policy action on a timeline that will be meaningful to bees and beekeepers. Our consideration of the evidence for the causes of bee decline and CCD is focused on pesticides as one of three leading factors identified by researchers. By our analysis, the weight of evidence demonstrates that pesticides are indeed key in explaining honey bee declines, both directly and in tandem with the other two leading factors, pathogens and poor nutrition. The science supporting this conclusion is presented in this report.

2 - Overview :: Colony Collapse Disorder in context

Honey bee populations have steadily declined in the U.S. since 1947 at a gradual rate averaging 1% per year[iv]. Steeper declines have been recorded since 1987, but the last four winters have seen extraordinary losses averaging 29 to 36% per year.[v,vi,vii,viii]

Most scientists agree that there is no single cause of CCD. Rather, recent population declines are likely caused by a combination of factors acting in concert to weaken bee colonies to the point of collapse;[ix] and emerging science points specifically to impaired immunity. Lead suspects in this causal complex include: nutritional stress, pathogens and pesticides.

What is Colony Collapse Disorder?

Colony Collapse Disorder, or CCD, was first described in the U.S. in 2006. Its symptoms are distinct from other loss epidemics and include the following:

- Colonies found suddenly empty of adult bees, leaving their brood unattended
- No sign of dead bees
- No hive pests or food robbers, despite surplus honey and pollen stores
- Common parasites not present at levels thought to cause population decline

- ***Nutritional stress ::*** Nutritional stress undermines colony health through a variety of mechanisms, including immune system harm and reduction in reproductive viability. One key component of nutritional stress for honey bees includes habitat loss that results in a less varied and therefore less nutritious diet. Habitat loss has been occurring steadily for the last 50 years with measurable effects on bee health. For instance, regional differences in ratios of open to developed land have been traced to higher colony losses. One key driver of recent habitat loss is the increased use of broad-spectrum herbicides that accompanies herbicide-resistant, genetically engineered crops.[x]

- ***Pathogens ::*** Pathogens like parasitic mites, viruses and a gut fungus have garnered the most media attention as causal factors in CCD. Multiple studies have confirmed, however, that there is no single pathogen associated with the disorder. In an analysis of studies published as of early 2009, two leading U.S. researchers noted that "…no single pathogen found in the insects could explain the scale of the disappearance. In other words, the bees were all sick, but each colony seemed to suffer from a different combination of diseases."[xi] Parasitic mites of the genus *Varroa* are the most important pest to honey bees globally[xii] and act as vectors to transmit a number of viruses that significantly weaken colonies. Deformed wing virus and a trio of related paralysis viruses have also emerged as important to colony losses, as has a fungal gut pathogen of the genus *Nosema*. Emerging microbiota research points to the possible disruption of normal, symbiotic bee gut cultures by a combination of stressors resulting in increased susceptibility to pathogens.

- ***Pesticides ::*** Pesticides have been known to cause large-scale bee deaths since the early 1900s, many through direct poisoning during aerial sprays. These types of acute bee die-offs are not at issue in CCD, although they do still happen. Regulations and phase-outs of acutely toxic pesticides have reduced the number of acute poisonings in most of Europe and North America, but bee exposure to multiple pesticides continues. Sub-lethal effects, less studied and understood than acute effects, have become a key concern as systemic neonicotinoid pesticides—present in small amounts throughout plant tissues from seed to harvest—have become an importantant and rapidly growing segment of the global insecticide market since their introduction in the 1990s.[xiii] Other pesticides of concern include those used by beekeepers to control pathogens, and certain fungicides thought to be safe for bees which have recently

been found to act synergistically with some neonicotinoids, increasing the latter pesticides' bee toxicity by 200- to 1,000-fold.[xiv]

The following four studies provide an overview of the factors involved in recent honey bee declines as well as a description of Colony Collapse Disorder.

1) vanEngelsdorp D, Meixner, MD. 2010. **A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them**. J Invertebr Pathology 103: S80-S95.

> This article is the most recent and comprehensive review of historical loss patterns and the data gaps that make these patterns difficult to describe. Researchers reviewed 110 years of census surveys, experimental studies, technical reports and review studies to arrive at an analysis of global population trends and the factors that affect managed honey bee populations. Various data inconsistencies notwithstanding, they determined that populations have increased worldwide over the last 50 years, but severe declines have occurred in the U.S. (61%), Mexico, and Europe (27% continent-wide) in the same time frame. In all of these declines, several interacting factors are likely. These include long-term reductions in bee foraging habitat, changing weather patterns, a contraction of the gene pool, weak queen bees, pathogens, pesticides and socioeconomic factors that affect beekeeping popularity and profitability.

2) vanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, et al. 2009. **Colony collapse disorder: A descriptive study.** PLoS ONE 4(8): e6481.

> This study looked at 91 managed honey bee colonies from 13 apiaries in California and Florida to confirm the definition of CCD, identify its potential causes and inform future research. Over 200 variables were quantified and compared between CCD-afflicted colonies and apiaries, and those not afflicted. While 61 of the 200 variables were found frequently enough to make worthwhile comparisons, no single one stood out as being significantly linked to CCD. The comparative results did suggest some important trends: 1) compromised immunity is likely playing a role; and 2) CCD is either a contagious condition or caused by exposure to a common risk factor. Recommendations for future research include: 1) longitudinal studies that monitor parasite, pathogen and pesticide loads while quantifying pesticide tolerance in the study populations; 2) studies on the interactions among pesticides and pathogen loads.

3) vanEngelsdorp D, Speybroeck N, Evans JD, Nguyen BK, Mullin C, Frazier M, et al. 2010. **Weighing risk factors associated with bee colony collapse disorder by classification and regression tree analysis**. J Econ Entomol 103(5): 1517-1523.

> To better understand the relative importance and relationships among different risk factors in explaining CCD, researchers performed a classification and regression tree (CART) analysis on the data set of the above epidemiological study (vanEngelsdorp et al. 2009). This was the first case of CART analysis being used to understand bee pathology. Overall, the results of the analysis provide further evidence for the prevailing consensus that CCD is caused by multiple factors acting together to decrease colony fitness and increase susceptibility to disease. Pesticides accounted for 6 of the 19 variables having greatest discriminatory power. Of note is the fact that, at a level above 66 ppb in developing bees, the *Varroa* miticide, coumaphos, was positively correlated to healthy colonies; this was the most predictive factor between the two populations. Results indicate that pesticides are very likely involved in the CCD causal complex. Among several areas recommended for further study, two were highlighted: the effect of sub-

lethal pesticide exposure on pathogen prevalence, and the relationship between varying tolerance to pesticides and colony survival.

3 - Understanding pesticides as a causal factor in colony collapse

Pesticide Prevalence

Pesticides are a prominent part of the honey bee environment, both in the hive and in the larger environment. This is especially true in the U.S., where 1,200 different pesticide active ingredients are approved and in use in 18,000 different product combinations. By contrast, France and Britain have each registered around 500 and 300, respectively.[xv]

Multiple surveys in the U.S. and Europe have shown that a mixture of pesticide formulations and types are present in bees, wax, stored food and the pollen and nectar on which bees forage. Field studies have found neonicotinoid pesticides in particular in soil, dust, planter exhaust, water (guttation) droplets exuded by treated plants and on nearby, untreated plants and fields. A cross-section of these studies follows.

1) Krupke C, Hunt G, Eitzer B, Andino G, Given K. 2012. **Multiple routes of pesticide exposure for honey bees living near agricultural fields.** PLoS ONE 7(1).

This field study established that bees near agricultural fields are exposed to a variety of pesticides via multiple routes at harmful levels throughout the foraging period. Pesticides found include the neonicotinoids clothianidin and thiomexotham, atrazine (an herbicide) and fungicides, including one known to synergize with neonicotinoids (propiconazole). Soils, pollen (bee-collected and directly from plants), dandelions, dead and healthy bees, and planter waste products were all examined as potential exposure routes. The authors looked specifically at corn, which occupies more arable land in North America than any other crop—88 million acres. Corn is planted throughout the U.S. Midwest from mid-April through early May when the energetic requirements of bees are increasing rapidly as hives prepare for colony growth, requiring increased foraging. Virtually all corn (excepting the 0.2% cultivated organically) in the U.S. is grown from treated seeds, and this study found that bees forage heavily on corn: corn pollen made up over 50% of the pollen collected by bees, by volume, in 10 of 20 samples. Authors also sampled dandelions, which are a preferred nectar and pollen source during this period. Dandelions in nearby, untreated fields were contaminated with clothianidin. Soil from fields which had not been planted with treated seeds in over two growing seasons tested positive for clothianidin as well, which authors interpreted as a feature of the chemical's persistence and mobility.

Pollen collected from the treated plants was contaminated by clothianidin as expected, but bee-collected pollen samples showed higher levels, indicating additional pathways of exposure. Levels of contamination in bee-collected corn pollen in this study were 10-fold higher than reported from an experiment on clothianidin-treated canola. This is significant because clothianidin was approved for use on corn and canola simultaneously based on the canola field test. The finding that bee-collected pollen contained neonicotinoids is of particular concern because clothianidin is even more toxic when ingested orally by a bee, and because of the potential for harm when developing bees are exposed to pesticides within the hive through stored pollen. During the period observed, nurse bees were emerging and they must feed on

pollen reserves in the form of royal jelly. Authors calculated that, at the levels observed, a new bee would consume 50% of the oral LD50 during the 10 days it spends as a nurse bee.

In sum, the study established multiple exposure routes, at harmful levels, at a critical time, on the most common crop. The authors' most salient finding, however, is the establishment of a new, especially mobile and toxic exposure route—planter exhaust material. Corn seeds are sown using an automated planting system that relies on air/vacuum mechanisms to space the seeds; in order to keep seeds treated with pesticides from sticking to one another, talc is used. This talc becomes contaminated and is then exhausted during planting, either down with the seed or into the air. Authors found "extremely high" levels of neonicotinoids and fungicides in planter exhaust material.

2) Mullin CA, Frazier M, Frazier J, Ashcraft S, Simonds R, vanEngelsdorp D, et al. 2010. **High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health**. PLoS ONE 5(3): e9754.

Researchers conducted the most extensive North American survey of pesticide residues in managed honey bee colonies to date in 23 states and one Canadian province during the 2007-2008 growing season. They used conventional (gas chromatography-mass spectrometry) and recently developed (liquid chromatography-tandem mass spectrometry) analytical techniques to detect any of 200 pesticides and their metabolites at concentration levels as low as 0.1 ppb in a representative cross-section of bees, pollen and wax. A total of 121 pesticides and metabolites comprising 5,519 total residues were detected and quantified in 887 samples. Wax samples averaged 8, pollen samples averaged 7.1, and bee samples averaged 2.5 different pesticide residues each, with at least two pesticides detected in 92% of all samples analyzed. Pyrethroids, a group of widely used pesticides that are toxic to bees, were the dominant class of insecticides detected in all samples. Nearly half (49.9%) of all samples contained at least one systemic pesticide. Pollen contained high levels of fungicides, which tended to co-occur with low levels of systemic pesticides, implicating possible synergistic effects.

The authors concluded that "The widespread occurrence of multiple residues, some at toxic levels for single compounds, and the lack of any scientific literature on the biological consequences of combinations of pesticides, argues strongly for urgent changes in regulatory policies regarding pesticide registration and monitoring procedures as they relate to pollinator safety. This further calls for emergency funding to address the myriad holes in our scientific understanding of pesticide consequences for pollinators."

3) vanEngelsdorp D, Evans JD, Donovall L, Mullin C, Frazier M, Frazier J, et al. 2009. **"Entombed pollen": a new condition in honey bee colonies associated with increased risk of colony mortality**. J Invertebr Pathol 101(2): 147-149.

Two U.S. longitudinal studies that sought to uncover causes of poor colony health (and CCD in particular), were begun in spring of 2007. During both studies, researchers discovered a phenomenon known as entombed pollen, in which bees responsible for managing food stores in the hive seal off some pollen with propolis and wax. This behavior is generally used by bees to quarantine microbial threats, such as an invading lizard or mouse that dies inside the hive. Entombed pollen had much higher levels of three specific pesticides (two miticides and one fungicide) than typical pollen, and notably had no detectable microbes. Hives with such pollen in late spring were twice as likely to die in mid-fall as hives with normal pollen. Incidence of entombed pollen was notably greater in reused wax comb regardless of any disinfection

treatments applied, which suggests there is a transmittable factor common to both entombing behavior and colony death. Researchers urged further study.

4) Bonmatin JM, Marchand PA, Charvet R, Moineau I, Bengsch ER, Colin ME. 2005. **Quantification of imidacloprid uptake in maize crops.** J Agr Food Chem 53: 5336-5341.

Researchers in France conducted a three-year, random-sample field study of environmental contamination by the most prevalent neonicotinoid, imidacloprid, in corn fields with active bee colonies. This study was part of a national research effort seeking to evaluate the environmental risk of imidacloprid to honey bees. Using a recently developed method capable of quantifying imidacloprid at 1 ppb and detecting it at 0.1 ppb (high pressure liquid chromatography–mass tandem spectrometry, HPLC/MS/MS), researchers established that imidacloprid is habitually present in flowering, treated corn at levels known from previous dose studies to induce a variety of harmful effects, including eventual death, for honey bees. Average levels were 6.6 ppb for pollen-producing flowers, 4.1 ppb for stems and leaves, and 2.1 ppb for pollen itself. These levels are similar to those previously studied for sunflower and canola. Imidacloprid-contaminated corn pollen made up 54% of pollen samples collected at hive entrances, reflecting a mixture of pollen sources and with a correspondingly lower average contamination of 0.6 ppb.

Neonicotinoids :: Acute, sub-lethal & chronic effects

Neonicotinoids are a relatively new, and very widely used class of insecticides that work on the central nervous system of sucking insects such as fleas and aphids.[xvi] They were introduced in the 1990s and have since become the fastest-growing class of insecticides in the history of synthetic pesticides. By 2005 neonicotinoids had gained a 16% total market share of the nearly € 8 billion global market, and a near-lock (77%) on the global seed treatment market which itself grew from a niche € 155 million to a € 535 million market.[xvii] Among their approved uses in the U.S. are topical flea treatments for pets, lawn and garden uses, and a variety of agricultural uses including stone fruits, nuts, canola, sunflowers and corn.

Neonicotinoids fall into two subclasses: nitroguanidines and cyanoamidines. The nitroguanidines, which are highly acutely toxic to honey bees, include imidacloprid, clothianidin, thiamethoxam and dinotefuran. The cyanoamidines are not as acutely toxic to honey bees and include thiacloprid and acetamiprid. Neonicotinoids are known to persist in soil for years and have the potential to accumulate in soil. These chemicals are also highly water soluble, and are present throughout treated plants from seed to harvest.

To date, most U.S. regulatory decisionmaking addressing the risks posed to honey bees by neonicotinoids has hinged, by default, on the establishment of acute toxicity exposure scenarios without requiring tests for sub-lethal effects.[xviii] The standard laboratory method for assessing pesticide risk is determine the median lethal dose (LD_{50}) required to kill half the tested population over a certain timeframe. In the U.S. this protocol remains the primary basis for risk assessment in pesticide registration. However, this approach to risk assessment only takes into account the survival of adult honey bees exposed to pesticides over a short time frame: the typical acute toxicity test is 48 hours although it may be extended to 96 hours. Acute toxicity tests establishing LD_{50} levels on adult honey bees may be particularly ill-suited for the testing of systemic pesticides because of the ways bees are exposed to systemics in the field—in smaller doses, over a long period of time, through a variety of routes. Chronic feeding tests using whole colonies have been suggested better way to quantify the effects of systemics.[xix]

Despite repeated calls for a reevaluation of pesticide testing protocols, regulatory processes in the U.S. and Europe have not been adapted to consider sub-lethal, chronic or synergistic effects of pesticides on pollinators.[xx,]

Key terms for describing pesticide impacts

- ***Acute toxicity ::*** The acute toxicity of a pesticide to bees (either by contact or ingestion) is quantified by noting the dose at which half of the insects die within a specific time period. This is known dose that is lethal to 50% of the test population or LD_{50}. "Acute" and "lethal" are often used synonymously, but a range of effects, such as general agitation, vomiting, wing paralysis, arching of the abdomen similar to a sting reflex and uncoordinated movement, have been observed in association with acute doses.[xxi] The acceptable risk for each pesticide is set by regulatory agencies based on acute toxicity tests and the expected rate and mode of pesticide application, both of which are determined by the manufacturer. The LD_{50} values of neonicotinoids are low compared to older classes of insecticides; they are considered highly toxic to honey bees.

- ***Sub-lethal toxicity ::*** The sub-lethal toxicity of neonicotinoid pesticides is of particular concern because the most common field exposure scenarios are likely at the sub-lethal rather than acute level. Sub-lethal effects of neonicotinoids on honey bees include behavioral disruptions such as disorientation, reduced foraging, impaired memory and learning, and shifts in communication behaviors. Other important sub-lethal effects might include compromised immunity, delayed development and a host of indirect, potentially cascading effects that impact the hive's ability to sustain itself.

- ***Cumulative and chronic effects ::*** Neonicotinoids function by binding to niconitic acetylcholine receptors in insects' brains, receptors which are particularly abundant in bees, increasing during development from larval to adult stages.[xxii] This binding leads to an over-accumulation of acetylcholine, resulting in paralysis and death. The most recent scientific observations point to a long-lasting effect in which molecules unbind from receptors, but remain in the bee brain, possibly rebinding multiple times before metabolization occurs.[xxiii] Whether this constitutes effectively irreversible, cumulative toxicity remains unclear; but chronic toxicity effects over time are a likely result.

Neonicotinoids at-a-glance

Neonicotinoids are a widely used class of systemic insecticides introduced in the early 1990s that have been of particular interest for their effects on honey bees.

They can be applied as a spray (foliar) or, more commonly, used as systemics. Systemic pesticides are applied as seed coatings or soil drenches and are taken up through the plant's vascular system, they are transmitted to all parts of the plant, including pollen and nectar. Neonicotinoids are very persistent and therefore accumulate over time in the environment.

Most neonicotinoids are classified as acutely toxic to bees. But single, high-dose (i.e. acute) exposures are likely less common than are the chronic, sub-lethal exposure levels faced by bees as they forage in the field.

Honey bee colony collapses in France in 1999 were the first to implicate imidacloprid, the most widely used neonicotinoid, in colony loss. Researchers have since found a range of sub-lethal effects caused by neonicotinoids: altered foraging and feeding behavior and impaired orientation, social communication, undermined immunity and delayed larval development.

Many independent studies in the U.S. and in Europe have shown that small amounts of neonicotinoids—both alone and in combination with other pesticides—can cause impaired communication, disorientation, decreased longevity, suppressed immunity and disruption of brood cycles in honey bees. A selection of these studies follow.

1) Medrzycki P, Montanari R, Bortolotti L, Sabatini AG, Maini S, Porrini C. 2003. **Effects of imidacloprid administered in sub-lethal doses on honey bee behaviour. Laboratory tests.** B Insectol 56(1): 59-62.

> In this study, researchers monitored the behavior of adult forager bees in highly controlled, laboratory conditions to observe effects of imidacloprid fed at sub-lethal doses in sugar solution. Four different contamination concentrations were tested: 100 ppb and 500 ppb as a single dose (20 microliters), and each concentration as a continuously available food source for 24 hours. Three replications of the experiment were performed. Researchers concluded that imidacloprid at all doses given caused significant reductions in mobility that lasted for one to several hours, with bees remaining stationary for longer periods as well as moving more slowly. They also noted that bees seemed to lose their communicative ability at all doses, failing to coordinate their activity with other bees. They recommended further study to better understand the length of time that these effects last, and to investigate the effects on social behavior essential to the proper functioning of a hive.

3) Colin ME, et al. 2004. **A method to quantify and analyze the foraging activity of honey bees: relevance to the sub-lethal effects induced by systemic pesticides**. Archives of Environmental Contamination and Toxicology 47: 387-395.

> This study investigated the sub-lethal effects of two insecticides in semi-field conditions on the foraging behavior of honey bees. Imidacloprid and fipronil were chosen because both behave systemically, were recently introduced, considered highly toxic to bees, had shown sub-lethal effects on bees in lab conditions and had been implicated in honey productivity declines in Europe. The primary aim was to address a gap in environmental assessment of systemic pesticides by improving on the methods used to quantify foraging behavior changes. Bee colonies were placed in enclosed tunnels and their feeding behavior video recorded over a period of five days, constituting a cumulative effects study much shorter than a bee or hive lifecycle study would be. With imidacloprid at 6.0 µg/kg, inactive bees—those visiting the feeder, but not feeding—increased over time in relation to active bees. With fipronil at 2.0 µg/kg, most bees stopped coming to the feeder by the last day, and the few that did tended to be inactive. Convulsions and paralysis were also observed in bees feeding on fipronil-contaminated food. Researchers concluded that both insecticides disturb the hive's primary activity, feeding, at sub-lethal levels 70 times below the referenced LD_{50}s. They also concluded that their experimental protocol "provided an indispensable interface between controlled conditions in the laboratory and the field," which suggests its adoption in regulatory testing of sub-lethal effects.

4) Decourtye A, Armengaud C, Renou M, Devillers J, Cluzeau S, Gauthier M, et al. 2004. **Imidacloprid impairs memory and brain metabolism in the honeybee (Apis mellifera L.)**. Pestic Biochem Phys 78: 83-92.

> This laboratory study shows sub-lethal effects at low concentrations of imidacloprid using four different methodologies. Researchers sought to build from prior studies that clarified the role of

nicotinic acetylcholine receptors (nAChR) in honey bee learning and memory capacities to investigate the specific effects of the neonicotinoid imidacloprid on those capacities. Bees were conditioned to respond to a specific floral scent in association with food, a behavior that demonstrates associative learning and memory formation. Behavioral results led researchers to conclude that imidacloprid at a dose of 12 ng/bee significantly inhibited associative learning as well as retention of successfully learned associations, whether learned before, during or after exposure. This impairment of retention notably affected medium-term, but not long- or short-term memory, appearing as a temporary amnesia. Metabolic activity in specific parts of the bee brain were also analyzed for changes in response to imidacloprid. These results indicated that at both doses, imidacloprid was acting detrimentally in the parts of the bee brain involved with associative and contextual memory.

5) Yang EC, Chuang YC, Chen YL, Chang LH. 2008. **Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae)**. J of Econ Entomol 101(6): 1743-1748.

Researchers conducted a semi-field experiment to follow on results of laboratory studies that had shown a number of detrimental effects from ingestion of imidacloprid-contaminated food. They tested the time intervals between individual worker bee visits to a feeder after ingestion of imidacloprid-contaminated sugar solution at concentrations ranging from 40 µg/L to 6,000 µg/L. Bees showed abnormal foraging behavior beginning at 50 µg/L (41.6 ppb) and worsening with higher contamination. Effects ranged from a statistically significant time delay between feeder visits, to disappearance (from feeder and hive) for a full day without return. At 1600 µg/L, more than 90% of bees went missing for a full day, but all returned the following day. Above this concentration, a portion of bees did not return, and above 800 µg/L, bees that returned from long delays continued to show abnormal foraging behavior. Based on an estimation of average meal size per feeder visit, abnormal foraging behavior began a dose per bee of 1.82-4.33 nanograms. Researchers concluded that abnormal foraging behavior could occur in the field through multiple visits to imidacloprid-contaminated flowers.

Synergistic + Combined Effects

Synergism is a phenomenon in which two or more factors produce a combined effect that is greater than the sum of their separate effects. As investigations into the causes of CCD have continued to point toward multiple factors working in concert to increase bees susceptibility to disease, synergism and combined effects have emerged as a critical area of research.

In 2004, a lab study (see # 1 below) showed that the acute toxicity of two neonicotinoid pesticides on honey bees dramatically increases when combined with either of two common fungicides. Four years after this finding was published, researchers established that these types of combinations are prevalent in bee hives.[xxiv] Between 2010 and 2012, three separate studies demonstrated synergism between the common parasite *Nosema* and pesticide exposure (thiacloprid, imidacloprid and the non-neonicotinoid systemic fipronil). Hives exposed to these pesticides were significantly more susceptible to infection.

"Chemical cocktails" :: Fungicides, pyrethroid insecticides, miticides

Neonicotinoids are but one class of pesticides, honey bees are exposed to dozens of different pesticides on a daily basis (see "Pesticide Prevalence," esp. Mullin 2010). Included among these are a mix, or

"chemical cocktail," of insecticides, herbicides and fungicides as well as the miticides used by beekeepers to control pathogens in the hive.

Non-neonicotinoid pesticides of special concern for their impacts on bees, and potential role in the causal complex of CCD are fungicides and pyrethroids—particularly in combination. Fungicides have long been thought to be relatively harmless to bees, but their recent, dramatic uptick in use (especially with corn, beginning around 2007)[xxv] coupled with their synergizing effect on certain insectides (including neonicotinoids and pyrethroids) have brought renewed attention. Pyrethroids are highly variable in their toxicity to bees, but have come under recent scrutiny both because their high fat solubility means that pyrethroids persist and bioaccumulate in bee wax, and because they are known to synergize with certain fungicides.

As bee detoxification mechanisms are increasingly understood, particularly toxic "chemical cocktails" composed of certain fungicides, pyrethroids and neonicotinoids are slowly coming into resolution. Scientists believe that part of bees' increased vulnerability to pesticides comes from their having relatively few genes that encode detoxification enzymes. For example, one such group of enzymes (P450s) mediate detoxification pathways in ways that can be inhibited by certain widely used fungicides (e.g. propiconazole), and are thought to be important for bees' ability to tolerate the common pyrethroid miticide, fluvalinate.[xxvi] Although current data are unclear, another hypothesis as to the physiological mechanisms by which synergistic effects between pathogens and pesticides may operate is that pathogen metabolites may interfere with the detoxification process.[xxvii]

1) Iwasa T, Motoyama N, Ambrose JT and Roe M. 2004. **Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera**. Crop Protection 23(5): 371.

> This laboratory study established a synergistic effect between neonicotinoids and fungicides. A neonicotinoid was found to be up to 1,141 times more toxic to bees when combined with a common fungicide. Researchers performed laboratory tests for acute toxicity of several neonicotinoid pesticides and metabolites, both alone and combined with each of several fungicides commonly used in crop production. These tests were for contact toxicity, rather than oral ingestion. They found that three combinations between a neonicotinoid and a fungicide were highly synergistic. Acetamiprid, a neonicotinoid that is much less acutely toxic than imidacloprid, becomes 244 times more so when combined with the fungicide triflumizole. Thiacloprid, also much less toxic than imidacloprid, becomes 559 times more so when combined with the fungicide propiconazole, and 1,141 times more toxic when combined with triflumizole. Though plants treated with the maximum recommended levels for agricultural use did not exhibit a statistically significant effect on honey bee mortality after three and 24 hours, further study was suggested before reaching conclusion as to in-field, synergistic toxicity.

2) Smodis Skerl MI, Kmecl V, Gregorc A. 2010. **Exposure to pesticides at sublethal level and their distribution within a honey bee (Apis mellifera) colony.** Bulletin of Environmental Contamination and Toxicology 85(2): 125-8.

> This study examined the role and prevalence of pesticides in honeybee colonies, both those introduced intentionally by beekeepers to control mites (acaricides) as well as agricultural chemicals found incidentally in the hive. The goal was to determine whether, and if so, how much, pesticides accumulated in the bodies of bees in the hive, in the royal jelly, and in bee larvae as well as how the chemicals are spread throughout the hive. The acaricides introduced into the colony were amitraz, coumaphos and fluvalinate, while the organophosphate diazinon served as a representative agricultural chemical. The acaricides were applied following

commonly accepted regimes to treat against the *Varroa destructor*, and the diazinon was introduced in accordance with a treatment plan that would be applicable to an apple orchard. Coumaphos and fluvalinate were the most prevalent throughout the colony. No diazinon was found in the samples tested and amitraz only in amounts below the level of detection. Coumaphos was found in royal jelly from nurse bees, while fluvalinate was found in the bodies of bees and in bee larvae. The presence of fluvalinate throughout the colony, especially in larvae, demonstrate that chemicals in the hive can be transmitted from bee to bee as well as to food and thus to larvae, spreading throughout the entire colony.

3) Dai P-L, Wang Q, Sun J-H, Liu F, Wang X, Wu Y-Y and Zhou T. 2010. **Effects of sub-lethal concentrations of bifenthrin and deltamethrin on fecundity, growth, and development of the honeybee Apis mellifera ligustica.** Environmental Toxicology and Chemistry 29: 644–649.

This study examined the sub-lethal effects of two pyrethroid insecticides, bifenthrin and deltamethrin, on honeybee health. The study was performed on colonies in the laboratory that were fed small doses of the chemicals. Effects measured included fecundity, growth and the development of individual bees. The importance of these particular issues for overall health of the colony and general bee population was emphasized. Data was taken over several years and results measured against control colonies not fed the insecticides. The authors found that bifenthrin is "highly toxic" and deltamethrin "moderately toxic" to honeybees. This was based on findings that exposure to the insecticides significantly reduced colony fecundity due to reduced rates of egg laying and impaired ability of the colony to transition to a new queen. Developmental effects on honeybee larvae were also observed. In comparing their findings to pesticide applications in the field, the study's authors note that commonly recommended bifenthrin application rates are higher than the insecticide's LC_{50} for honey bees and deltamethrin application rates are about the same as the LC_5.

Pathogen interactions :: Nosema + pesticides

Nosema, a family of fungal gut parasites, and the *Varroa destructor* mite are two relatively recent honey bee pathogens. A particularly virulent and newly emergent (ca. 2005) strand of *Nosema*, *Nosema ceranae,* has become an area of research and concern around the world, especially in Spain. Both pathogens have been shown to interact with pesticides to weaken colony health more than either does alone. *Nosema* is a fast-spreading fungal gut pathogen that is thought to interfere with honey bees' ability to absorb nutrients (infected bees consume significantly more calories), and known to suppress immune response.[xxviii] *Varroa* mites act as vectors, transmitting disease across and within colonies.

With the relatively recent observation that CCD-affected hives are marked by an overall increased and variable pathogen load, but with no one pathogen found to consistently correlate with hive loss, researchers have begun looking for what is making the bees susceptible to disease to begin with.[xxix] Three separate studies between 2010 and 2012 (below) have demonstrated a synergistic effect between pesticides and the pathogen *Nosema.* The most recent study by leading USDA bee researchers found that bees with undetectable levels of imidacloprid—to which they were exposed only indirectly in brood food as developing larva—faced significantly more *Nosema* infections than did their control counterparts.

The overall pattern for bees exposed both to systemic pesticides (neonicotinoids and fipronil) and *Nosema* infection in these studies is that bees get sick more easily and die sooner as a result of both stressors in combination than either in isolation.

1) Pettis JS, vanEngelsdorp D, Johnson J, Dively G. 2012. **Pesticide exposure in honey bees results in increased levels of the gut pathogen Nosema.** Naturwissenschaften.

This study demonstrated increased pathogen growth among individual bees reared in colonies exposed to imidacloprid at levels below those considered to have sublethal impacts. Researchers exposed honey bee colonies during three brood generations to sub-lethal doses of a widely used pesticide, imidacloprid, and then challenged newly emerged bees with *Nosema*. They used GC/MS (gas chromatography-mass spectrometry) with a limit of detection of 0.1 ppb to analyze the bees, and verified pesticide exposure to colonies by measuring the weekly consumption of the treated protein patties and by analyzing imidacloprid in stored bee bread. *Nosema* infections increased significantly in the bees from pesticide-treated hives when compared to bees from control hives. Newly emerging bees which tested negative for imidacloprid, but had been exposed in the hive, were also significantly lighter in weight. As noted by the authors, this study is distinct from previous studies establishing this synergistic effect (esp. Vidau et al. and Alaux et al., below) in its focus on larva exposed only indirectly via brood food tended by nurse bees that had eaten imidacloprid-spiked protein. "Our test bees could have only received pesticide exposure during larval development."

The finding that individual bees with *undetectable levels* of imidacloprid, after being reared in a sub-lethal pesticide environment within the colony, had higher *Nosema* is significant in itself. It also has suggestive implications that the authors do not tease out beyond noting that future research should be conducted at the hive level over multiple generations rather than at the individual bee level. Authors do "suggest new pesticide testing standards be devised that incorporate increased pathogen susceptibility into the test protocols." Their conclusion: "Interactions between pesticides and pathogens could be a major contributor to increased mortality of honey bee colonies, including colony collapse disorder, and other pollinator declines worldwide."

2) Vidau C, Diogon M, Aufauvre J, Fontbonne R, Viguès B, et al. 2011. **Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*.** PLoS ONE 6(6): e21550.

In this laboratory study individual bees were infected with *Nosema ceranae*, exposed 10 days later to systemic pesticides fipronil and thiacloprid (a neonicotinoid). The main finding was that infected bees have a much higher mortality rate than uninfected bees when exposed to the same sublethal level of fipronil or thiacloprid (71% - 82% vs. 47%). Infected bees also appeared to be generally more sensitive to sublethal pesticide poisoning, "After exposure to insecticides, uninfected honey bees did not display any signs of intoxication. By contrast, at this level of exposure, insecticides triggered aggressiveness and tremors in infected honey bees during the first days of exposure." This study also confirmed previous findings that energetic stress was the main symptom of *N. ceranae* infection in itself: infected bees consumed much more sucrose than uninfected bees. Authors note that while the synergistic effect observed by Alaux et al. (2010) seemed to be linked to increased sucrose consumption, their findings were not.

A secondary, and by this data unconfirmed, hypothesis pursued by this study was that the mechanism of the synergistic effect between *Nosema* and these pesticides was linked to a decrease in bees' detoxification capacity as mediated by two groups of enzymes. Noting that data on the mechanisms underlying synergistic effects between pesticides and pathogens is poorly understood, authors note, "susceptibility of insects to pesticides is a more complex phenomenon than previously thought. The influence of parasitism in the ecosystem must be considered in

toxicological studies….[especially] since *N. ceranae* spreads rapidly and can affect more than 80% of honey bee colonies."

3) Alaux C, Brunet J, Dussaubat C, et al. 2010. **Interactions between Nosema microspores and a neonicotinoid weaken honeybees (Apis mellifera)**. Environmental Microbiology 12(3): 774–782.

> Researchers conducted a laboratory experiment to test an emerging, but unstudied hypothesis that high colony losses might be attributable to a combination of two factors each known to have effect on honey bee colony health: a fungal gut-pathogen (*Nosema ceranae*) and a neonicotinoid (imidacloprid). Their results indicate that a synergistic interaction is occurring that significantly weakens bees, both individually and socially. Concentrations of orally administered imidacloprid were 0.7, 7.0, and 70 µg/kg, made available for 10 hours per day over the study period of 10 days. These concentration levels are based on several studies that showed environmental levels of imidacloprid in the honey and pollen of treated crops reached 5.0 µg/kg. The highest individual death rates and energetic stress occurred with the combination of both agents compared to each alone and a control group. The enzymatic activity that correlates to bees' ability to sterilize the hive food for adults and larvae significantly decreased only when the two agents were combined. This suggests a synergistic interaction that could threaten the colony's ability to withstand a broad range of pathogens in the long-term.

4) Wu JY, Anelli CM, Sheppard WS. 2011. **Sub-lethal effects of pesticide residues in brood comb on worker honey bee (Apis mellifera) development and longevity**. PLoS ONE 6(2): e14720.

> This was the first study to establish sub-lethal effects on worker bees from pesticide residues in contaminated brood comb. Observed effects in this laboratory study included: delayed development of larval worker bees, delayed adult emergence and reduced adult longevity in larvae reared in cells contaminated with the miticides fluvalinate (a pyrethroid) or coumaphos. These effects can impact colony viability indirectly by causing premature shifts in hive roles, foraging activity and population dynamics, and by creating increased developmental time for *Varroa* mites which in turn can render a hive more susceptible to this common parasite and disease vector. Pesticide residues rapidly migrated from treatment to control comb, with a corresponding change in developmental and longevity effects: broods raised in treatment comb gradually had reduced effects while those raised in control comb (successively more contaminated with each brood cycle) had increased effects. Brood deaths followed this trend, with high rates occurring after multiple cycles in progressively more contaminated control comb, and significantly lower rates occurring in treatment comb as its contamination level lessened.

Microbiota out of balance :: Gut cultures, immunity + nutrition

Unintentional disruption of natural, symbiotic bee microbial cultures is one way in which hive health may be critically undermined by pesticides as well as other stressors in the contemporary, commercial beekeeping environment.

Honey bee microbiota (including fungi, bacteria, viruses, etc.) exists at two major levels: within the individual bee "gut" culture and throughout the hive considered as an extended organism. While very little is understood about the honey bee's complex and diverse microbial community, scientists do know enough to describe a co-evolved, minimally functioning, or "core," honey bee microbial community as well as hypothesize about key functions susceptible to disruption—specifically nutrition and immunity. For example, within the larger hive environment, beebread is the most microbially active, although whether this activity serves primarily to preserve or process nutrients is not well understood. Scientists

studying CCD have also recently found a consistent difference in the microbial abundance profile of affected vs. healthy hives.[xxx]

Emerging research in this area has been made possible in part through recent breakthroughs in new, high-throughput metagenomic sequencing technologies. These tools allow scientists to both better characterize insect microbial life in ways that focus on how hosts and symbionts interact functionally, at the epigenetic level.

1) Anderson, K.E., Sheehan, et al. 2011. **An emerging paradigm of colony health: microbial balance of the honey bee and hive (*Apis mellifera*).** Insectes Sociaux 58: 431-444.

> Taking CCD as its critical context, this review article argues that research attention should be paid to the symbiotic microbial communities that play critical roles in bee nutrition and pathogen defense. Authors note that most important immune-related function of a gut microbiota may be the ability to obstruct colonization by pathogens, thereby preventing infections. In this context, the well-known pathogen *Nosema ceranae,* is listed as an infectious microbe that interferes with digestion mid-gut.
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> Noting the "microbial frontier" opened by recent advances in high-throughput metagenomic sequencing technologies, the authors review existing literature and then argue for increased, systems-oriented research into the role of microbiota in bee colony health. With specific regard to pesticides, the authors note that broad spectrum antibiotics and fungicides applied directly to control disease are also known to destroy beneficial, non-target fungi and bacteria in ways that may disruption of the hive's beneficial microbial balance. Quoting Mullin et al., (2010), they flag as a concern the fact that synergistic effects of multiple, commonly found pesticides on the hive's microbiota are "entirely unknown." Authors conclude: "The road to sustainable honey bee pollination may eventually require detoxification of agricultural systems and in the short term, the integrated management of honey bee microbial systems."

4 - Research Challenges

In the context of multiple, interacting factors, methodological challenges are expected. Some are endemic to the task of epidemiological research and therefore unavoidable. Others are the result of equipment limitations, poor study design or regulatory framework failures.

Equipment + detection sensitivity

Until 2003, analytical techniques were not sensitive enough to detect systemic pesticide residues in plant tissue below a level of 20-50 ppb—much higher than the levels now known to be typical. Pollen had also never been analyzed. Detection of pesticides at very low levels is key for our understanding of the actual pesticide load in bee hives, bees and foraging habitat, including soil. This challenge remains an issue even in the most extensive studies, with limits of detection achieved between 1.0 and "a few" ppb while chronic effects have been observed at concentrations as low as 0.1 ppb. Over the last nine years more sensitive analytical techniques and tools such as high performance liquid chromatography coupled with tandem mass spectrometry (HPLC/ACPI-MS/MS or LC-MS/MS) have been developed, allowing sublethal and chronic exposure via pollen and beebread to be measured.[xxxi]

1) Bonmatin JM, Moineau I, Charvet R, Colin ME, Fleche C, Bengsch ER. 2005. **Behaviour of Imidacloprid in Fields. Toxicity for Honey Bees.** Environmental Chemistry: Green Chemistry and Pollutants in Ecosystems; Springer-Verlag Berlin Heidelberg, 483-494.

> Using the recently developed method for detecting imidacloprid at levels as low as 0.1 ppb (high pressure liquid chromatography–mass tandem spectrometry, HPLC/MS/MS), researchers showed the long persistence and slight accumulation of imidacloprid in soils (treated and untreated) as well as its uptake in non-treated crops. Sunflowers were shown to be particularly capable of recovering imidacloprid from untreated, contaminated soils, as were corn and several other adventitious plants. Untreated wheat, barley and rape recovered less imidacloprid from contaminated soils. The average values of imidacloprid found in sunflower and corn pollen corresponds to a range of concentrations in which sub-lethal effects in bee foraging behavior had been observed.

2) Bonmatin JM, Moineau I, Charvetet R, et al. 2003. **Method for analysis of imidacloprid in soils, plants and pollens.** Analytical Chemistry 75(9): 2027-2033.

> In this study, researchers set out to address a limitation in then-current research methods by developing a technique to quantify systemic pesticide residues in the field at levels known, in the lab, to have sub-lethal effects on honey bees. Using rigorous protocols, they developed a method to consistently detect imidacloprid and similar pesticides at levels below 1.0ppb. A new extraction method was paired with a known analytic method already in recent use, high performance liquid chromatography coupled to tandem mass spectrometry (HPLC/ACPI-MS/MS or LC-MS/MS). Their limit of detection was 0.1 ppb in soil and plants (stems, leaves and flowers) and 0.3 ppb in pollen, which had never undergone analysis for imidacloprid. To validate the method, researchers conducted small field tests in sunflower and corn fields. They found that most samples from treated fields, as well as fields that had been treated a full year earlier, had detectable levels, most of which were high enough to have sub-lethal effects on bees according to studies cited.

Study design

Designing studies that accurately assess pollinators' exposure to pesticides under field (i.e. outdoor) conditions is especially difficult because of the wide variety of factors in the natural environment. Multiple exposure pathways, synergistic and combined effects from multiple chemicals (i.e. the "chemical cocktail" effect), timing, relative levels of existing pathogens, variabilities of weather and genetic predispositions all run the risk of confounding any experiment designed to measure pesticide exposure and toxicity in the honey bee environment.

Laboratory vs. field studies

Studies seeking to determine the effects of pesticides on honey bees typically begin in the lab with a single pesticide and a sample of adult honey bees. Once several studies achieve similar results, the relationship between the tested substance and the organism is informed with an initial understanding of potential effect. Conditions in the lab are highly controlled to eliminate the possibility that observed effects might actually be caused by some other factor than the tested substance. To further understand that relationship, subsequent studies typically create "semi-field" conditions that more closely resemble the natural bee environment, but still partially control the parameters to limit the possibility of errors in the results. Full field experiments are used to assess the effects of the substance as it occurs in the bee's environment, but tend to have less consistent results because conditions are not as readily controllable. It tends to take a larger population of subjects and a longer period of experiment time to achieve results that correlate to either semi-field or lab studies. Lab results and semi-field results are not always replicable in full-field studies even with these necessary allowances.

It is inherently more difficult to track an individual bee—to measure the pesticide concentrations to which each bee is exposed and then its subsequent behavior. Many other factors also play a role in bee behavior and colony health, such as the presence of other pesticides in the hives and the foraging environment (the latter of which can be several to many tens of square miles), weather conditions, genetic predispositions, the age and health of the queen, and the relative presence of parasites and pathogens. One of these many variabilities specific to field conditions that has recently come into focus is the impact of relative humidity on the toxicity of neonicotinoids to bees.

1) Decourtye A, Devillers J, Aupinel P, Brun F, Bagnis C, Fourrier J, Gauthier M. 2011. **Honeybee tracking with microchips: a new methodology to measure the effects of pesticides.** Ecotoxicology 20:429-437.

> This study shows disorientation by pesticides and illustrates the difficulties of measuring such effects under field conditions. Few studies have investigated the impact of pesticides on homing flight due to the difficulty of measuring the flight time between the food source and the hive. The aim of this study was to show how the RFID (radio frequency identification) device can be used to study the effects of pesticides on both the behavioral traits and the lifespan of bees. Researchers developed a method to automatically record the disorientation of individual foragers and to detect the alteration of the flight pattern between an artificial feeder and the hive. Fipronil was selected as test substance due to the lack of information on the effects of this insecticide on the foraging behavior of free-flying bees. It was shown that oral treatment of 0.3 ng of fipronil per bee (LD_{50}/20) reduced the number of foraging trips.

Multiple exposure pathways

Contact (by touch) toxicity :: Dust, soil and planter exhaust/talc

Noting the correlation between bee losses and corn planting season in Italy and Europe, scientists there began exploring the possibility that bees were being poisoned by the dust emitted from pneumatic drilling machines used to plant neonicotinoid-coated seeds around 2003.[xxxii] More recent studies have confirmed that this route of exposure is indeed lethal, and is exacerbated by humidity. The leading hypothesis is that bees flying through contaminated dust are "powdered" with acutely toxic levels of neonicotinoids as their abdomens collect airborne fragments of treated seed coating.

1) Tapparo A, Marton D, Giorio C, et. al. 2012. **Assessment of the environmental exposure of honeybees to particulate matter containing neonicotinoid insecticides coming from corn coated seeds.** Environmental Science & Technology.

> This field study investigates planter exhaust during corn sowing as an exposure pathway for bees foraging in and around those fields. The core finding is that bees flying over sowing fields are directly exposed to neonicotinoids at lethal levels significantly higher than the contact LD_{50} values (18, 22, and 30 ng/bee for imidacloprid, clothianidin and thiamexotham, respectively). Also confirmed are recent findings that a) high quantities of neonicotinoids from seed coating particles are emitted by drills used during corn sowing; b) lethal levels of neonicotinoid-contaminated planter exhaust can land on bees' abdomens as they fly through the dust; c) bee mortality is higher under humid conditions, supporting the hypothesis that particles are more likely to stick to bees' abdomens in humid conditions. Study authors note that bees seem to remove the seed coating particles during subsequent foraging or in the hive under normal humidity conditions, and find a "significant decrease in the insecticide content" when bees are sampled after death. They hypothesize that metabolic degradation (probably also effective post-mortem) may affect concentrations found in samples.
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> The study's authors also explicitly address two methods of harm reduction proposed by industry: thicker, more adhesive seed coating, and modifications of the drills intended to reduce airborne release of contaminated planter dust. They conclude that neither presents a likely solution. In drawing these conclusions, they point to the fact that following the European introduction of seed coatings meant to be more resistant to abrasion in 2009-2010, Austrian, Slovenian and German (prior to the ban) beekeepers continued to report colony loss in conjunction with corn sowing. No such colony losses were observed in Italy after the ban there. These conclusions were also supported by direct observation differences in toxic emissions between modified and unmodified drills: modified drilling machines emit large amounts of contaminated dust at acute levels.

2) Marzaro, M., Vivian, L., et al. 2011. **Lethal aerial powdering of honey bees with neonicotinoids from fragments of maize seed coat**. Bulletin of Insectology 64 (1): 119-126.

> This field study investigated two possible mechanisms through which bees can come into lethal contact with neonicotinoid-contaminated fragments, or dust, emitted as treated seeds are sown: 1) direct aerial "powdering" of the bees as they come into contact; or, 2) indirect exposure through the nearby contaminated vegetation, dew and guttation droplets. Authors also tested the synergistic effect of relative humidity levels on bee mortality. Conducting chemical analyses of contaminated dew, guttation droplets, dust from abraded seed coatings, and dead bees, they found that bees were not lethally poisoned by drinking dew and guttation droplets, but that direct contact with airborne, contaminated dust is acutely toxic to bees under conditions of high humidity. Dust from abraded seed coatings expelled during sowing were shown to contain more

than 20% neonicotinoid, a concentration at least 2,600 times greater than what is used in spray applications. Study authors also conducted trials with seeds treated with a fungicide but not a neonicotinoid, under both humidity conditions. They found that the fungicide-treated seed coating dust was not lethal to bees under either condition. Using these trials as de facto controls, they concluded that humidity alone does not cause mortality; rather, humidity has a synergistic influence on the contact toxicity of neonicotinoids.

3) APENET. 2010 & 2011. **Effects of coated maize seed on honey bees.** Reports based on results obtained from the second and third years' activity of the APENET project. Consortium for research and experimentation in agriculture.

A group of Italian scientists from various institutions began publishing annual results from an ongoing monitoring network (APENET) in 2009. Their purpose has been to investigate the "effects of coated maize [corn] seed on honey bees." Main findings consist in establishing that bees are exposed to acutely toxic levels of pesticide-contaminated dust from automatic planters depositing treated seed in the field, and that relative humidity has a synergistic effect mortality. These studies look at the neonicotinoids clothianidin, imidacloprid, and thiamexotham, as well as fipronil, which is a systemic insectide commonly used in treated corn seed. They have also conducted field trials in methods for dust and drift abatement. 2010 and 2011 reports are 97 and 123 pages respectively, and are structured as six and eight chapters. Each of these chapters can be treated as a distinct study with its own research agenda, methods and results section. Three relevent chapters published in both years' reports are treated below.

"Dust dispersal during coated maize seed sowing and estimated effects on bees."

In field trials, dispersal of pesticide-contaminated dust was found to depend on a number of procedures: seed coating procedure; use of a modified drill (deflector) to reduce dust dispersal during planting; weather and environmental conditions. In 2010, modification of the seeders' pneumatic drills with an air deflector succeeded in reducing the dispersal of pesticide-contaminated dust by around 50%. These results, however, depended on seed quality. In 2011, experimenters devised a filter to attach to air deflectors with the intention of further reducing dustiness. This combined modification reduced dust dispersal by 90% - 95%, excepting very fine dust particles. In both situations bees die at higher rates: 30%-60% with the filter + deflector, 85% with deflectors alone.

"Effects in bees by contact with dust during flight over a field sown with coated maize seed."

Researchers confirmed a hypothesis that bees flying over a seeder that is sowing treated seed may be exposed to a lethal dose in a single flight via contact toxicity without the poisoning being mediated by ingestion of contaminated food. Humidity was further found to significantly synergize with the pesticides. Authors noted that bees' abdomens (or integuments) are shaped to catch pollen and are thus extremely likey to trap dust, and that dust had an exceptionally 20% (by weight) concentration of the active ingredient when compared to spray formulations.

"Sub-lethal effects of neonicotinoids and fipronil on learning and memory of odors and spatial orientation."

Studying the proboscis extension reflex (PER) as an index of certain cognitive processes, researchers found sub-lethal effects of neonicotinoids and fipronil on learning and memory of odors. Researchers also designed a testing protocol to measure bees' orientation capacity. Bees

exposed to clothianidin at 0.7 and 0.47 ng/bee showed marked impairment in homing ability and foraging frequency after a single dose at either level in 2010. Disorientation and disrupted olfactory memory and learning can significantly impact bees' foraging abilities and social life as both are mediated heavily by scent. Results from 2011, although not yet complete, appear to confirm 2010 findings.

Oral (ingestion) toxicity :: Pollen, nectar + guttation droplets

Established oral toxicity levels of neonicotinoids for bees are significantly higher than are contact toxicity levels. Potential oral exposure routes that have been recently studied include pollen, nectar and guttation droplets. Guttation droplets are a kind of dew exuded by plants during the night and in the early morning; they have been shown to contain lethal levels of neonicotinoid pesticides. While bees readily consumed guttation droplets in lab conditions, subsequent field studies have failed to establish that bees readily use these droplets as a water source. Field studies have shown that bees collect and bring back to the hive pollen and nectar contaminated with neonicotinoid pesticides both from directly treated crops (corn), and from nearby untreated plants known to serve as nutrition sources for bees (dandelions).

1) Girolami V, Mazzon L, Squartini A, et al. 2009 **Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees**. Journal of Economic Entomology 102(5): 1808-1815.

Researchers investigated the levels of pesticides present in the droplets exuded by plants grown from treated seeds, and the toxicity effects of those droplets to honey bees that consumed them. Study showed that the concentration of imidacloprid in guttation drops can be near those in the active ingredient that is applied in field sprays. Bees die within minutes after consuming guttation drops from imidacloprid-treated seeds. Four different pesticides were tested, all grown from treated corn seed, both open-field and in-lab: three neonicotinoids (imidacloprid, clothianidin and thiamexotham) and one non-systemic pesticide (fipronil). All treated seeds also included two fungicides, fludioxonil and metalaxyl-m, reflecting commercially available seeds. The experimental controls consisted of untreated seeds and seeds treated only with fungicides. The neonicotinoid pesticides were found to be consistently present in guttation drops. Levels of contaminant varied by pesticide and by replication as expected, but in all cases were above 1,000 µg/L. Imidacloprid-treated corn was the most efficient translocator, concentrating up to 200,000 µg of active substance per liter, but clothianidin and thiamexotham were more toxic (maximum concentrations found at 100,000 µg/L). Drops remained on the young plants throughout most of the day due to collecting in the cup formed at the base of the young plants. Bees readily consumed field-grown guttation drops presented to them in lab conditions. In all cases for neonicotinoids, bees experienced acute toxicity, dying within two to 44 minutes (concentration-dependent).

2) Greatti M, et al. 2006. **Presence of the a.i. imidacloprid on vegetation near corn fields sown with Gaucho® dressed seeds**. Bulletin of Insectology 59 (2): 99-103.

Following European beekeeper assertions of a link between imidacloprid-dressed corn seed and large-scale honey bee deaths, research efforts were made to determine whether the pesticide was becoming airborne during seed-sowing. This study showed that imidacloprid can be released at levels that are toxic to bees during sowing operations and that nearby plants can be contaminated by imidacloprid in dust from sowing operations. Three different seed treatments containing imidacloprid, one with a new ingredient intended to improve pesticide adherence, and two without imidacloprid were tested. Results clearly demonstrated that imidacloprid from

all three treated seeds can contaminate nearby grasses and flowers on the day of sowing and for at least four days afterward, in amounts relevant to both sub-lethal and lethal effects on honey bees. Sunny, warm weather was observed to correlate to higher amounts of imidacloprid detected, and for longer periods compared to cold and rainy weather. The amount of soil dust generated by pneumatic seed drills used in the trial was also implicated in potentially wide dispersal of imidacloprid particles through wind. Based on known patterns of corn-seed sowing in Northern Italy, researchers concluded that imidacloprid could accumulate on vegetation surrounding corn fields during successive periods of sowing for a period of 3-4 weeks, representing a longer-term contamination than was observed in the study.

Time + Timing

Understanding the effects of pesticides and other stressors on hive health is complicated by issues of time: duration, sequencing and developmental stages of a bee can all play a role. Studying the effects of pesticide exposure over too short a time scale is perhaps the most critical blindspot of most research to date. Current U.S. regulatory guidelines specify that honey bee toxicity testing be done within a timeframe of 48 - 96 hours, which is too short to observe many chronic or sublethal effects—particularly when those effects are indirect or cascading. Recent research into synergistic effects of pesticides and *Nosema* has surfaced a potential sequencing issue whereby bees exposed first to infection, then to pesticides show signs of poisoning at sublethal levels when pesticide exposure alone (without previous infection) at the same levels do not appear to have a toxic effect.[xxxiii]

Hive + Bee Lifecycles

Hives contain overlapping generations in which each plays a critical and interdependent role in maintaining hive health. The complex social structure of the hive is an ecosystem (some scientists characterize it as an organism or superorganism) in which individual bees can be categorized by a combination of age and hive function. Each category of bee consumes different types and amounts of food: pollen, nectar, and bee-made food containing one or both, such as bee bread, honey and royal jelly. They also differ in their activities, some spending most of their lives in the hive while others spend a majority of time foraging in flowers a few miles away from the hive.

Contact exposure to pesticides accordingly varies—larvae spend their first three days of life floating in nectar, and foraging bees are in contact with pollen for many hours. Larvae and adult bees, like human infants and adults, have differing detoxification capacities and nutritional needs. The queen, nurse bees and drones all likewise exist in different milieus. These variations all make a difference in how a hive or individual bee would be affected by pesticides.

1) Rortais A, et al. 2005. **Modes of honeybee exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees**. Apidologie 36: 71–83.

In this study, researchers demonstrated the importance of bee life cycle to the study of pesticide effects. Researchers used well-established bee biology data to quantify imidacloprid ingestion by bees whose hives are near treated sunflowers, according to each bee category. Their estimates focus on those categories that consume the highest amounts of pollen (nurse bees) and nectar (wax-producing, brood-attending, winter and foraging bees). Individuals within these categories potentially consume between 0.5 to 3.8 nanograms of imidacloprid, over a period of time that is

most relevant to their age and activities (from 5 to 90 days). These cumulative doses are consistent with amounts now known from both prior and subsequent feeding experiments to be lethal, or to cause a variety of sub-lethal effects that can result in premature death within hours or days.

5 - Structural Bias

Bias, sources of which scientists seek to minimize and eliminate, appears to be playing a role in our collective understanding of pesticide effects on honey bees. The prominent role of pesticide manufacturers in conducting and funding studies has generated controversy and concern among independent researchers, beekeepers and citizen groups.

The following critical reviews examine how conflicts of interest in honey bee research impact research findings, yield citation bias (where contradictory studies are excluded from introductory literature reviews), and exert undue influence on pesticide policymaking decisions.

1) Maini S, Medrzycki P, Porrini C. 2010. **The puzzle of honey bee losses: a brief review**. B Insectol 63(1): 153-160.

> The authors reviewed 84 studies that address the question of whether pesticides are adversely affecting honey bees and other non-pest insects, within the context of scientific and public controversy around CCD. They provide a focused, critical review of the most recent experimental and review studies addressing CCD, especially the effects of imidacloprid in the field (the most-used neonicotinoid that has been banned to varying degrees in four European countries). They also critically discuss venues for research publication and presentation. They compellingly demonstrate that science funded by agrochemical companies (including Bayer CropScience, the maker of several neonicotinoids including imidacloprid), have: 1) focused CCD research more on parasites and pathogens than on pesticides; 2) published the most favorable among all results on studies of pesticide effects on honey bees (no significant effects or effects at dose levels that do not correlate to environmental levels); and thus 3) potentially influenced policy decisions made to protect bees from pesticides toward less rigorous risk assessments and less cautious regulations.

2) Kindemba V. 2009. **The impact of neonicotinoid insecticides on bumblebees, honey bees and other non-target invertebrates** (revised version). Buglife: The Invertebrate Conservation Trust.

> This report presents a critical analysis of scientific research, technical reports and regulatory process documents relevant to the effects on non-target organisms of all five neonicotinoids registered for use in the U.K. Largely focused on imidacloprid's effects on honey bees according to the bulk of research available, the author makes a strong case for the existence of structural bias and regulatory inadequacy. Among studies showing that imidacloprid has negligible sub-lethal or chronic toxicity to honey bees, or that the effects seen are not relevant to amounts found in the bee environment, most were funded or carried out by the manufacturer. Conversely, a longer list of industry-independent research tends toward opposite results: imidacloprid being sub-lethally and chronically toxic at lower amounts, which are indeed relevant to environmental levels. In some cases, this was noted to be related to equipment sensitivity for pesticide detection, but the overall, patterned discrepancy indicates that results were influenced by factors related to the agendas of those who funded and conducted the studies as well as the regulatory reviewers. The regulatory process is found to be deficient in its assessment for a variety of other reasons: lack of standard methodology for investigating sub-lethal effects, failure to investigate long-term, seasonal, conditional, or synergistic effects in the

face of compelling evidence for doing so, negligence in requiring studies on larvae, lack of validation criteria for reviewing study methodologies and failure to investigate all possible routes of bee exposure.

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