Maine Board of Pesticides Control

Miscellaneous Pesticides Articles and Studies

July–August 2013

(***identified by Google Alerts or submitted by individuals***)

PAUL R. LEPAGE
GOVERNOR

STATE OF MAINE
DEPARTMENT OF AGRICULTURE, CONSERVATION AND FORESTRY
BOARD OF PESTICIDES CONTROL
28 STATE HOUSE STATION
AUGUSTA, MAINE 04333-0028

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COMMISSIONER

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Public Advisory

For Immediate Release
Date: August 13, 2013

Contact: Henry Jennings, 207-287-2731
henry.jennings@maine.gov

Mainers Urged to Sign Up for Free Disposal of Banned, Unusable Pesticides

AUGUSTA—Mainers are urged to take advantage of a free opportunity to dispose of banned or unusable pesticides that they may have in their homes or elsewhere on their properties. This October, the Maine Board of Pesticides Control (BPC) will team up with the Maine Department of Environmental Protection (DEP) to dispose of banned pesticides or pesticides that have become caked, frozen, or otherwise rendered unusable.

This free disposal program is open to homeowners, family-owned farms and greenhouses. All people need to do is **register by September 27, 2013**.

It's not unusual for homes and farms to have unintentional hazardous waste—old or unusable pesticides sitting around in basements, garages, or barns. Old chemicals like DDT, lead arsenate, 2,4,5-T, and chlordane, can be difficult and expensive to dispose of properly.

While removal of these pesticides can seem daunting, it's important for the protection of public, wildlife, and environmental health that they are dealt with properly and not thrown in the trash or down the drain, where they can contaminate land and water resources, including drinking water.

"We urge people holding these chemicals to contact us immediately to register," said BPC Director, Henry Jennings. "There will be four sites throughout the state where participants will be able to bring their obsolete pesticides and dispose of them conveniently and at no cost."

The collected chemicals go to out-of-state disposal facilities licensed by the federal Environmental Protection Agency where they are incinerated or reprocessed.

> **Registration by September 27, 2013, is mandatory—drop-ins are not permitted.** To register, get details, and learn important information about the temporary storage and transportation of obsolete pesticides, go to the BPC Web site at thinkfirstspraylast.org, or call 207-287-2731.

The Maine Obsolete Pesticides Collection Program, jointly sponsored by the BPC and DEP, and paid for entirely through pesticide product registration fees, has kept more than 90 tons of pesticides out of the waste stream since its start in 1982.

- *For more information on the Maine Board of Pesticides Control, go to: thinkfirstspraylast.org.*
- *For more information on the Maine Department of Environmental Protection, go to: maine.gov/dep.*

# # #

Important Note to the Media: Since registration is required, please post any information from this release as soon as possible. This will allow adequate time for participants and the Board of Pesticides to process applications. *Thank you for your help in getting the word out!*

Pilot

REGISTER BY SEPT. 27 FOR FREE DISPOSAL PROGRAM

Have old, banned pesticides kicking around the home or farm? State will take them this October

Offered to to homeowners, family-owned farms and greenhouses

Posted: Friday, August 23, 2013 - 9:00am

AUGUSTA — Maine residents are able to dispose of banned or unusable pesticides that they may have in their homes or elsewhere on their properties. This October, the Maine Board of Pesticides Control will team up with the Maine Department of Environmental Protection to dispose of banned pesticides or pesticides that have become caked, frozen, or otherwise rendered unusable. The free disposal program is open to homeowners, family-owned farms and greenhouses. Those interested must register by Sept. 27.

Banned and unusable pesticides can be disposed of free-of-charge through a state-sponsored program this October. (Below): Old metal, cardboard or paper pesticide containers eventually degrade and rupture and require proper disposal to possible contamination of land and water resources.

According to Board of Pesticides Control (BPC), it is not unusual for homes and farms to have unintentional hazardous waste — old or unusable pesticides sitting around in basements, garages, or barns. Old chemicals like DDT, lead arsenate, 2,4,5-T, and chlordane, can be difficult and expensive to dispose of properly.

While removal of these pesticides can seem daunting, it's important for the protection of public, wildlife, and environmental health that they are dealt with properly and not thrown in the trash or down the drain, where they can contaminate land and water resources, including drinking water.

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Maine Resource Recovery Association

FYI:

Sign Up Free Disposal of Banned, Unusable Pesticides

Please forward, as appropriate. Thank you.

Maine Department of Agriculture, Conservation and Forestry

Public Advisory

Contact: Henry Jennings, 207-287-2731

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For more information on the Maine Department of Environmental Protection, go to:

maine.gov/dep.

If someone forwarded this email to you and you wish to be added to our list please **join our mailing list!**

For more information please call MRRA at 207-942-6772 or visit our website: ***mrra.net***

John Albertini, Program Coordinator

Maine Resource Recovery Association

PO Box 1838, Bangor ME 04402-1838

Maine Runs Free Pesticide Disposal Program

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08/16/2013 10:09 AM ET

The Maine Board of Pesticides Control and Department of Environmental Protection are running a program in October to dispose of old chemicals like DDT.

AUGUSTA, Maine (AP) _ Maine is urging residents to take advantage of a free program to safely dispose of pesticides this fall.

The Maine Board of Pesticides Control and Department of Environmental Protection are running a program in October to dispose of old chemicals like DDT and lead arsenate Maine residents might have in their homes. The department is looking to dispose of any banned pesticides or pesticides that have become caked or frozen and are no longer usable.

It says improperly getting rid of these chemicals can contaminate drinking water.

Home, farm or greenhouse owners will be able to bring the pesticides to four locations throughout Maine. Residents must register for the program by Sept. 27.

Return!

Maine

Maine plans free pesticide disposal program

The Associated Press

Maine | Friday, August 16, 2013 at 5:40 am

AUGUSTA (AP) — Maine is urging residents to take advantage of a free program to safely dispose of pesticides this fall.

The Maine Board of Pesticides Control and Department of Environmental Protection are running a program in October to dispose of old chemicals like DDT and lead arsenate Maine residents might have in their homes. The department is looking to dispose of any banned pesticides or pesticides that have become caked or frozen and are no longer usable.

It said improperly getting rid of these chemicals can contaminate drinking water.

Home, farm or greenhouse owners will be able to bring the pesticides to four locations throughout Maine. Residents must register for the program by Sept. 27.

Maine offers pesticide disposal opportunity

Registration deadline is Sept. 27

UPDATED 11:34 AM EDT Aug 17, 2013

AUGUSTA, Maine -

Mainers are being urged to take advantage of an opportunity to get rid of banned and unusable pesticides for free.

The Maine Board of Pesticides Control is teaming up with the Department of Environmental Protection on a program that allows homeowners, family-owned farms and greenhouse operators to dispose of old pesticides they have hanging around. Officials say it's not unusual for people to have chemicals such as DDT, lead arsenate and chlordane that are no longer usable or legal.

Officials plan to set up four sites around the state in October where participants can take their unwanted pesticides, which will then be sent to out-of-state disposal facilities.

The registration deadline is Sept. 27. Click here for more information.

From: Jennings, Henry
Sent: Thursday, August 22, 2013 3:19 PM
To: Jennings, Henry
Cc: Fish, Gary; Bills, Anne; Schlein, Paul B; Additon, Ellis
Subject: Free Collection of Unusable Pesticides

Dear Licensed Pesticide Applicator:

I am writing to alert you that the Maine Board of Pesticides Control (BPC) will sponsor a free collection of unusable pesticides during the week of October 21 through 25, 2013. There will be four drop off points in Presque Isle, Bangor, Augusta and Portland. Only farmers (including greenhouse operators) and homeowners may legally participate in the program. If you would like to participate in the 2013 collection, you must complete an inventory form and submit it to the BPC by September 27. The form and supporting information may be found at this link, http://www.maine.gov/agriculture/pesticides/public/obsolete.htm , or you may call the BPC main office 207-287-2731 to request a paper copy of the form. Approximately two weeks before the collection, registered participants will receive a letter with directions to the drop off point, and the time of the collection. Keep in mind that the BPC only accepts pesticides through this program.

Sincerely,

Henry Jennings, Director
Maine Board of Pesticides Control
207-287-7543
henry.jennings@maine.gov

Maine Government News

Back to current news.

Second Mosquito Pool Test Positive for Eastern Equine Encephalitis

August 20, 2013
Human Services

Maine Center for Disease Control and Prevention has confirmed the presence of Eastern Equine Encephalitis (EEE) in a mosquito pool from the town of York in York County.

AUGUSTA – Maine Center for Disease Control and Prevention has confirmed the presence of Eastern Equine Encephalitis (EEE) in a mosquito pool from the town of York in York County. This is the second mosquito pool from Maine to test positive for EEE in 2013.

According to Dr. Sheila Pinette, Director of Maine CDC, additional positive tests are likely. “We still have plenty of warm weather ahead in the next few weeks and this increases the possibility of additional positive pools.”

EEE is a virus that is transmitted through the bite of an infected mosquito. It can cause serious illness in humans, large animals like horses and some species of birds. Maine confirmed EEE in a flock of pheasants during 2012 and experienced unprecedented EEE activity during 2009 with multiple animals and mosquito pools testing positive for the virus.

Regionally, all of our surrounding states have also identified EEE in 2013 including mosquito pools in New Hampshire, Vermont, and Massachusetts. Two horses have tested positive for EEE in Massachusetts as well.

“EEE is a very serious illness” says Dr. Stephen Sears, State Epidemiologist, “Mainers need to take appropriate precautions against mosquitoes to prevent this illness.” Maine CDC recommends the following preventative measures to protect against EEE and other mosquito-borne illnesses:

- Use an Environmental Protection Agency-approved repellent when outdoors, especially around dawn and dusk. Always follow the instructions on the product’s label;
- Wear protective clothing when outdoors, including long-sleeved shirts, pants and socks;
- Keep window and door screens down to keep mosquitoes out of the home;
- Limit time outdoors at dawn and dusk when many species of mosquitoes are most active;
- Remove containers holding water in and around the home, as water can attract mosquitoes.

Maine's Health and Environmental Laboratory (HETL) routinely performs testing for EEE and West Nile virus (WNV) in mosquitoes, large animals and humans. Maine stopped testing individual dead birds for mosquito-borne illnesses in 2006 and no longer uses them as an indicator for disease.

Maine CDC will continue to update information on mosquito-borne disease surveillance in Maine on a weekly basis. These reports are posted every Monday from May through September at http://www.maine.gov/dhhs/mecdc/infectious-disease/epi/vector-borne/arboviral-surveillance.shtml

Future positive tests will be announced through this report.

Information on pesticides and repellents is available at the Maine Board of Pesticides Control website at: http://www.maine.gov/agriculture/pesticides/public/index.htm#mosquito

Boston

First EEE Death of the Year

The Norfolk resident, a women in her 80s, had been hospitalized in mid-Aug. and died three days later.

By Melissa Malamut | Hub Health | August 21, 2013 10:33 am

The Massachusetts Department of Public Health (DPH) has announced the first human case of Eastern Equine Encephalitis (EEE) in a Massachusetts resident. EEE was discovered this season in mosquitoes in our area in July. The woman, who was in her 80s, was hospitalized in mid-August. She was a Norfolk resident, and died several days after being hospitlaized.

The DPH is conducting an epidemiological investigation to figure out where the woman was exposed to the infected mosquitoes and how she contracted the disease. After the investigation, the area will be assessed for more possible danger from infected mosquitoes.

"Our condolences go out to this individual's family and friends," said DPH Commissioner Cheryl Bartlett in a statement. "This underscores the serious nature of EEE and the need for vigilance. While the investigation is ongoing, this is a reminder to continue to use personal protection against mosquito bites, including covering exposed skin when outdoors, limiting outdoor activities between dusk and dawn, and using approved insect repellants."

There were seven cases of EEE in Massachusetts last year. And Mass. residents were warned to take precaution from mosquitoes due to EEE and another mosquito-borne disease, West Nile Virus, through the fall until the first hard frost.

Dr. Asim Ahmed from the Division of Infectious Diseases at Boston Children's Hospital says that EEE will often develop other very severe symptoms in two to three days like seizures, altered mental status, confusion, coma, and even death. He also says that the past few years have seen a change in where EEE is found in the state. "Historically within Massachusetts, EEE has mostly been found in the corridor southeast of Boston in close proximity to the wetland swamps that straddle Plymouth and Bristol counties," Ahmed says. "[The past couple of years], however, has been an unusual for EEE, which has been found in several uncharacteristic parts of the state: Franklin, Worcester, Essex, and Middlesex counties."

The DPH put out this statement to share the symptoms of EEE.:

> EEE is spread to humans through the bite of an infected mosquito. EEE is a serious disease in all ages and can cause death. The first symptoms of EEE are fever (often 103° to 106°F), stiff neck, headache, and lack of energy. These

> symptoms show up three to ten days after a bite from an infected mosquito. Inflammation and swelling of the brain, called encephalitis, is the most dangerous and common serious complication. The disease generally worsens quickly, and some patients may go into a coma within a week.

The best way to avoid mosquito-borne illness to to protect your self from mosquitoes. The DPH offers these tips.:

Be Aware of Peak Mosquito Hours. The hours from dusk to dawn are peak biting times for many mosquitoes. Consider rescheduling outdoor activities that occur during evening or early morning.

Clothing Can Help Reduce Mosquito Bites. Wearing long-sleeves, long pants and socks when outdoors will help keep mosquitoes away from your skin.

Mosquito-Proof Your Home. Drain Standing Water. Mosquitoes lay their eggs in standing water. Limit the number of places around your home for mosquitoes to breed by either draining or discarding items that hold water. Check rain gutters and drains. Empty any unused flowerpots and wading pools, and change water in birdbaths frequently.

Source URL: http://www.bostonmagazine.com/health/blog/2013/08/21/first-eee-death-of-the-year/

First 2013 case of EEE in Mass. found in Belchertown horse

The Lowell Sun Lowell Sun
Updated:

LowellSun.com

A Belchertown horse has been diagnosed with the first case of Eastern Equine Encephalitis in a horse this year, the Massachusetts Department of Public Health announced Wednesday.

This is Belchertown's second EEE infected horse in two years. Based on this finding, the EEE threat level has been raised to "Critical" in Belchertown and to "Moderate" in Granby, Ludlow, New Salem, Palmer, Pelham and Ware. DPH urges communities designated as "Critical" to cancel evening outdoor events for the remainder of the mosquito season.

"Today's finding significantly raises our concern for the area. It's important that people in high risk areas protect themselves from getting bitten by mosquitoes," DPH State Public Health Veterinarian Dr. Catherine Brown said in a press release. "Use insect repellant, cover up exposed skin, and avoid outdoor activities at dusk and nighttime, when mosquitoes are at their most active. DPH is working closely with several towns in the area to trap and test mosquitoes and we will use that information to help people understand the risk."

There have been no human cases of West Nile virus (WNV) or EEE so far this year. There were seven cases of EEE last year acquired by Massachusetts residents. EEE is usually spread to humans through the bite of an infected mosquito. EEE is a serious disease in all ages and can even cause death.

No matter where they live, individuals should continue to take personal precautions to avoid mosquito bites and the illnesses that can be caused, the DPH wrote in a press release. Precautions include:

Avoid Mosquito Bites

* Apply Insect Repellent when Outdoors: Use a repellent with DEET (N, N-diethyl-m-toluamide), permethrin, picaridin (KBR 3023), oil of lemon eucalyptus [p-methane 3, 8-diol (PMD)] or IR3535 according to the instructions on the product label. DEET products should not be used on infants under two months of age and should be used in concentrations of 30% or less on older children. Oil of lemon eucalyptus should not be used on children under three years of age.

* Be Aware of Peak Mosquito Hours: The hours from dusk to dawn are peak biting times for many mosquitoes. Consider rescheduling outdoor activities that occur during evening or early morning.

* Clothing Can Help Reduce Mosquito Bites: Wearing long-sleeves, long pants and socks when outdoors will help keep mosquitoes away from your skin.

Mosquito-Proof Your Home

* Drain Standing Water: Mosquitoes lay their eggs in standing water. Limit the number of places around your home for mosquitoes to breed by either draining or discarding items that hold water. Check rain gutters and drains. Empty any unused flowerpots and wading pools, and change water in birdbaths

* Install or Repair Screens: Keep mosquitoes outside by having tightly-fitting screens on all of your

windows and doors.

Protect Your Animals

* Animal owners should reduce potential mosquito breeding sites on their property by eliminating standing water from containers such as buckets, tires, and wading pools - especially after heavy rains. Water troughs provide excellent mosquito breeding habitats and should be flushed out at least once a week during the summer months to reduce mosquitoes near paddock areas.

* Horse owners should keep horses in indoor stalls at night to reduce their risk of exposure to mosquitoes.

* If an animal is diagnosed with WNV or EEE, owners are required to report to Department of Agriculture (DAR), Division of Animal Health by calling 617-626-1795 and to the Department of Public Health (DPH) by calling 617-983-6800.

More information, including all WNV and EEE positive results from 2013, can be found on the Arbovirus Surveillance Information web page at www.mass.gov/dph/mosquito or by calling the DPH Epidemiology Program at 617-983-6800.

Printed from: *Boston Herald* (http://bostonherald.com)

2nd Mass. horse found with EEE, precautions urged

Wednesday, August 7, 2013 -- The Associated Press

Local Coverage

Thursday, August 8, 2013

Author(s):
Associated Press

BOSTON — The state health department says Eastern equine encephalitis has been found in a second horse in Belchertown.

Officials are urging more precautions against mosquitoes in nearby Amherst.

The health department said Wednesday the threat level for the mosquito-borne virus has been raised to "high" in Amherst because the infected horse was stabled near the town border. Belchertown has already been designated at "critical" risk. Both designations mean communities are urged to cancel outdoor events during the evening when mosquitoes are most active.

The health department said it's raising the threat level to "moderate" in Hadley and South Hadley.

West Nile virus has been found in mosquitoes in several communities.

No human cases of EEE or West Nile virus have been reported this year.

Source URL:
http://bostonherald.com/news_opinion/local_coverage/2013/08/2nd_mass_horse_found_with_eee_precautions_urged

BANGOR DAILY NEWS

Researchers at Biddeford campus seek to study, drive away mosquitoes

Seth Koenig | BDN

University of New England senior Brendan Emanuel looks in on the nest inside of a birdhouse placed on the perimeter of a school soccer field. *Buy Photo*

By Seth Koenig, BDN Staff
Posted Aug. 16, 2013, at 6:09 p.m.

BIDDEFORD, Maine — University of New England researchers are partnering with school carpenters and landscapers in an ambitious effort to reclaim their Biddeford campus from some of nature's most reviled pests: Mosquitoes.

In addition to driving the insects away from high-traffic locations with strategically chosen and located birds, bats and plants, students and faculty advisers plan to trap mosquitoes and find out which species are bugging the local humans.

Noah Perlut, assistant professor of environmental studies at the university, said that while most people see all mosquitoes the same — as tiny, buzzing, blood-sucking irritants — there are actually 40 different species of mosquitoes in Maine.

Perlut said the UNE research team is realistic about its goals.

“We’re not trying to get rid of all the mosquitoes,” he said. “That’s not possible. I think there are a lot of places in humanity where they already would have done that if it was possible.”

The project began a year ago, when state officials found mosquitoes carrying the deadly Eastern equine encephalitis in York County. The disease — commonly referred to as EEE — was detected again in insects in Maine’s southernmost county this week.

“We were getting athletes back [on campus for preseason activities] and there was more and more concern about their safety,” Ronnie Souza, UNE director of environmental health and safety, said of the situation on campus a year ago.

Souza said the school administration did not want to spray pesticides campuswide and initially handed out individual bug spray containers to the returning student athletes. Even that didn’t seem like a sustainable solution long-term, Perlut said.

“One of the reasons this is such a unique project is that the administration engaged the faculty and students in trying to solve the problem,” he said.

Seeking a first semester project for one of their classes, students Caitlin Spaeth, Brendan Emanuel and Sam Fields took the reins, ultimately delivering to faculty and facilities managers a 60-page document laying out a strategy for driving mosquitoes away from places where students most often walk outside.

“We ended up with a three-pronged approach: Birds, bats and plants,” said Fields.

Working with university staff carpenters and landscapers, the students installed 22 bat boxes and 24 birdhouses — built to the specifications of tree swallows and bluebirds, which, like bats, like to eat mosquitoes — around the Biddeford campus.

Then the team turned its attention to what members discovered to be proven mosquito-repelling plants such as mints, citronella, sweet fern and monarda flowers, among others.

“What we’ve tried to do is target places where there’s a lot of student traffic, like the dormitories and meeting places,” said Phil Taschereau, UNE’s landscaping head and a certified master gardener. “Apparently, the students have been finding that it’s made a difference.”

Fields got the honor of being a test case. During the first of what will be a string of experiments recently, she found herself covered by more than 50 mosquitoes while standing 50 feet from one of the plant arrangements. When she moved to within 10 feet of the plants, the number of mosquitoes dropped to fewer than 30, and when she stood next to the vaguely orange- and mint-smelling leaves, her mosquito problem all but disappeared.

“If students just rub these plants as they’re coming or going from these buildings, it releases the oils into the air and gets it onto their skin,” Taschereau said of the natural repellent.

The next phase of the project, started this month, involves setting cannisterlike mosquito traps around the campus. When classes begin in the coming weeks, Emanuel said, he and Fields will view the captured pests under microscopes to determine which species of mosquitoes are nearby.

Spaeth graduated last spring.

Of the 40 species in Maine, only 12 can carry EEE or the similarly dangerous West Nile virus, Perlut said.

“What does it mean when the state says, ‘We found a mosquito with this disease’? Is that [a species that represents] 1 percent of the population? Or 90 percent?” posed Souza. “That’s what we’ll be trying to find out.”

New Lyme disease estimate: 300,000 cases a year

By MIKE STOBBE / AP Medical Writer / August 19, 2013

ATLANTA (AP) — Lyme disease is about 10 times more common than previously reported, health officials said Monday.

As many as 300,000 Americans are actually diagnosed with Lyme disease each year, the Centers for Disease Control and Prevention announced.

Usually, only 20,000 to 30,000 illnesses are reported each year. For many years, CDC officials have known that many doctors don't report every case and that the true count was probably much higher.

The new figure is the CDC's most comprehensive attempt at a better estimate. The number comes from a survey of seven national laboratories, a national patient survey and a review of insurance information.

"It's giving us a fuller picture and it's not a pleasing one," said Dr. Paul Mead, who oversees the agency's tracking of Lyme disease.

The ailment is named after Lyme, Conn., where the illness was first identified in 1975. It's a bacteria transmitted through the bites of infected deer ticks, which can be about the size of a poppy seed.

Symptoms include a fever, headache and fatigue and sometimes a telltale rash that looks like a bull's-eye centered on the tick bite. Most people recover with antibiotics. If left untreated, the infection can cause arthritis and more severe problems.

In the U.S., the majority of Lyme disease reports have come from 13 states: Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Vermont, Virginia and Wisconsin.

The new study did not find anything to suggest the disease is more geographically widespread, Mead said.

Online:

CDC report: http://www.cdc.gov/lyme/

The New York Times

August 26, 2013

Dangerous Ticks

By THE EDITORIAL BOARD

Residents of the Northeast and the Midwest know that ticks can carry the bacteria that cause Lyme disease. What most don't know is that the same family of black-legged ticks can also cause other diseases that are even more dangerous.

The worst is Powassan disease, which generally kills about 10 percent of its victims and leaves half the survivors with permanent neurological damage. Only 15 cases of this rare disease have been found in New York State in the last nine years, but there is no treatment and 5 of the patients have died. Fortunately, only a small percentage of ticks in New York are infected with the Powassan virus — between 4 percent and 6 percent at sites in the hardest-hit counties, Dutchess, Putnam and Westchester. By contrast, the Lyme bacterium has been found in 35 percent to 75 percent of the ticks at sites in those areas.

Three other diseases — anaplasmosis, babesiosis and illnesses caused by a newly detected pathogen, Borrelia miyamotoi — are transmitted by the same ticks and can, to varying degrees, cause severe disability and sometimes death. In rare cases a single tick could make a person sick with several diseases at the same time, greatly complicating diagnosis.

Senator Charles Schumer, Democrat of New York, recently urged the Centers for Disease Control and Prevention to allocate resources for research to understand the Powassan virus, create a treatment and learn how to prevent further spread. It would make sense to control this virus before it becomes a major threat.

Meanwhile, Lyme disease remains the most widespread tick-borne disease in the United States. Some 30,000 cases are reported annually to the C.D.C., but most cases go unreported because the symptoms are mild or mimic other diseases. The C.D.C. recently estimated that there may be 300,000 cases a year in this country, making Lyme "a tremendous public health problem."

There are huge gaps in what scientists know about how best to diagnose, treat or prevent the disease. Researchers have identified the bacteria, known as spirochetes, that cause Lyme and the black-legged ticks that can transmit the disease from rodents and deer to humans. But they know very little about prevention. There is no vaccine on the market, so health authorities can do little but advise taking sensible precautions against tick bites, like wearing long sleeves and pants, using insect repellent and looking closely for ticks to remove after spending time outdoors. (Even infected ticks don't always transmit the bacterium, especially if they are removed quickly.)

Lyme disease is best treated if caught early, when antibiotics can head off the worst consequences. But the early flulike symptoms, like fever, headache and fatigue, are so common that people may not realize they

have Lyme. There is no reliable diagnostic test to identify Lyme disease within the first month after the tick bite.

Although most cases are relatively mild and easily cured, some victims are left with lasting injuries, like joint pain, persistent fatigue or neurological damage. There is controversy over whether these long-lasting effects can be attributed to Lyme disease or may have been caused by something else that does not respond to the antibiotics used against Lyme disease.

Senator Richard Blumenthal, Democrat of Connecticut, has introduced a bill that would establish an advisory committee in the Department of Health and Human Services to help set priorities and coordinate federal programs for Lyme and other tick-borne diseases.

With little certainty about the best ways to address the problem, researchers ought to conduct trials of promising approaches to controlling rodents and deer that harbor the Lyme bacteria, study ways to kill the ticks themselves with fungi and plant extracts that are lethal to black-legged ticks but safe for wildlife, and develop new tests that can identify patients at the early stages of Lyme disease.

Meet The New York Times's Editorial Board »

[Article submitted by Nancy Oden (complete study follows this article).]

Print - Possible breakthrough in efforts to save U.S. honeybees | thv11.com

11:39 AM, Aug 13, 2013

UNDATED (CBS) -- The plight of the honeybees is growing more serious each year. The nation lost 31-percent of its colonies last winter. And as they die off, the threat to $15 billion worth of U.S. agriculture is becoming a major concern. Researchers may have a breakthrough in their search for what's killing the bees.

Dr. Jeff Pettis is the government's top expert on what's killing the honeybees. And his latest research turned up an unexpected culprit: common fungicides may be a bee's worst enemy. Pettis says, "That was the surprising part - that the fungicides, normally applied to flowering plants to protect against fungus were actually harming bees."

Pettis found that fungicides make bees more vulnerable to parasites. In fact, the same parasites have been linked to the syndrome known as Colony Collapse Disorder, which has wiped out millions of hives. Together with insecticides and foreign pests, the toll has been devastating. Pettis says, "For the past 7 years we've been losing honeybees at the rate of about 30 to 33 percent per year."

The public is starting to take notice. On a rooftop in the heart of New York City, Guillermo Fernandez tends a row of busy hives, making sure the queen bees are doing their job.

He's part of a new generation of private beekeepers, hoping to turn around the plight of the pollinators. He says, "I'm hoping that I'm contributing to our city and our agricultural system by helping to keep the bees, even if it's just a hive or two, alive."

The stakes are high. Honeybees pollinate roughly a third of what we eat from fruit to nuts to vegetables.

Now that the government is closing in on what's killing them, the thornier question may be what to do about it.

In light of the latest research, the USDA says it needs a closer look at how fungicides in particular are affecting bee safety. The challenge is to find the right balance - to protect crops from pests, without killing the pollinators.

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PLoS one

Colony Collapse Disorder: A Descriptive Study

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Abstract

Background: Over the last two winters, there have been large-scale, unexplained losses of managed honey bee (*Apis mellifera* L.) colonies in the United States. In the absence of a known cause, this syndrome was named Colony Collapse Disorder (CCD) because the main trait was a rapid loss of adult worker bees. We initiated a descriptive epizootiological study in order to better characterize CCD and compare risk factor exposure between populations afflicted by and not afflicted by CCD.

Methods and Principal Findings: Of 61 quantified variables (including adult bee physiology, pathogen loads, and pesticide levels), no single measure emerged as a most-likely cause of CCD. Bees in CCD colonies had higher pathogen loads and were co-infected with a greater number of pathogens than control populations, suggesting either an increased exposure to pathogens or a reduced resistance of bees toward pathogens. Levels of the synthetic acaricide coumaphos (used by beekeepers to control the parasitic mite *Varroa destructor*) were higher in control colonies than CCD-affected colonies.

Conclusions/Significance: This is the first comprehensive survey of CCD-affected bee populations that suggests CCD involves an interaction between pathogens and other stress factors. We present evidence that this condition is contagious or the result of exposure to a common risk factor. Potentially important areas for future hypothesis-driven research, including the possible legacy effect of mite parasitism and the role of honey bee resistance to pesticides, are highlighted.

Citation: vanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, et al. (2009) Colony Collapse Disorder: A Descriptive Study. PLoS ONE 4(8): e6481. doi:10.1371/journal.pone.0006481

Editor: Justin Brown, University of Georgia, United States of America

Received March 6, 2009; **Accepted** June 29, 2009; **Published** August 3, 2009

Funding: Funding was provided by the National Honey Board and the USDA-ARS Areawide Program on bee health; additional finding for DvE was provided by the Pennsylvania Department of Agriculture, Penn State Hatch funds and for DRT by the North Carolina Agriculture Foundation, a grant from the North Carolina Department of Agriculture & Consumer Services, and the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service grant number 2007-02281. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The winter of 2006/2007 witnessed large-scale losses of managed honey bee (*Apis mellifera* L.) colonies in the United States [1]. Those losses continued into the winter of 2007/2008 [2]. In the U.S., a portion of the dead and dying colonies were characterized *post hoc* by a common set of specific symptoms: (1) the rapid loss of adult worker bees from affected colonies as evidenced by weak or dead colonies with excess brood populations relative to adult bee populations (Figure 1); (2) a noticeable lack of dead worker bees both within and surrounding the affected hives; and (3) the delayed invasion of hive pests (e.g., small hive beetles and wax moths) and kleptoparasitism from neighboring honey bee colonies [3]. Subsequently, this syndrome has been termed Colony Collapse Disorder, or CCD.

Large-scale losses are not new to the beekeeping industry; since 1869, there have been at least 18 discrete episodes of unusually high colony mortality documented internationally [4]. In some cases, the descriptions of colony losses were similar to those described above. For example, a condition named "May Disease" occurred in Colorado in 1891 and 1896, where large clusters of bees completely disappeared or significantly declined over a short period of time [5].

Numerous causes of CCD have been proposed, often with little or no supporting data [6]. In an attempt to identify the potential cause(s) of CCD, we conducted an epizootiological survey of CCD-affected and non-affected apiaries. In doing so, we set an operational case definition that we verified by taking measurements of colony populations (brood and adult bees) and collecting samples of adult bees, wax comb, beebread (stored and processed pollen), and brood to test for known honey bee parasites (i.e., varroa mites, *Varroa destructor*, and honey bee tracheal mites, *Acarapis woodi*), pathogens (i.e., bee viruses and *Nosema* spp.), pesticide residues, protein content, genetic lineage, and morphological measurements. The results of an initial metagenomic analysis of some of the samples collected from this effort have already been reported [3].

Broadly defined, epizootiological studies are the study of disease occurrence in animal (in this case honey bee) populations. A primary function of epizootiology is to provide clues as to the

Figure 1. Frames of brood with insufficient bee coverage, indicating the rapid loss of adult bees.
doi:10.1371/journal.pone.0006481.g001

etiology of disease [7] as defined in the broadest sense - a departure from perfect health [8]. Descriptive epizootiological studies attempt to elucidate the cause(s) of disease by comparing health and risk factors in "diseased" and "non-diseased" populations [8]. A hallmark of these studies is that they are performed without a specific hypothesis, but they require an ability to classify the surveyed population into "diseased" and "non-diseased" individuals (in this case, colonies) based on a case definition.

Case definitions, especially when little is known about the disease, are often inductive and based on shared readily observable clinical characteristics [9]. Clinical characteristics, such as those used to classify colonies as suffering from CCD, are based on readily available (albeit sometimes broad) characteristics easily identified by "clinicians", which are often referred to as operational case definitions [8]. The operational case definition of CCD, used in this study, may have a low level of specificity and, thus, runs the risk of misclassifying individual colonies, which in turn can bias results [10]. Some of the characteristics used to define CCD, such as the lack of kleptoparasitism or the rapid loss of adult bees, are not easily quantified yet are readily identified by experienced beekeepers. Such ambiguity often results in skeptics dismissing the described condition as too vague to warrant recognition. The human medical literature, however, is filled with examples of such broadly defined disease (e.g., Gulf war syndrome [11]). Studies based on initially broad operational definitions permit the refinement of the case definition as more knowledge is gained about the condition [8]. Thus, the use of a sensitive, potentially overly inclusive definition is typical when investigating conditions for which the inclusion of suspect cases cannot be validated (e.g., by using laboratory test) and is common when investigating apparently new disease events, particularly when that event may be a new outbreak or epidemic.

The current study aimed to (a) characterize the spatial distribution of strong, weak, and dead colonies in apiaries containing colonies with and without CCD symptoms; (b) quantify and compare measurements among populations suspected to be suffering from CCD with apparently healthy colonies; and (c) gain insight into the cause of CCD. By physically mapping dead and weak colonies within CCD-affected and non-affected apiaries, we determined whether colonies graded with the same "condition" were randomly distributed within apiaries. A non-random distribution (e.g., dead colonies tending to neighbor other dead colonies) would suggest that an infective agent or exposure to a common risk factor may underlie the disorder.

We recognized, up front, that our characterization of CCD is not without bias; many measures, such as quantifying the colony population, are confounded with the overt symptom of CCD (i.e., lack of adult bee population). Other confounding measures are those that quantify colony stress. For example, whole-bee protein levels can serve as an indirect measure of developmental stress [12]. Honey bee larvae require sufficient protein in their brood food to ensure proper development and to optimize their activities during the winter. Farrar [13] showed that the quantity of stored pollen within a colony in the fall is significantly correlated with its spring adult bee population. Measures of mass, total protein, and protein-mass ratio can therefore act as an indirect measure of colony nutrition [13–19], parasitism [20–23], or both. Differences in these measures may be a consequence (i.e., collapsing colonies are less able to acquire sufficient forage to maintain proper colony health and function) or a contributing cause of the syndrome (e.g., nutritionally stressed colonies are more susceptible to pathogen attack). Another indirect measure of developmental stress is fluctuating asymmetry (FA). FA is defined as random differences in the shape or size of a bilaterally symmetrical character [24], which can be an indicator of individual fitness [25] because organisms exposed to stress during early development show less symmetry than unstressed organisms [26–33].

Some factors quantified and compared in this study have known impacts on colony health. Elevated populations of varroa mites, *Nosema* spp., and honey bee tracheal mites (HBTM) are known to damage colonies and may contribute to CCD. Both the HBTM and the varroa mite were introduced into the U.S. in the 1980's and are now widespread. While the number of managed honey bee colonies has been in decline in the U.S. since the 1940's, these mites have been implicated in drastic losses of colonies since their introduction [34]. Similarly, two species of *Nosema* are now widespread across the continental U.S. Historically, nosema disease was thought to be caused by the gut parasite *Nosema apis*, which can be particularly problematic for overwintering colonies [35,36]. However, a recent survey of historical samples collected

from across the U.S. suggests that *N. apis* has been largely displaced by *N. ceranae* over the past decade [37]. While the etiology of *N. ceranae* is poorly understood, it has been implicated with recent large-scale losses experienced by Spanish beekeepers [38,39]. Other pathogens, including bacteria, fungi, trypanosomes, and viruses, can also significantly impact colony health. An extensive survey of declining and healthy honey bee populations, using metagenomics and targeted polymerase chain reaction (PCR), helped to identify several microbial associates of CCD colonies, the most informative of which was the discistrovirus Israeli acute paralysis virus (IAPV) [4]. In the current study, we assayed colonies for the presence of 12 organisms spanning these different groups using sensitive PCR-based techniques [3,40,41]. Moreover, using established protocols testing mitochondrial DNA markers [42], we were able to assign the sampled colonies as either European in origin (Eastern vs. Western) or as African in origin (Northern vs. Southern). If certain mitotypes are found to be more affected by CCD, it could pin-point specific genetic strains of interest for future analyses [43,44] as well as induce future explorations into unique host-pathogen interactions.

Pesticide exposure is also a risk factor that was quantified in this study. Honey bees can contact and collect pesticides when foraging on crops that have been treated to control pest insects, pathogens, or weeds. In addition, since the late 1980's, U.S. beekeepers have been using miticides within their beehives to control parasitic mites (primarily Varroa mites). A diverse range of pesticides, both grower- and beekeeper-applied, have been detected in hive matrices [45–47], and many of these products are known to adversely affect colony health [48–50]. Here, we compare both the prevalence and load of different pesticides in the wax, beebread, brood, and adult bees in a subset of CCD-affected and non-affected populations.

Materials and Methods

Apiary selection and CCD assessment

In January and February 2007, we selected colonies resident in Florida and California distributed across 13 apiaries owned by 11 different beekeepers. Apiaries were classified as (1) having no colonies with CCD symptoms ('control') or (2) having colonies with CCD symptoms ('CCD'). The operational case definition employed to classify CCD cases verses non-cases were qualitative and made in the field by researchers experienced in clinical bee disease diagnosis. This was as follows (1) the apparent rapid loss of adult worker bees from affected colonies as evidenced by weak or dead colonies with excess brood populations relative to adult bee populations; (2) the noticeable lack of dead worker bees both within and surrounding the hive; and (3) the delayed invasion of hive pests (e.g., small hive beetles and wax moths) and kleptoparasitism from neighboring honey bee colonies. In those CCD colonies where some adult bees remained, there were insufficient numbers of bees to cover the brood, the remaining worker bees appeared young (i.e., adult bees that are unable to fly), and the queen was present. Notably, both dead and weak colonies in CCD apiaries were neither being robbed by bees (despite the lack of available forage in the area as evidenced by the lack of nectar in the comb of strong colonies in the area and by conversations with managing beekeepers) nor were they being attacked by secondary pests (despite the presence of ample honey and beebread in the vacated equipment).

The physical locations of the hives in a subset of the visited apiaries (n = 9) were mapped. We classified these colonies as either 'alive' or 'dead' (i.e., no live bees) and we classified the living colonies as either 'weak' or 'acceptable' based on the number of frames of bees (with those having four or fewer frames of bees being considered 'weak').

Colony strength and sample collection

In all, 91 colonies were sampled and used in subsequent analyses. The populations of adult bees and brood were measured in living colonies (n = 79) through the estimation of the total area of comb covered by adult bees or brood [after 51].

At the time of sampling, the presence of overt brood infections (pathogens) was noted. The condition of the quality of the brood pattern was also noted with areas of capped brood containing less than 80% viable brood (as indicated by cells empty of brood) were considered "spotty" while those brood patterns that had less than 20% brood mortality were considered "solid".

Samples of adult bees (~150 bees) were removed from a central brood frame, placed into a 50 ml centrifuge tube, and temporarily stored on dry ice before being frozen at –80°C for future processing. A subset of these bees was used for pathogen, protein, and pesticide analyses. An additional sample of ~320 bees, collected from the same frame, was placed in 75% ethanol in a 125 ml sampling container and used for quantification of varroa mite mean abundance, HBTM prevalence, and *Nosema* spp. spore prevalence and load. Finally, all live and dead (n = 12) colonies had ~15 cm×15 cm sections of brood comb removed from them, which contained wax and often (but not always) bee brood and beebread. Sampled comb was stored on dry ice before long-term storage at −20°C.

Physiological and morphological measures

Body mass and protein analyses. We used BCA Protein Assay kits (Pierce Scientific, Rockford, IL) to quantify protein content from six separate adult worker honey bees from each of the sampled colonies containing live bees (n = 79). This process uses bicinchoninic acid for the colorimetric detection and quantification of soluble protein (Bradford assay), which indicates the developmental nutrition of bees within a colony during larval feeding [52].

We removed each bee from −80°C storage onto ice and separated its head, gaster (abdomen), and thorax with a razor blade. After the wings and legs were removed from the thorax (because, during shipping, many bees did not have a full complement of appendages), we weighed each body segment to the nearest 0.1 mg using a Metler digital scale. Immediately after weighing, each segment was placed into a separate 1.5 ml microcentrifuge tube on ice. We then added 150 µl, 600 µl, and 500 µl of extraction buffer (1×PBS+0.5% Triton X-100) to the head, abdomen, and thorax tubes, respectively. Each sample was homogenized using a clean plastic pestle, placed on ice for 30 min, and centrifuged at 14,000 g for 5 min. The supernatant was then transferred from each tube to a separate 0.5 ml microcentrifuge tube and frozen at −20°C until further analysis.

We performed the BCA tests by adding 18 µl of 1x phosphate-buffered saline, 2 µl thawed protein extract, and 100 µl BCA working reagent (Pierce Scientific, Rockford, IL) to individual PCR reaction tubes, vortexing and spinning the tubes to homogenize the reagents, and incubating them for 30 min at 37°C on a thermocycler. We then cooled the tubes on ice for 15 min and immediately read their absorbance using a Nanodrop® spectrophotometer. Following the Bradford assay, we calculated the final levels of soluble protein using a standard curve generated from known concentrations of Bovine Serum Albumen.

Morphometric measures. From each living colony from which adult bees were sampled into ethanol (n = 76), both forewings from 10 workers were removed and mounted on microscope slides

using transparent tape. The wings were then scanned at 600 dpi using a Hewlett Packard ScanJet ADF flatbed scanner. The centroid size of each wing was calculated by determining the relative position (landmark) of 12 vein intersections [after 53] and then calculating the square root of the sum of squared distances between each landmark and the centroid of each forewing [54]. The relative position of each landmark was determined using a script written for UTHSCSA Image Tool software (downloaded from http://ddsdx.uthscsa.edu/dig/itdesc.html) and the resulting data were imported into SAS [55] to automate the centroid-size calculation.

To distinguish between true measures of FA and measurement error, a randomly selected sub-sample of up to 10 bees from 24 colonies (n = 216) had their centroid sizes recalculated from the original scanned image. A two-way ANOVA (repeated measures) revealed that the mean square of the interaction between individual bees and wing side was significantly larger than the mean square of the error term ($F = 4.66$, df = 215, 432; $P<0.0001$), suggesting that measurement error was not a significant source of centroid size variation [56].

A simple linear regression was conducted [57] comparing centriod size and FA. As no association was found ($F = 0.085$, df = 1, 7, $P = 0.7714$), no correction for scale effect was warranted [56]. Consequently, FA_1 [58] measures were calculated by determining the absolute difference in centroid size between an individuals left and right wings.

Risk exploratory variables

Macro-parasite and pathogen quantification. The mean abundance of varroa mites (mites per bee, or mpb) was determined by separating mites from the entire sample of bees stored in ethanol by shaking them in soapy water and then counting both the number of mites and bees in the sample [59–61]. Thirty of these bees also had their abdomens removed to measure the mean abundance of *Nosema* spp. spores (spores per bee) following Cantwell [62]. Finally, using the methods outlined by Delfinado-Baker [63], the prevalence of honey bee tracheal mites (*Acarapis woodi*) was determined by examining thoracic slices of 16 bees per colony, which is the number suggested for differentiating highly infested colonies (prevalence >30%) and colonies with low infestation (prevalence<10%) [64]. For all of these tests, colonies were additionally classified as being affected or not affected by the parasite or pathogen, regardless of the load.

Pathogen analyses. We determined the prevalence (proportion of colonies affected) of several pathogens, including bacteria, trypanosomes, *Nosema* species, and numerous viruses: Acute Bee Paralysis Virus (ABPV), Black Queen Cell Virus (BQCV), Chronic Bee Paralysis Virus (CBPV), Deformed Wing Virus (DWV), Israeli Acute Paralysis Virus (IAPV), Kashmir Bee Virus (KBV), and Sacbrood Virus (SBV). Each pathogen was targeted with a single diagnostic primer [3, 40, 41; Table 1] except IAPV, for which we employed three distinct primer pairs as a means of capturing all members of this diverse lineage. For IAPV, we present relative transcript abundances based on each primer pair separately and an aggregate (arithmetic mean; $IAPV_{Avg}$) from all primer pairs. We extracted total RNA from pooled abdomens of eight worker bees from each colony (n = 76) by grinding abdomens in 1 ml guanidine thiocyanate lysis buffer, pelleting debris, and then extracting RNA from the supernatant using the RNAqueous procedure (Ambion). We then generated cDNA from approximately 500 ng of total RNA using a mixture of poly-dT primers [40] and Superscript II reverse transcriptase (Roche). We carried out quantitative PCR on individual samples and targets using the fluorescent intercalating dye SYBR Green and a Bio-Rad Icycler thermal cycler. We optimized primer pairs for each pathogen target (Table 1) and conducted all PCR reactions using a thermal profile of 3 min at 94°C, followed by 40 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s), and 78°C (20 s). The 78°C step was used to avoid background signals from potential primer-dimer artifacts. We normalized the estimates of pathogen transcript abundance by the ddC_T method [65], using the geometric mean C_T value of three honey bee housekeeping genes (actin, RPS5, and mGsT) as a reference for pathogen transcript abundance.

Pesticide analyses. Multi-residue pesticide analysis was conducted by the USDA-AMS-NSL at Gastonia, NC, using a modified QuEChERS method [66]. Of the 22 samples of brood comb that contained beebread, 7 had insufficient quantities (<3 g) to analyze on their own, so samples were pooled with other colonies within the same apiary having the same condition (CCD

Table 1. Quantitative-PCR primers for measuring transcript abundances of honey bee pathogens.

Locus	Forward Primer	Reverse Primer
ABPV	ACCGACAAAGGGTATGATGC	CTTGAGTTTGCGGTGTTCCT
BQCV	TTTAGAGCGAATTCGGAAACA	GGCGTACCGATAAAGATGGA
DWV	GAGATTGAAGCGCATGAACA	TGAATTCAGTGTCGCCCATA
KBV	TGAACGTCGACCTATTGAAAAA	TCGATTTTCCATCAAATGAGC
IAPV_B4SO427	CGAACTTGGTGACTTGAAGG	GCATCAGTCGTCTTCCAGGT
IAPV-F1a	GCGGAGAATATAAGGCTCAG	CTTGCAAGATAAGAAAGGGGG
IAPVpwF16	ACCCCCAACTGCTTTCAACAG	CTGGATATAGTACATTAATGTCCTGC
SBV	GGGTCGAGTGGTACTGGAAA	ACACAACACTCGTGGGTGAC
N. apis	CAATATTTTATTGTTCTGCGAGG	TATATTTATTGTATTGCGCGTGCT
N. ceranae	CAATATTTTATTATTTTGAGAGA	TATATTTATTGTATTGCGCGTGCA
Trypanosome	CTGAGCTCGCCTTAGGACAC	GTGCAGTTCCGGAGTCTTGT
Bact774	GTAGTCCACGCTGTAAACGATG	GACGGGCGGTGTGTRCA
RPS5	AATTATTTGGTCGCTGGAATTG	TAACGTCCAGCAGAATGTGGTA
Am actin	TTGTATGCCAACACTGTCCTTT	TGGCGCGATGATCTTAATTT
MGST	TTGCTCTGTAAGGTTGTTTTGC	TGTCTGGTTAACTACAAATCCTTCTG

doi:10.1371/journal.pone.0006481.t001

or control) (n = 18). Comb wax, beebread, brood, or adult bees (3 g) were extracted with 27 ml of 44% water, 55% acetonitrile, and 1% glacial acetic acid, after which 6 g of anhydrous magnesium sulfate and 1.5 g anhydrous sodium acetate were added. A 1–2 ml portion of the supernatant was then treated with primary secondary amine, anhydrous magnesium sulfate, and C18 (LC only) or graphitized carbon black (GC only). The resulting supernatant was analyzed by both high-performance liquid chromatography/tandem mass spectroscopy (LC/MS-MS) on a Thermo-Fisher TSQ triple quadrupole MS and gas-liquid chromatography/mass spectroscopy (GC/MS) on an Agilent 5975 triple quadrupole MS for up to171 pesticides and related metabolites [46]. Choices of insecticides, fungicides, and herbicides to analyze were based largely on their frequency of use where bees may be exposed (e.g., in-hive miticides, plant systemics), and their potential for bee toxicity. Limit of detections were in the low part per billion (ppb) range.

Genetic analyses. We extracted the DNA from three adult worker bees from each sampled colony (n = 73) using Puregene DNA extraction kits (Gentra systems, Inc.). We then employed an established mitotyping protocol as outlined in Nielsen et al. [42]. This procedure amplifies small (≈1 kb) sections of mitochondrial DNA from the COI and rRNA gene sequences and then subjects them to restriction enzyme digests using *HimfI*, *EcoRI*, and *HincII*. Splicing and banding patterns of the resultant amplified PCR product determined the maternal origin of the bees as either West European (subspecies including *Apis mellifera mellifera*), East European (subspecies including *A. m. ligustica*), North African (*A. m. lamarkii*), or South African (*A. m. scutellata*) after they were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide.

Statistical analyses

Neighboring colony strength ratings. The colonies in all of the mapped CCD apiaries were managed on palletized systems, with either four or six colonies per pallet. Should CCD be caused by an infectious condition or exposure to a common risk factor, we would not expect that colonies in dead or weakened states to be randomly distributed within an apiary but rather be in closer proximity to one another. We tested this hypothesis by comparing the expected and observed frequencies of neighboring colonies (those sharing the same pallet and those with entrances facing in the same direction) with the same or different classifications (dead, weak, or acceptable). As is common in epizootiological studies (e.g. [67]), we examined possible relationships between apparently healthy and diseased colonies by comparing the expected (the number of categorized colonies expected to neighbor one another based on the overall frequency of that condition within an apiary) and observed frequencies of colonies sharing the same strength classification in mapped apiaries using a Chi-square test. The degree (or risk) associated with neighbouring weak or dead colonies in CCD-affected and non-affected apiaries was quantified by calculating odds ratio (95% confidence intervals (logarithmic approximation)). Each neighbor-to-neighbor rating is compared to the reference group as "Adequate – Adequate" neighbor pairings. A P value$\leq$0.05 was considered significant.

CCD characterization. For statistical purposes, we used two methods to compare CCD and control populations. First, we grouped all colonies within an apiary, and thus compared apiary averages for a given measure in CCD vs. control apiaries. This approach averages the measurements from colonies regardless of whether any particular colony showed signs of collapse and so may include data from colonies not suffering from CCD. However, as sampled apiaries contained colonies that were actively collapsing, colonies graded as "adequately strong" or "control" in CCD apiaries could have been at an early, asymptomatic stage of collapse. Comparing CCD vs. control apiaries reduced the sample size and, consequently, the power of statistical analysis.

The second approach compared adequately strong colonies (control) with colonies that were obviously suffering from CCD (or had presumably died from CCD, such as those that had wax samples analyzed for pesticides; n = 11). While this approach increased the statistical power of analysis, it risked including colonies that were at the early stages of collapse in the control group. We performed and report both types of exploratory comparisons; CCD vs. control populations classified at the apiary- and individual-colony level.

Risk explanatory variables analyses. We compared individual- and colony-level measurements between CCD and control apiaries and colonies using Wilcoxon rank sum tests. Nonparametric tests were employed because the basal assumptions of parametric tests (i.e., normality and constant variance) were not satisfied [68]. We assumed that the observations in the two independent samples are representative of the populations of interest. We also compared the incidence (proportion of colonies affected) of the fungal disease chalkbrood (*Ascosphaera apis*), European foulbrood (*Melissococcus pluton*), and spotty brood patterns between the two groups using a Chi-square test or Fisher's exact test when the observed frequency in any cell was less than 5.

Unless otherwise noted, all statistical analyses were carried out using SAS JMP 9.0 [57] When risk factor prevalence data is presented, 95% confidence intervals on the point estimate were calculated by hand to adjust for incident rates based on 100 or fewer cases [8].

Results

Colony strength measurements

As the operational case definition for CCD was based, in part, by a clinical assessment that adult bee populations were in rapid decline, differences between non-affected and CCD-affected colony strength measures are not surprising (Table 2 and 3). These results verify that the application of the operational case definition was able to segregate the two populations in a discreet and non-random way.

Comparison of apiaries and ratings of neighboring colony strength

CCD-affected apiaries contained 3.5 times the number of dead colonies compared to control apiaries. Similarly, CCD apiaries contained 3.6 times more weak colonies compared to control apiaries (Table 4). In CCD apiaries, neighbouring colonies that were both of adequate strength ('acceptable') were 2.3 times less frequent than would have been expected, while neighboring colonies that were both 'weak' or both 'dead' were approximately 1.3 times more frequent than expected (Table 5). The opposite was true in control apiaries, where adequately strong colonies were 2.6 times more likely to neighbor other colonies of adequate strength. Moreover, the odds ratio demonstrated that in CCD apiaries there was an increased risk of colonies being weak or dead when they neighbored other weak or dead colonies (Table 5). This suggests that CCD is either a contagious condition or results from exposure to a common risk factor.

Comparison of protein and mass measurements

None of the measurements of soluble protein, mass, or protein-to-mass ratio were different when colonies from CCD apiaries were compared to colonies from control apiaries (Wilcoxon rank sum test; P>0.10; Table 2). Similarly, no measures of mass, soluble

Table 2. Strength and mean physiological and morphometric measurements of bees from colonies (N_t) located in CCD and control apiaries.

Variable		CCD Apiaries	Mean±S.E.	Median (25th & 75th percentiles)	Control Apiaries	Mean±S.E.	Median (25th & 75th percentiles)	Wilcoxon rank sum test
		N_t			N_t			*P*
Strength	Frames of brood	56	2.0±0.24	2.0 (0.3–3.0)	18	1.7±0.45	1.3 (0.8–1.9)	0.46
	Frames of bees	60	5.4±0.68	4.0 (2.0–8.0)	18	7.8±1.26	6.0 (4.0–9.8)	0.02*
	Ratio bees/brood	53	4.7±0.89	2.0 (1.0–4.0)	17	7.5±1.44	4.5 (4.0–10.0)	0.00*
Proteins#	Proteins in the head [A]	60	2.2±0.18	1.3 (1.1–3.4)	18	1.7±0.27	1.3 (1.1–1.7)	0.48
	Proteins in the abdomen [B]	61	12.7±0.82	10.2 (5.6–12.7)	18	10.0±0.98	10.2 (6.0–12.7)	0.21
	Proteins in the thorax [C]	61	4.1±0.87	4.2 (3.4–4.2)	18	4.4±0.18	4.3 (3.9–4.9)	0.19
	Total proteins [D]	60	16.4±0.82	15.4 (12.2–18.4)	18	14.8±1.21	15.4 (10.3–18.3)	0.71
	Mass of the head [E]	60	12.1±0.13	12.1 (11.3–13.1)	18	12.1±0.21	12.1 (11.4–12.9)	0.91
	Mass of the abdomen [F]	61	64.9±1.99	61.6 (55.2–72.3)	18	59.4±3.36	61.1 (47.8–67.4)	0.27
	Mass of the thorax [G]	61	33.5±0.33	33.8 (31.8–35.6)	18	34.1±0.44	34.3 (32.7–35.6)	0.46
	Total mass [H]	60	103.6±2.43	102.5 (92.3–113.4)	18	101.7±3.97	99.9 (91.5–113.2)	0.78
	Ratio [A]/[E]	60	0.10±0.003	0.10 (0.09–0.11)	18	0.11±0.01	0.11 (0.09–0.12)	0.11
	Ratio [B]/[F]	61	0.18±0.007	0.18 (0.15–0.22)	18	0.16±0.01	0.18 (0.15–0.22)	0.22
	Ratio [C]/[G]	61	0.12±0.003	0.12 (0.11–0.14)	18	0.13±0.01	0.13 (0.12–0.14)	0.41
	Ratio [D]/[H]	60	0.15±0.005	0.15 (0.12–0.17)	18	0.14±0.01	0.14 (0.12–0.17)	0.43
Morphological measures	Centroid size	58	59.7±0.79	58.8 (56.6–61.3)	18	60.9±0.73	60.7 (58.4–63.3)	0.08
	FA	58	1.7±0.116	1.48 (1.30–1.98)	18	1.9±0.11	1.9 (1.5–2.2)	0.04*

FA: Fluctuating asymmetry.
#A total of 6 heads or abdomens or thoraces from one colony were used.
*$P<0.05$.
doi:10.1371/journal.pone.0006481.t002

protein, or protein-to-mass ratio differed between the two types of colonies (Wilcoxon rank sum test; $P>0.06$; Table 3).

Comparison of morphometric measurements

The average forewing centroid size in bees from colonies sampled in CCD apiaries was no different than bees from colonies sampled in control apiaries ($P=0.08$). In contrast, a comparison of the absolute difference between the centroid size in right and left wings (FA_1) revealed that bees from colonies in CCD apiaries were more symmetrical than those in control apiaries (Wilcoxon rank sum test; $P=0.04$; Table 2).

Similarly, the average centroid size in bees sampled from CCD and control colonies was not different ($P=0.34$). Bees from CCD colonies, however, were more symmetrical than those in control colonies (Wilcoxon rank sum test; $P=0.01$; Table 3).

Comparison of overt signs of disease and brood pattern

Six percent of colonies from CCD apiaries had clinical infections of chalkbrood disease (CB) and 8% had clinical infections of European foulbrood (EFB; Table 6). While none of the colonies in control apiaries had clinical infections with these common brood diseases, the incidence of colonies affected did not differ significantly between apiary types (Fisher's exact test: $P>0.50$). Fifty-five percent of colonies from CCD apiaries had spotty brood patterns, which was not different than the 43% of colonies in control apiaries that had the same condition ($P=0.41$).

Colonies suffering from CCD did not have a higher incidence rate of either CB or EFB, nor did they have a greater incidence of poor brood patterns when compared to colonies not apparently suffering from CCD ($P>0.35$; Table 7).

It is of interest to note that EFB-infected larvae found in one apiary suffering from CCD were distinctly corn-yellow in appearance (Figure 2A) as opposed to the usual beige appearance of infected larvae (Figure 2B). Microscopic examination of smears from these samples revealed nearly pure cultures of EFB's causal agent *Melissococcus pluton*. This is unusual, as EFB smears usually reveal high levels of opportunistic bacteria such as *Paenibacilus alvei*, *Brevibacillus laterosporus*, and *Enterococcus faecalis* with little or no evidence of the causal agent *M. pluton* [60].

Comparison of macro-parasite and pathogen prevalence and load

Neither the proportion of colonies affected nor the mean abundance of varroa mites or *Nosema* spp. spores differed between CCD apiaries and control apiaries ($P>0.05$; Table 6). HBTM infection was more than three times as prevalent in control apiaries as compared to CCD apiaries (43% vs. 14% of colonies affected, respectively; $\chi^2=6.41$, $P=0.01$; Table 6). The mean prevalence of HBTM in bees from infected colonies was higher in control apiaries than CCD apiaries (8% vs. 1%, respectively; $\chi^2=7.71$, $P=0.01$; Table 6).

Neither the prevalence of colonies with varroa mites, *Nosema* spp. spores, or HBTM, nor the load of infection for these macro parasites/pathogens differed between CCD and control colonies ($P>0.05$; Table 7).

Comparison of pathogen prevalence

None of the screened pathogens showed higher prevalence or load in colonies from CCD apiaries when compared to colonies from control apiaries (Table 6).

Table 3. Strength and mean physiological and morphometric measurements of bees from colonies considered to be normal (control) or affected by CCD (N_t).

Variable		CCD Colonies	Mean±S.E.	Median (25th & 75th percentiles)	Control Colonies	Mean±S.E.	Median (25th & 75th percentiles)	Wilcoxon rank sum test
		N_t			N_t			*P*
Strength	Frames of brood	38	1.5±0.23	1.0 (0.3–3.0)	36	2.4±0.34	1.9 (0.6–3.5)	0.04*
	Frames of bees	39	3.6±0.64	2.0 (1.0–4.5)	39	8.3±0.86	8.0 (4.0–11.00)	0.00*
	Ratio bees/brood	35	4.9±1.15	2.0 (1.0–5.0)	35	6.0±1.00	4.0 (2.3–8.0)	0.05*
Proteins#	Proteins in the head [A]	39	2.2±0.24	1.3 (1.0–3.5)	39	1.9±0.19	1.3 (1.1–2.7)	0.96
	Proteins in the abdomen[B]	39	13.4±1.11	10.9 (9.6–16.6)	40	10.7±0.77	10.3 (6.7–13.4)	0.12
	Proteins in the thorax [C]	39	4.1±0.111	4.2 (3.5–4.6)	40	4.3±0.16	4.2 (3.7–4.8)	0.40
	Total proteins [D]	39	17.1±1.14	15.4 (12.8–18.4)	39	14.9±0.76	15.4 (10.3–18.4)	0.53
	Mass of the head [E]	39	12.1±0.18	11.9 (11.2–13.2)	39	12.2±0.13	12.1 (11.6–12.9)	0.48
	Mass of the abdomen [F]	39	67.2±2.58	63.9 (57.6–72.7)	40	60.2±2.19	58.9 (49.8–70.0)	0.06
	Mass of the thorax [G]	39	33.2±0.41	33.4 (31.7–35.5)	40	34.1±0.34	34.5 (33.0–35.7)	0.12
	Total mass [H]	39	105.6±3.31	102.7 (91.9–116.7)	39	100.8±2.46	101.5 (92.1–112.6)	0.38
	Ratio [A]/[E]	39	0.10±0.004	0.09 (0.08–0.11)	39	0.10±0.003	0.10 (0.09–0.11)	0.20
	Ratio [B]/[F]	39	0.19±0.008	0.18 (0.16–0.23)	40	0.17±0.008	0.18 (0.13–0.20)	0.16
	Ratio [C]/[G]	39	0.12±0.003	0.12 (0.11–0.14)	40	0.13±0.005	0.13 (0.11–0.14)	0.69
	Ratio [D]/[H]	39	0.16±0.006	0.15 (0.14–0.18)	39	0.14±0.005	0.15 (0.11–0.17)	0.22
Morphological measures	Centroid size	36	59.9±1.17	58.8 (56.5–61.1)	40	60.0±0.59	60.0 (56.9–62.4)	0.34
	FA	36	1.5±0.06	1.4 (1.3–1.8)	40	2.0±0.16	1.9 (1.4–2.2)	0.01*

FA: Fluctuating asymmetry.
#A total of 6 heads or abdomens or thoraces from one colony were used.
*$P<0.05$.
N_t: Number of colonies tested.
doi:10.1371/journal.pone.0006481.t003

Kashmir Bee Virus (KBV) was more prevalent in colonies suffering from CCD as compared to control colonies (42% vs. 8%, respectively; Fisher's exact test $P=0001$; Table 7). KBV virus titers were higher in CCD colonies when compared to control colonies ($P=0.01$; Table 7).

Table 4. Percentage of adequately strong, weak and dead colonies in apiaries containing colonies with symptoms of CCD and apparently healthy (control) apiaries.

Apiary	Location	N	Dead (%)	Weak (%)	Strong (%)
CCD	FL	66	18.1	39.4	42.2
	FL	88	30.6	69.3	0.0
	FL	200	41.0	47.0	12.0
	CA	76	7.9	42.1	50.0
	CA	28	25.0	57.1	17.9
	CA	48	20.8	35.4	43.8
Subtotal		506	28.4	48.6	22.9
Control	FL	64	0	0	100
	CA	34	23.4	38.2	38.2
	CA	88	7.9	13.6	78.4
Subtotal		186	8.1	13.4	78.5

doi:10.1371/journal.pone.0006481.t004

Overall, 55% of CCD colonies were infected with 3 or more viruses as compared to 28% of control colonies (Table 8: $\chi^2=5.4$, $P=0.02$). Both *Nosema* species were equally prevalent in CCD and control colonies (Table 7). However, 34% of CCD colonies were found to be co-infected with both *Nosema* species as compared to 13% of control colonies (Fisher's exact test, $P=0.05$).

CCD colonies were co-infected with a greater number of known pathogenic organisms (viruses and *Nosema* species) than control colonies (4.34±0.37 vs. 3.0±0.37, respectively; Wilcoxon rank sum test $P=0.026$).

Comparison of pesticide prevalence and residue levels

In all, 50 different pesticide residues and their metabolites were found in the 70 wax samples tested, 20 were found in the 18 pollen (beebread) samples tested, 5 in the 24 brood sampled tested, and 28 in the 16 adult bees tested.

There are some notable constraints with this pesticide data set. The number of beebread and adult-bee samples in control apiaries was low. This was largely a result of insufficient amounts of pollen collected from CCD-affected colonies (n = 7), leading to combining colony samples to obtain a sufficient quantity for analysis (n = 3). After adult bees had been distributed for protein and pathogen analysis, there was only one adult bee sample from a colony in a control apiary available for pesticide analysis. Another issue is that pesticides and metabolites were added to the screen as they became identified within samples. Because the beebread samples were analyzed earlier than the adult bee or brood samples, potentially important pesticides (such as chlorothalonil,

Table 5. Observed and expected frequencies of neighboring colonies with similar or different strength ratings in CCD and control apiaries.

Strength Rating		CCD (N = 6)		Control (N = 3)		OR (95% CI)#
Colony 1	Colony 2	Observed	Expected	Observed	Expected	
Adequate	Adequate	28	65	60	23	–
Adequate	Weak	26	26	9	9	5.98 (2.52–14.2)*
Adequate	Dead	15	14	4	5	7.38 (2.36–23.1)*
Weak	Weak	59	44	0	15	255 (15.2–4273)*
Weak	Dead	64	50	3	17	39.5 (12.3–126.5)*
Dead	Dead	25	18	0	7	109.3 (6.42–185.9)*

*$P<0.05$.
#OR: odds ratio; CI: confidence interval.
doi:10.1371/journal.pone.0006481.t005

amitraz metabolites, and the coumaphos metabolite, chlorferone) were left out of the former but not the latter analyses. Also, a majority of the wax samples were not analyzed for amitraz metabolites, the fungicides boscalid and iprodione, and the coumaphos metabolites chlorferone, coumaphos oxon, and potasan. Where only some of the samples in a given matrix were analyzed for coumaphos metabolites, only coumaphos (and not 'total coumaphos' levels - coumaphos plus metabolites) were compared. Lastly, a lack of detection of some chemicals does not necessarily rule out potential exposure. Chemicals that metabolize or break down quickly may have been removed from the various matrixes tested. Alternatively, some chemicals may have been consumed (in the case of beebread) before samples were collected.

There were no differences in the mean number of pesticides detected in the wax of colonies from CCD apiaries (5.96 ± 0.63) compared to colonies from control apiaries (4.87 ± 0.48; $\chi^2=0.125$, $P=0.72$). Similarly, there were no differences in the number of detections in beebread (CCD: 4.18 ± 0.62 vs. control: 7.50 ± 0.62; $\chi^2=1.83$, $P=0.175$) or brood (CCD: 2.15 ± 0.08 vs. control: 2.00 ± 0.00; $\chi^2=0.65$, $P=0.42$).

None of the pesticides detected in more than 20% of the samples in a given matrix was more prevalent in CCD apiaries than in control apiaries (Table 9). There were, however, higher levels of coumaphos in the wax of control apiaries than was detected in CCD apiaries (Wilcoxon rank sum test, $P=0.05$, Table 9).

There were neither differences in the mean number of pesticides detected in the wax of CCD-affected colonies (5.92 ± 0.84) compared to control colonies (5.67 ± 0.84; $\chi^2=0.001$, $P=0.97$) nor the number of detections in beebread (CCD: 5.09 ± 0.71 vs. control: 5.14 ± 1.14; $\chi^2=0.038$, $P=0.85$), brood (CCD: 2.18 ± 0.12 vs. control: 2.07 ± 0.07; $\chi^2=0.57$, $P=0.44$), or adult bees (CCD: 4.37 ± 1.73 vs. control: 9.00 ± 3.88; $\chi^2=0.89$, $P=0.34$).

Esfenvalerate was more prevalent in the wax of control colonies (32%) when compared to CCD colonies (5%) (Fisher's exact test, $P=0.001$; Table 10). Mean levels of this product were also higher in both the wax and adult bees from control colonies when compared to CCD colonies ($P=0.002$ and 0.04, respectively; Table 10). Coumaphos levels in wax, brood, and adult bees were higher in control colonies than in CCD colonies ($P=0.009$, 0.04, and 0.03, respectively; Table 10).

Comparison of mitotypes

Only one of the 98 colonies screened for mitotype was found to be Western European in matrilineal origin. The remaining colonies were all found to be of Eastern European origin. None were positively detected as being African in origin.

Discussion

This descriptive epidemiological study was initiated to better characterize CCD and compare risk-factor exposure between control and afflicted populations in hopes of identifying factors that cause or contribute to Colony Collapse Disorder. Of the more than 200 variables we quantified in this study, 61 were found with enough frequency to permit meaningful comparisons between populations. None of these measures on its own could distinguish CCD from control colonies. Moreover, no single risk factor was found consistently or sufficiently abundantly in CCD colonies to suggest a single causal agent. Nonetheless, our results help to elucidate this poorly understood affliction of the honey bee colonies and provide insight into the planning of hypothesis-driven research.

CCD apiaries contained more dead and weak colonies than did control apiaries and the distribution of dead and weak colonies in CCD apiaries was not random. Dead and weak colonies were more likely to neighbor each other in CCD apiaries as compared to control apiaries (Table 3), suggesting that an infectious agent or the exposure to a common risk factor may be involved in colony collapse.

While no single pathogen or parasite was found with sufficient frequency to conclude a single organism was involved in CCD, pathogens seem likely to play a critical (albeit secondary) role. CCD colonies generally had higher virus loads and were co-infected with a greater number of disease agents than control colonies. Elevated virus and *Nosema* spp. levels potentially explain the symptoms associated with CCD. One possible way honey bees regulate pathogen and parasite loads within a colony is for infected individuals to emigrate from their hive [69]. This behavior has been proposed to explain the rapid loss of adult populations in colonies collapsing from *N. ceranae* [39]. Whether infected individuals die away from the hive as the result of an evolved response (suicidal pathogen removal [69]) or from a sudden debilitating process by which forager bees cannot return to the hive [39] is irrelevant to understanding how colony collapse can unfold. Premature loss of worker bees does not preclude non-pathogenic causes; recent work has shown that worker bee longevity can be reduced when they are exposed to sub-lethal levels of coumaphos during the larval and pupal stages (Pettis, unpublished). The premature loss of forager bees, the older cohort in a colony, results in younger bees prematurely becoming forager bees [70]. If

Table 6. Parasite and pathogen loads of bees from colonies (N_t) located in CCD and control apiaries.

Variable		CCD Apiaries		Load		Control Apiaries		Load		Prevalence		Load (Wilcoxon rank sum test)
		N_t	Prevalence** (95% CI)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence** (95%CI)	Mean±S.E.	Median (25th & 75th percentiles)	χ^{2*}	*P*	*P*
Brood condition	Spotty Brood	49	55 (41–73)			14	43 (23–72)			0.66	0.41	
	Chalkbrood	51	6 (4–8)			17	0				0.56	
	European foulbrood	51	8 (6–11)			17	0				0.57	
Parasites	Varroa†	51	64 (48–84)	0.086±0.0284	0.007 (0–0.038)	17	53 (31–84)	0.020±0.014	0.003 (0–0.014)	0.74	0.39	0.24
	HBTM‡	51	14 (10–18)	1±0.6	0 (0–0)	17	43(25–69)	8±2.9	0 (0–16)	6.41	0.01*	0.01*
	Nosema♠	51	55 (41–72)	1.82±0.486	0.7 (0.0–1.80)	17	35 (20–56)	0.34±0.201	0.0 (0.0–0.15)	1.96	0.16	0.09
Pathogens♥	ABPV	58	45 (34–58)	4.4±0.86	0.0 (0.0–7.5)	18	33 (20–52)	2.3±0.90	0.0 (0.0–5.20)	0.75	0.38	0.29
	Bacteria	58	93 (70–100)	12.7±0.80	13.4 (9.1–18.0)	18	100 (59–100)	13.3±1.25	14.2 (7.7–18.0)		0.57	0.86
	BQCV	58	72 (54–93)	9.6±1.02	10.2 (0–13.8)	18	78 (46–100)	7.7±1.67	5.5 (1.1–15.4)		0.77	0.40
	CBPV	58	33 (25–43)	2.4±0.54	0.0 (0.0–3.8)	18	50 (30–79)	1.5±0.42	0.5 (0–3.27)	1.75	0.19	0.66
	DWV	58	44 (34–57)	5.7±0.99	0.0 (0.0–10.1)	18	66 (39–100)	5.6±1.21	5.50 (0–8.5)	2.62	0.11	0.56
	IAPVF1a	58	22 (17–28)	2.0±0.56	0.0 (0.0–0.0)	18	17 (10–27)	1.6±1.06	0.0 (0.0–0.0)		0.74	0.63
	IAPVpw1617	58	16 (12–21)	1.8±0.62	0.0 (0.0–0.0)	18	6 (3–9)	1.2±1.15	0.0 (0.0–0.0)		0.43	0.32
	IAPV_B4SO427	58	10 (8–13)	1.5±0.60	0.0 (0.0–0.0)	18	6 (3–9)	1.1±1.09	0.0 (0.0–0.0)		1	0.58
	IAPVAvg	58	28 (21–36)	1.8±0.52	0.0 (0.0–1.2)	18	17 (10–27)	1.3±1.03	0.0 (0.0–0.0)		0.53	0.36
	KBV	58	29 (22–37)	3.0±0.76	0.0 (0.0–4.8)	18	2 (1–3)	0.7±0.53	0.0 (0.0–0.0)		0.21	0.11
	SBV	58	16 (12–21)	0.8±0.29	0.0 (0.0–0.0)	18	28 (16–44)	2.0±0.84	0.0 (0.0–4.5)	1.37	0.24	0.21
	Nosema ceraneae	58	47 (36–61)	5.8±1.00	0.0 (0.0–12.9)	18	72 (42–100)	6.0±1.60	0.0 (0.0–14.6)	3.63	0.06	0.31
	Nosema apis	58	28 (21–36)	3.5±0.83	0.0 (0.0–6.47)	18	11 (7–17)	0.09±0.07	0.0 (0. 0–0.0)		0.21	0.09
	Trypanasomes	58	76 (58–98)	8.3±0.84	8.8 (0.0–13.6)	18	94 (56–100)	10.6±1.54	8.17 (5.5–17.7)		0.10	0.26

*Where no statistic is presented, Fisher's exact test was used as some cells had fewer than 5 responses.

**% of colonies infected with organism.

†Load = mean abundance (number of varroa mites per bee) in colonies.

‡Load = the percentage of bees infested with HBTM per colonies.

♠Load = mean abundance (number of spores per bee ($\times 10^6$)) in colonies.

♥♥All pathogen loads are scaled relative to the geometric mean of honey bee housekeeping genes RPS5, MGsT, and actin [37].

doi:10.1371/journal.pone.0006481.t006

Table 7. Parasite and pathogen loads of bees from colonies considered to normal (control) or affected by CCD (N_t).

Variable		CCD Colonies		Load		Control Colonies		Load		Prevalence		Load (Wilcoxon rank sum test)
		N_t	Prevalence **(95% CI)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence **(95% CI)	Mean±S.E.	Median (25th & 75th percentiles)	X^{2*}	*P*	*P*
Brood condition	Spotty Brood	35	60 (42–83)			27	52 (34–76)			0.04	0.85	
	Chalkbrood	38	5 (4–7)			39	3 (2–4)				0.62	
	European foulbrood	38	8 (6–11)			39	3 (2–4)				0.36	
Parasites	Varroa†	32	53 (36–75)	0.054±0.020	0.002 (0.0–0.037)	36	70 (49–97)	0.084±0.0372	0.007 (0.0–0.0 29)	1.91	0.17	0.37
	HBTM‡	32	19 (13–27)	0±0.0	0 (0–0)	36	22 (15–30)	10±2	0 (0–0)	0.13	0.72	0.44
	Nosema♠	32	63 (43–89)	1.9±0.59	0.1 (0.0–1.9)	36	39 (27–54)	1.0±0.47	0.0 (0.0–0.3)	3.79	0.05*	0.06
Pathogens♥	ABPV	38	47 (33–65)	5.1±1.23	0.0 (0.0–8.7)	38	37 (26–51)	2.6±0.62	0.0 (0.0–6.1)	0.86	0.35	0.22
	Bacteria	38	97 (69–100)	12.9±0.91	13.6 (9.2–17.6)	38	92 (65–100)	12.8±0.98	14.1 (8.4–18.0)		0.61	1.00
	BQCV	38	79 (56–100)	10.8±1.31	11.6 (3.5–14.6)	38	68 (48–93)	7.4±1.09	6.3 (0.0–13.7)	1.09	0.30	0.07
	CBPV	38	37 (26–51)	2.6±0.70	0.0 (0.0–3.8)	38	37 (26–51)	1.9±0.51	0.0 (0.0–3.3)	0.00	1.00	0.73
	DWV	38	61 (43–84)	7.7±1.3	6.5 (0.0–12.9)	38	40 (28–55)	3.6±0.86	0.0 (0.0–7.0)	3.37	0.07	0.02*
	IAPVF1a	38	24 (17–33)	2.1±0.69	0.0 (0.0–0.8)	38	18 (13–25)	1.7±0.71	0.0 (0.0–0.0)	0.32	0.57	0.57
	IAPVpw1617	38	16 (11–22)	2.0±0.82	0.0 (0.0–0.0)	38	11 (8–15)	1.3±0.71	0.0 (0.0–0.0)		0.74	0.48
	IAPV_B4SO427	38	11 (8–15)	1.6±0.80	0.0 (0.0–0.0)	38	8 (6–11)	1.2±0.69	0.0 (0.0–0.0)		1.00	0.67
	IAPVAvg	38	29 (21–40)	1.9±0.68	0.0 (0.0–1.5)	38	21 (15–29)	1.4±0.63	0.0 (0.0–0.0)	0.63	0.43	0.42
	KBV	38	42 (30–58)	4.4±1.10	0.0 (0.0–7.0)	38	8 (6–11)	0.5±0.29	0.0 (0.0–0)		0.00*	0.00*
	SBV	38	18 (13–25)	1.1±0.41	0.0 (0.0–0.0)	38	18 (13–25)	1.1±0.44	0.0 (0.0–0.0)	0.00	1.00	0.98
	Nosema ceranae	38	55 (39–76)	6.9±1.30	2.3 (0.0–13.8)	38	50 (35–67)	4.8±1.09	0.7 (0.0–9.8)	0.21	0.65	0.34
	Nosema apis	38	29 (21–40)	3.5±1.03	0.0 (0.0–6.5)	38	18 (13–25)	1.8±0.80	0.0 (0.0–0.0)	1.16	0.28	0.25
	Trypanosomes	38	82 (58–100)	9.6±1.00	9.8 (5.0–14.1)	38	79 (56–100)	8.2±1.09	7.0 (1.1–13.1)	0.08	0.77	0.31

*Where no statistic is presented, Fisher's exact test was used as some cells had fewer than 5 responses.
**% of colonies infected with organism.
†Load = mean abundance (number of varroa mites per bee) in colonies.
‡Load = the percentage of bees infested with HBTM per colonies.
♠Load = mean abundance (number of spores per bee ($\times 10^6$)) in colonies.
♥♥All pathogen loads are scaled relative to the geometric mean of honey bee housekeeping genes RPS5, MGsT, and actin [37].
doi:10.1371/journal.pone.0006481.t007

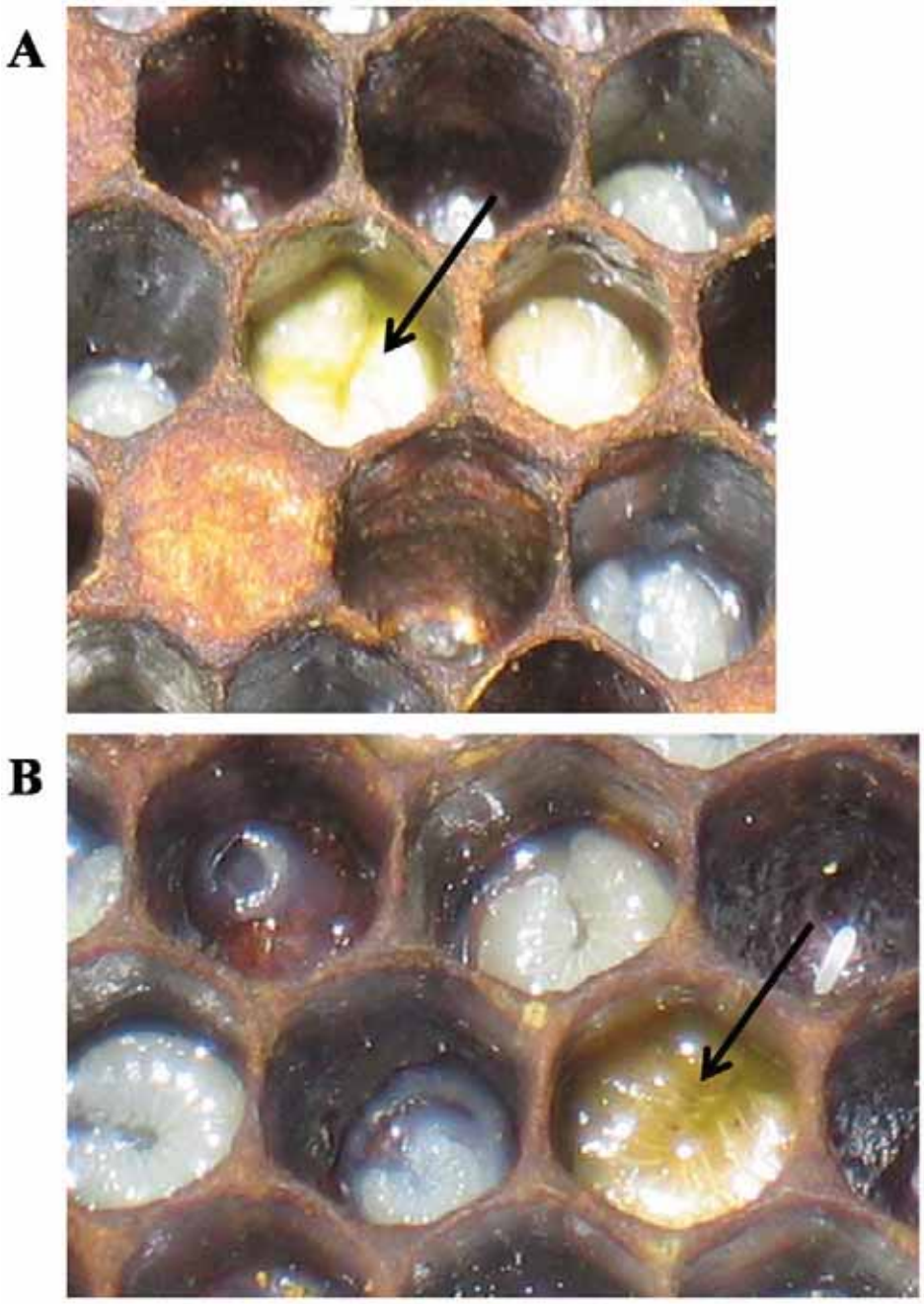

Figure 2. EFB-infected larvae (←) in some CCD-affected colonies were ''corn yellow'' (A) rather than the typical ''beige yellow'' (B).
doi:10.1371/journal.pone.0006481.g002

these replacement bees die at a rate that exceeds the colony's ability to replace them, the result would be rapid depopulation, a reduction in the bee-to-brood ratio, and eventually colony failure.

This study verified initial field observations [1] that there was a difference in the bee-to-brood ratio between CCD-affected populations when compared to controls. If the bees in colonies undergoing CCD collapse are young bees (as field observations suggest), we would expect to find indirect evidence of this in the measures of parasite loads with known associations to bee age. Tracheal mite loads increase as bees age [71], possibly explaining why HBTM incidence and prevalence were higher in control apiaries than in CCD-affected apiaries. Alternatively, HBTM levels may be lower in CCD colonies because infested individuals left the colony.

An unavoidable bias that results from sampling colonies in the midst of collapse is that only surviving bees are collected. These bees, arguably, are the least sick or most fit individuals. Asymmetry is expected to increase when stressful conditions disturb the normal development of insects [72]. In honey bees specifically, increased levels of symmetry correlates to increased fitness [53]. Bees from colonies suffering from CCD were consistently more symmetrical than those from control colonies. It is therefore reasonable to assume that bees surviving in CCD colonies, while young, were the fittest bees, surviving longer than their less-fit sisters. While this assumption needs to be verified experimentally, a comparison of the ranges of FA in populations of bees from CCD colonies versus control colonies provides tacit support to this hypothesis. The lower ranges of FA

Table 8. Percentage of Control and CCD colonies infected with Y or more viruses.

Colony classification	n	Percentage (%)				
	Y	1	2	3	4	5
Control		81.6	60.5	28.9	15.8	7.9
CCD		84.2	71.1	55.3	31.6	23.7
	X^2	0.09	0.94	5.4	2.6	Fisher's
	P	0.76	0.33	0.02	0.10	0.05

doi:10.1371/journal.pone.0006481.t008

measures were comparable between CCD and control populations (25^{th} percentile: 1.3 vs. 1.4 for CCD and control colonies, respectively), while the upper range of FA measures was notably higher in control colonies when compared to CCD colonies (75^{th} percentile: 2.2 vs. 1.8, respectively), suggesting that bees in CCD colonies under the most development stress (and with the greatest FA) had left or been removed from colonies before sampling.

Recently, *N. ceranae* was linked to colony losses in Spain [73], and a subsequent study documented how pathogen levels developed over time. In the final stages of collapse, the young bees remaining in the colony became heavily infected with this agent [39]. Our survey found only about half of the colonies sampled, both in CCD and control populations, were infected with *N. ceraneae*, and while some colonies had levels of infection that likely contributed to colony loss, this was not the case for the majority.

In a previous study using subsamples from the same colonies sampled here, IAPV was identified as highly correlated to CCD [3]. This expanded study did not replicate those results. The overall incidence of IAPV reported here was generally lower than found in the prior survey. This result might reflect decreased sensitivity of the assay used here, although prevalence of other viruses generally was comparable to prior results. Alternatively, the discrepancy in findings might reflect unappreciated genetic variation across lineages of IAPV, to the extent that primers poorly matched template cDNA. To minimize this risk, we estimated transcript levels using three published primer pairs for three regions of the genome, and we found broadly concordant results (Tables 6 and 7). As in [3], we treated products for any of the three used primer pairs as evidence for IAPV presence. Finally, the current survey included more colonies and covered a wider geographical range than the previous survey. IAPV shows strong geographical patterns (Evans JD et al., unpublished), and it is expected that surveys for this and other pathogenic viruses will differ across apiaries and regions [74].

The intrinsic bias associated with sampling only surviving (and presumably the least-sick) bees did not prevent us from establishing that workers in CCD colonies were more ill than those in control colonies. Co-infection with both *Nosema* species was 2.6 times greater in CCD colonies when compared to control colonies, and colonies co-infected with 4 or more viruses were 3.7 times more frequent in CCD colonies than in control colonies. While honey bee colonies are commonly infected with one or more pathogens, often without exhibiting overt signs of illness [75], the greater prevalence and abundance of infectious agents in CCD colonies does suggest that either they were exposed to a greater number of pathogens or their ability to fight infection had been compromised.

Several factors are known or suspected to be able to compromise the honey bee immune response. One proposed factor is poor nutrition. In this study, we measured protein content as a surrogate for evidence of poor nutrition in CCD colonies, and these results suggest that nutrition does not play a decisive factor. However, caution is needed in drawing strong inferences from these findings, as nutritional deficiencies may have much more subtle effects on bee development and immunity than can be detected with our methods.

Chronic or sub-lethal exposure to agricultural- or beekeeper-applied pesticides can weaken the honey bee immune system [48], hampering the ability of bees to fight off infection. This study found no evidence that the presence or amount of any individual pesticide occurred more frequently or abundantly in CCD-affected apiaries or colonies. In fact, the opposite was true; two products, esfenvalerate in wax, and coumaphos in wax, brood, and adult bees were found more frequently and at higher levels in control colonies than in CCD colonies.

Esfenvalerate or fenvalerate (racemic form), a pyrethroid insecticide, is considered to be highly toxic to bees [76], but its threat to honey bees is thought to be minimal as it tends to repel them. Exposed forager bees are thought to die in the field before returning to the hive [77], so detection of this product in wax is curious. Finding this product more frequently and at higher levels in control colonies may be spurious, however, similar residue levels in both CCD and control apiaries suggest uniform in-field exposure between populations.

Coumaphos is a product used by beekeepers to control varroa mites. Elevated levels of this product in control apiaries suggest that beekeepers managing those apiaries had more aggressively controlled for this parasitic mite than beekeepers managing CCD apiaries. In addition, control apiaries tended to have higher levels of fluvalinate ($P = 0.06$), another approved acaricide. Regardless of these differences in mite-control compounds, we were unable to detect differences in varroa mite levels in CCD- compared to control apiaries or colonies, suggesting that this mite was not the immediate cause of CCD. This does not necessarily mean that mite infestations have no role in collapse. It is possible that some of the sampled colonies had their mite populations controlled by miticides a few months prior to our sampling. Thus, while mite populations were comparable between the two groups at the time of sampling, there may have been a difference in the mite populations prior to mite treatment applications. Varroa mite parasitism is known to weaken the bees' immune system [78] and facilitate the transmission of viruses to brood and adult bees [79]. Further, high virus levels resulting from high populations of varroa mites are not always immediately suppressed by effective mite control [80]. The potential "legacy" effect of high mite populations in CCD-affected colonies should be the focus of future longitudinal epidemiological studies prior to the categorical dismissal of varroa mites as a causal or contributing agent in CCD.

Coumaphos, an organophosphate, is lipophilic, and so accumulates in wax. Increased levels of the compound in wax have been shown to decrease survivorship of developing queens [81,82]. Similar results with worker bees have also been recorded (Pettis, unpublished). A quick method to assess larval survival is to quantify the number of empty brood cells in an area of capped brood or, to

Table 9. The pesticide residue prevalence and load in wax, beebread, and brood from colonies (N_t) located in CCD and control apiaries.

Matrix	Chemical	CCD Apiaries		Load		Control Apiaries		Load		Prevalence (Fisher's exact test)	Load (Wilcoxon rank sum test)
		N_t	Prevalence (%)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence (%)	Mean±S.E.	Median (25th & 75th percentiles)	*P*	*P*
Wax	Boscalid	30	40 (27–57)	29.7±8.46	0.0 (0.0–0.0)	5	0			0.14	0.09
	Chlorothalonil	62	33 (25–42)	24.0±9.11	0.0 (0.0–7.75)	8	13(7–27)	4.9±1.72	0.0 (0.0–0.0)	0.42	0.25
	Chlorpyrifos	62	71 (54–91)	6.3±1.23	4.1 (0.0–7.5)	8	100 (43–100)	5.8±0.64	6.4 (4.1–7.4)	0.11	0.20
	Coumaphos	62	100 (77–100)	3373±552	1750 (719–4085)	8	100 (43–100)	6398±1815	6050 (2110–8992)	1.00	0.05*
	Dicofol	62	17 (13–22)	4.1±3.33	0.0 (0.0–0.0)	8	0			0.34	0.20
	Endosulfan	62	25 (19–32)	4.4±2.16	0.0 (0.0–0.33)	8	13(7–27)	0.6±0.55	0.0 (0.0–0.0)	0.67	0.42
	Esfenvalerate	62	13 (10–17)	1.7±0.97	0.0 (0.0–0.0)	8	38(16–75)	1.0±0.52	0.0 (0.0–2.10)	0.11	0.09
	Fluvalinate	62	100 (77–100)	12508±1718	8530 (2452–15608)	8	100 (43–100)	41737±23748	19800 (6510–42575)	1.00	0.06
	Iprodine	35	21 (15–30)	48.9±20.97	0.0 (0.0–0.0)	5	0			0.56	0.26
Beebread	Atrazine	16	32 (18–52)	8.4±16.61	0.0 (0.0–4.57)	2	0	0.0±0.0	0.0 (0.0–0.0)	1.00	0.37
	Chlorpyrifos	16	88 (50–100)	1.8±0.74	0.65 (0.32–1.75)	2	0	0.8±0.05	0.8 (0.7–0.8)	1.00	0.62
	Coumaphos	16	50(29–81)	18.5±7.7	2.1 (0.0–26.3)	2	50 (6–100)	3.6±3.6	0.0 (0.0–4.5)	1.00	0.89
	Dicofol	16	19 (11–31)	0.2±0.49	0.0 (0.0–0.0)	2	100 (12–100)	0.6±0.15	0.0 (0.0–0.7)	0.07	0.08
	Endosulfan1	16	32 (18–52)	0.2±0.38	0.0 (0.0–56)	2	50 (6–100)	0.4±0.4	0.4 (0.0–0.8)	1.00	0.50
	Fenpropathrin	16	50 (29–81)	1.0±0.30	0.4 (0.0–1.6)	2	100 (12–100)	1.5±0.70	1.5 (0.8–2.2)	0.47	0.30
	Fluvalinate	16	94 (54–100)	276±162.5	76 (8–270)	2	100 (12–100)	68±56	68 (12–124)	1.00	0.78
	Malathion	16	19 (11–31)	0.7±0.48	0.0 (0.0–0.0)	2	50 (6–100)	1.8±1.8	1.8 (0–3.6)	0.41	0.29
	Tebuthiuron	16	38 (22–62)	5.2±2.57	0.0 (0.0–2.85)	2	50 (6–100)	24±24	24 (0–48)	1.00	0.42
Brood	Coumaphos	20	100 (61–100)	51.8±12.83	27.5 (5.6–101)	4	100 (27–100)	92.4±32.2	114 (24–139)	1.00	0.33
	Fluvalinate	20	100 (61–100)	844±315	282 (149–930)	4	100 (27–100)	887±418	817 (127–1720)	1.00	0.53

Only pesticides found in 20% or more of samples are reported.

doi:10.1371/journal.pone.0006481.t009

Table 10. The pesticide residue prevalence and load in wax, beebread, brood and adult bees from colonies considered to be normal (control) or affected by CCD (N_t).

Matrix	Chemical	CCD Colonies		Load		Control Colonies		Load		Prevalence (Fisher's exact test)	Load (Wilcoxon rank sum test)
		N_t	Prevalence (%) (95%CI)	Mean±S.E.	Median (25th & 75th percentiles)	N_t	Prevalence (%) (95%CI)	Mean±S.E.	Median (25th & 75th percentiles)	*P*	*P*
Wax	Boscalid	20	40 (24–62)	35.9±11.96	0.0 (0.0–66.0)	15	27 (15–45)	11.59±5.62	0.0 (0.0–0.0)	0.48	0.27
	Chlorothalonil	42	36 (26–49)	22.5±7.53	0.0 (0.0–16.15)	28	21 (14–30)	19.88±17.04	0.0 (0.0–0.0)	0.28	0.16
	Chlorpyrifos	42	73 (53–99)	6.5±1.62	4.1 (0.0–7.52)	28	75 (50–100)	5.83±1.25	5.6 (0.25–7.4)	1	0.68
	Coumaphos	42	100 (72–100)	2645±500	1335 (524–3320)	28	100 (67–100)	5330±1053	4090 (1435–6270)	1	0.0*
	Dicofol	42	19 (14–26)	5.3±4.92	0.0 (0.0–0.0)	28	11 (7–16)	0.9±0.58	0.0 (0.0–0.0)	0.51	0.43
	Endosulfan	42	21 (15–28)	2.0±0.94	0.0 (0.0–0.0)	28	25 (17–36)	6.88±4.57	0.0 (0.0–1.2)	0.77	0.67
	Esfenvalerate	42	5 (4–7)	1.4±1.33	0.0 (0.0–0.0)	28	32 (21–46)	1.98±0.84	0.0 (0.0–1.6)	0.00*	0.00*
	Fluvalinate	42	100 (72–100)	11825±1906	9420 (3067–15275)	28	100 (67–100)	21844±7350	9565 (2570–29675)	1	0.53
	Iprodine	24	21 (13–31)	32.5±15.83	0.0 (0.0–0.0)	14	14 (8–24)	59.5±42.6	0.0 (0.0–0.0)	1	0.75
Beebread	Atrazine	11	36 (18–64)	8.8±5.31	0 (0–4.7)	7	16 (6–33)	5.42±5.42	0.0 (0.0–0.0)	0.60	0.32
	Chlorpyrifos	11	91 (45–100)	2.14±1.03	0.7 (0.3–1.8)	7	86 (35–100)	1.0±0.47	0.7 (0.4–0.8)	1	0.82
	Coumaphos	11	45 (22–81)	24.02±10.73	0 (0–67)	7	57 (23–100)	5.4±2.87	4.2 (0.0–7.2)	1	0.81
	Dicofol	11	18 (9–32)	0.22±0.16	0 (0–0)	7	42 (17–87)	0.29±0.15	0.0 (0.0–0.7)	0.32	0.39
	Endosulfan1	11	27 (12–48)	0.18±0.10	0 (0–0.5)	7	43 (17–89)	0.37±0.18	0.0 (0.0–0.8)	0.63	0.39
	Fenpropathrin	11	54 (27–48)	1.45±0.41	0.8 (0–1.6)	7	57 (23–100)	0.83±0.36	0.8 (0.0–2.0)	1	0.85
	Fluvalinate	11	100 (50–100)	351±235	94 (7.4–339)	7	86 (35–100)	99±42.1	59 (12–193)	0.38	0.75
	Malathion	11	27 (13–48)	0.96±0.70	0 (0–0.9)	7	14 (6–29)	0.51±0.51	0 (0–0)	1	0.57
	Tebuthiuron	11	27 (13–48)	4.69±3.07	0 (0–1.6)	7	57 (23–100)	11.42±7.12	0 (0–27)	0.33	0.19
Brood	Coumaphos	13	100 (53–100)	30.76±11.88	21 (3.9–40.5)	11	100 (50–100)	91.39±18.26	111 (17–137)	1	0.04*
	Fluvalinate	13	100 (53–100)	1044±479	279 (135–1043)	11	100 (50–100)	623±197	364 (149–1270)	1	0.67
Adults	Chlorothalonil	9	33 (15–63)	4.56±4.07	0.0 (0.0–2.0)	7	29 (12–60)	0.81±0.53	0.0 (0.0–2.5)	1	0.89
	Chlorpyrifos	9	33 (15–63)	0.37±0.19	0.0 (0.0–1.0)	7	29 (12–60)	0.34±0.22	0.0 (0.0–1.0)	1	0.94
	Coumaphos	9	78 (36–100)	48.3±40.12	7.4 (0.5–22.5)	7	100 (40–100)	57.2±15.53	65 (11–100)	0.48	0.03*
	Esfenvalerate	9	43 (20–82)	0±0	0.0 (0.0–0.0)	7	11 (4–23)	3.75±1.77	0.0 (0.0–8.5)	0.26	0.04*
	Endosulfan (total)	9	0	0.9±0.9	0.0 (0.0–0.0)	7	29 (12–60)	0.8±0.8	0.0 (0.0–0.0)	0.18	0.92
	Fluvalinate	9	100 (46–100)	1769±814.6	238 (88.5–4540)	7	100 (40–100)	333±215.0	142 (91–185)	1	0.22

Only pesticides found in 20% or more of samples are reported.

doi:10.1371/journal.pone.0006481.t010

use the beekeeper colloquial term, brood "spottiness". We found no evidence that bees from control colonies had a greater frequency of spotty brood than CCD colonies despite the elevated levels of coumaphos in wax in the control colonies. This suggests that bees in control colonies had developed a tolerance to coumaphos exposure. Coumaphos-tolerant bees may be afforded protection through several routes. First, by living on wax comb with elevated miticide levels, varroa mite populations may remain lower than they would in colonies with lower levels of coumaphos residues in their brood nest. However, as coumaphos-resistant mites are widespread in the U.S. [81], this explanation seems unlikely unless coumaphos-resistant mites are less fit than non-resistant mites. Even a small reduction in the reproductive fitness of varroa mites could have a pronounced effect on their population growth and thus their effect on colony health [83]. Second, coumaphos (and/or fluvalinate) tolerance in bees provides cross-resistance to pesticide exposures from other organophosphates and pyrethroids [84] which may be affecting CCD-afflicted bees at sub-lethal doses. Honey bees, as compared to other insects, are notably lacking in detoxification enzymes which provide moderate levels of cross-resistance to pesticides [85]. Any enhancement in these enzyme levels may greatly improve the ability of bees to tolerate the numerous pesticides they encounter in-hive or while foraging.

When unexplained disease outbreaks occur, epidemiologists use descriptive studies to help identify possible cause(s). By definition, descriptive studies are non-hypothesis driven but rather highlight differences between diseased and non-diseased populations in an effort to inform future research.

This descriptive study looked for differences in colony strength, morphometrics, and risk factors in CCD and control colonies. Like all descriptive studies, we cannot make any definitive statement concerning which factors do or do not contribute to or cause CCD. However, our results permit some valuable inferences to be drawn, as the distribution of CCD-infected colonies was not random in infected apiaries and thus the underlying factor is likely contagious or caused by exposure to a common risk factor(s). As no one disease agent was found in all CCD colonies, and because bees derived from CCD colonies were infected with more pathogens then their control colony counterparts, we suspect that while pathogen infection may cause the symptoms of collapse, these infections are secondary and are the result of some other factor or combination of factors that reduce the bees' ability to mitigate infection. As mentioned throughout the text, these inferences must be considered in concert with the limitations and assumptions that are intrinsic to epidemiological studies.

For practical reasons, quantifying most factors in honey bee colonies (e.g., parasite loads, physiological measures, pesticide and pathogen loads) involves testing a sub-sample of colonies in a population. While increasing sample size would obviously result in increased test specificity, this was not always logistically possible. Moreover, our approach assumes that the factor(s) responsible for CCD would occur with high frequency in the affected population. Should this not be the case, our efforts may not have been resolute enough to detect it. Our study also assumes that the factor(s) responsible for CCD were present in the colonies at the time of sample collection, which also may not have been the case. For example, if pollen contaminated with a pesticide were responsible for CCD, contaminated pollen would have been consumed prior to sample collection and thus would not have been detected in the samples collected. Similarly, bees infected with the causative disease agent could have died away from the colony and thus not collected. Finally, Varroa mites or other parasites could have differed among populations prior to sampling, but effective control measures masked these differences at the time of sample collection.

Descriptive studies rely on operational case definitions. The case definition used in this study was applied by experienced bee clinicians using easily observable characteristics [9]. While the application of the case definition may have misdiagnosed colonies, our finding that colony strength measures differed between CCD and control colonies suggests the classification of colonies into affected and non-affected groups was not random. As with other descriptive studies based on case definitions, our findings enable us to propose refining the operational case definition of CCD [8]. In addition to the characteristics of CCD colonies previously described—(1) no dead bees in the colonies or apiary, (2) adult populations rapidly declined leaving brood poorly or completely unattended, and (3) the absence of robbing or kleptoparasitism in collapsed colonies—we now propose that the operational case definition for CCD include (4) at the time of collapse, varroa mite and nosema populations are not at levels known to cause economic injury or population decline. This additional characteristic should assist in distinguishing diminishing populations associated with elevated levels of varroa mites (and virus) [86] and *N. ceranae* [39] from collapsing populations associated with CCD.

The primary aim of descriptive studies is to help narrow future efforts that attempt to identify the cause of disease. This study suggests that future, longitudinal studies should focus on monitoring parasite (varroa mite), pathogen, and pesticide loads while quantifying pesticide tolerance in study populations. More specific studies that investigate potential interactions among pesticides and pathogen loads are also warranted.

This is the first descriptive epizootiological survey of honey bee colonies that provides evidence that the condition known as CCD is consistent with a contagious condition or reflective of common risk factors within apiaries Of the 61 variables quantified (including adult bee physiology, pathogen loads, and pesticide levels), no single factor was found with enough consistency to suggest one causal agent. Bees in CCD colonies had higher pathogen loads and were co-infected with more pathogens than control populations, suggesting either greater pathogen exposure or reduced defenses in CCD bees. Levels of the miticide coumaphos were higher in control populations than CCD-affected populations. Potentially important areas for future hypothesis-driven research, including the possible legacy effect of mite parasitism and role of honey bee resistance to pesticides, are highlighted.

Acknowledgments

We would like to thank Nathan Rice for field assistance; Anne Marie Trimble, Sara Ashcraft, Dawn Lopez, Michele Hamilton, and Vic Levi for laboratory assistance; Michael Andree, Sharon McDonald, Karen Roccasecca, and Linda Wertz for sample analysis; Joel Caren, John Harman, Winnie Lee, Kelly Hyland, and Flora Lee for their assistance in performing the protein and mitotype analyses; Yonglian Fan and Christina Grozinger for providing the spectrophotometer; and Roger Simonds for the supervision of chemical analyses.

Author Contributions

Conceived and designed the experiments: Dv JSP. Performed the experiments: Dv JDE CM MF JF DRT. Analyzed the data: Dv CS EH BKN RMU. Contributed reagents/materials/analysis tools: JDE CM MF JF DLCF YPC DRT. Wrote the paper: Dv JDE CS CM EH BKN RMU DRT JSP.

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News Releases By Date

New Pesticide Labels Will Better Protect Bees and Other Pollinators

Release Date: 08/15/2013
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WASHINGTON – In an ongoing effort to protect bees and other pollinators, the U.S. Environmental Protection Agency (EPA) has developed new pesticide labels that prohibit use of some neonicotinoid pesticide products where bees are present.

“Multiple factors play a role in bee colony declines, including pesticides. The Environmental Protection Agency is taking action to protect bees from pesticide exposure and these label changes will further our efforts,” said Jim Jones, assistant administrator for the Office of Chemical Safety and Pollution Prevention.

The new labels will have a bee advisory box and icon with information on routes of exposure and spray drift precautions. Today’s announcement affects products containing the neonicotinoids imidacloprid, dinotefuran, clothianidin and thiamethoxam. The EPA will work with pesticide manufacturers to change labels so that they will meet the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) safety standard.

In May, the U.S. Department of Agriculture (USDA) and EPA released a comprehensive scientific report on honey bee health, showing scientific consensus that there are a complex set of stressors associated with honey bee declines, including loss of habitat, parasites and disease, genetics, poor nutrition and pesticide exposure.

The agency continues to work with beekeepers, growers, pesticide applicators, pesticide and seed companies, and federal and state agencies to reduce pesticide drift dust and advance best management practices. The EPA recently released new enforcement guidance to federal, state and tribal enforcement officials to enhance investigations of beekill incidents.

More on the EPA’s label changes and pollinator protection efforts: http://www.epa.gov/opp00001/ecosystem/pollinator/index.html

View the infographic on EPA’s new bee advisory box: http://www.epa.gov/pesticides/ecosystem/pollinator/bee-label-info-graphic.pdf

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NATURE

24 JUL

GMOs May Feed the World Using Fewer Pesticides

By Amy Maxmen on Wed, 24 Jul 2013

Walter De Jong shouts over the roar of fans in the greenhouse. He's telling me about the seedlings beside him, which pepper the dark soil in a grid of small planting pots. De Jong, a potato breeder and geneticist at Cornell University in Ithaca, New York, hopes that at least one of the plants will yield a best seller, but it's far more likely that they'll amount to compost.

De Jong produced the plants in the same old, laborious way that his father did before him. He collected pollen from a plant that produces potatoes that fry as potato chips should and then sprinkled the pollen on the flower of a potato plant that resists viruses. If the resulting potatoes bear their parents' finest features—and none of the bad ones—De Jong will bury them in the ground next year and test their mettle against a common potato virus. If they survive—and are good for frying and eating—he and his team will repeat this for 13 years to ensure that problematic genes did not creep in during the initial cross.

Walter De Jong, a second-generation potato breeder, walks the fields in upstate New York.

Each year, the chance of failure is high. Potatoes that resist viruses, for example, often have genes that make them taste bitter. Others turn an unappetizing shade of brown when fried. If anything like that happens, De Jong will have to start from scratch. Tedious as it is, he loves the work. Kicking up dirt in the furrows that cascade along the hillsides of upstate New York, he says, "I'm never stressed in the potato fields."

De Jong has some serious cred in the agriculture world. Not only was his father a potato breeder, he's also descended from a long line of farmers. The potato farmers he works with appreciate this deeply, along with his commitment to the age-old craft of producing new potato varieties through selective breeding. They even advocated on his behalf during his hiring and when he was up for tenure at Cornell, a school with a long history of agriculture research. "All of our farmers like Walter," says Melanie Wickham, the executive secretary of the Empire State Growers organization in Stanley, New York. Often, he's in the fields in a big hat, she says. Other times "you'll see him in the grocery store, looking over

the potatoes."

De Jong has been working with farmers long enough to know that our food supply is never more than a step ahead of devastating insect infestations and disease. Selective breeders like De Jong work hard to develop resistant crops, but farmers still have to turn to chemical pesticides, some of which are toxic to human health and the environment. De Jong enjoys dabbing pollen from plant-to-plant the old-fashioned way, but he knows that selective breeding can only do so much.

Seedlings from De Jong's selective breeding experiments poke their shoots above the soil in a greenhouse.

So while De Jong still devotes most of his time to honing his craft, he has recently begun to experiment in an entirely different way, with genetic engineering. To him, genetic engineering represents a far more exact way to produce new varieties, rather than simply scrambling the potato genome's 39,000 genes the way traditional breeding does. By inserting a specific fungus-defeating gene into a tasty potato, for example, De Jong knows he could offer farmers a product that requires fewer pesticides.

"We want to make food production truly sustainable," De Jong says, "and right now I cannot pretend that it is."

The need to protect crops from ruin grows more vital every day. By 2050, farmers must produce 40% more food to feed an estimated 9 billion people on the planet. Either current yields will have to increase or farmland will expand farther into forests and jungles. In some cases, genetically modified organisms (GMOs) would offer an alternative way to boost yields without sacrificing more land or using more pesticides,

De Jong says. But he fears this approach won't blossom if the public rejects GMOs out of hand.

When It All Began

In the late 1990s, the agriculture corporation Monsanto began to sell corn engineered to include a protein from the bacteria *Bacillus thuringiensis*, better known as Bt. The bacteria wasn't new to agriculture—organic farmers spray it on their crops to kill certain insects. Today more than 60% of the corn grown within the United States is Bt corn. Farmers have adopted it in droves because it saves them money that they would otherwise spend on insecticide and the fuel and labor needed to apply it. They also earn more money for an acre of Bt corn compared with a conventional variety because fewer kernels are damaged. Between 1996 and 2011, Bt corn reduced insecticide use in corn production by 45% worldwide (110 million pounds, or roughly the equivalent of 20,000 Olympic swimming pools).

Between 1996 and 2001, Monsanto also produced Bt potato plants. Farmers like Duane Grant of Grant 4D Farms in Rupert, Idaho, welcomed the new variety. Grant grew up on his family's farm, and his distaste for insecticides started at a young age. As a teenager, he recalls feeling so nauseous and fatigued after spraying the fields that he could hardly move until the next day. Today, pesticides are safer than those used 40 years ago, and stiffer U.S. federal regulations require that employees take more precautionary measures when applying them, but Grant occasionally tells his workers to head home early when they feel dizzy after spraying the fields. He was relieved when GM potatoes were introduced because he didn't have to spray them with insecticide. He was warned that pests might overcome the modification in 15 to 20 years, but that didn't deter him—he says the same thing happens with chemicals, too.

Unlike Bt corn, you can't find any fields planted today with Bt potatoes. Soon after the breed hit the market, protestors began to single out McDonald's restaurants, which collectively are the biggest buyer of potatoes worldwide. In response, McDonald's, Wendy's, and Frito-Lay stopped purchasing GM potatoes. In 2001, Monsanto dropped the product and Grant returned to conventional potatoes and the handful of insecticides he sprays on them throughout the summer.

"There is not a single documented case of anyone being hurt by genetically modified food, and yet this is a bigger problem for people than pesticides, which we know have caused harm," he says. "I just shake my head in bewilderment at the folks who take these stringent positions

that biotech should be banned."

Even some organic farmers bristle when asked about the anti-GMO movement.

In the decade after Monsanto pulled their GM potatoes from the market, dozens of long-term animal feeding studies concluded that various GM crops were as safe as traditional varieties. And statements from science policy bodies, such as those issued by the American Association for the Advancement of Science, the U.S. National Academy of Sciences, the World Health Organization, and the European Commission, uphold that conclusion. Secondly, techniques to tweak genomes have become remarkably precise. Specific genes can be switched off without lodging foreign material into a plant's genome. Scientists don't necessarily have to mix disparate organisms with one another, either. In cisgenic engineering, organisms are engineered by transferring genes between individuals that could breed naturally.

Even some organic farmers bristle when asked about the anti-GMO movement. Under the U.S. Organic Foods Production Act, they are not allowed to grow GMOs, despite their ability to reduce pesticide applications. Organic farmers still spray their crops, just with different chemicals, such as sulfur and copper. Amy Hepworth, an organic farmer at Hepworth Farms in Milton, New York, says that they, too, can take a toll on the environment.

Hepworth would like to continuously evaluate new avenues towards sustainable agriculture as technology advances. However, her views often clash with her customers' in the affluent Brooklyn, New York, neighborhood of Park Slope. Many of them see no benefit in GMOs' ability to reduce pesticides because they say farmers should rely strictly on traditional farming methods.

"What people don't understand is that without pesticides there is not enough food for the masses," Hepworth says. "The fact is that GM is a tool that can help us use less pesticide."

A Chemical World

Back when Monsanto introduced its GM potatoes, De Jong did not argue with people who opposed them. Although the potatoes seemed practical, he felt that Monsanto was powerful enough to defend itself. Indeed, he frequently voices his distaste how the company has used its monopoly over agriculture worldwide. (De Jong is not affiliated with Monsanto.) However, Monsanto's association with GMOs—the company sells many such seed lines—means public opinion against it has morphed into a hatred of GMOs in general. Myths about the health dangers of GMOs

abound, which upsets De Jong as a scientist.

But what really catalyzed De Jong's concerns over misguided fears of GMOs was a recent trip he took to Indonesia. As De Jong's van rumbled through the countryside late last April, he watched men in T-shirts and no facemasks spraying chemicals on their crops. While touring a potato farm, he snapped a photograph of insecticide powder blanketing crates of stored potatoes like snow. Insects thrive in Indonesia's tropical climate, and farmers spray certain crops heavily to prevent damage. The chemicals they use tend to be more toxic because newer, safer chemicals cost more. Plus, chemical regulations that are common in U.S. and Europe are rarely enforced in the developing world. "I just felt awful because some of this is really nasty stuff," De Jong says. "We can pretend that this doesn't happen, but it does."

De Jong spotted these potatoes covered in insecticide on his recent trip to Indonesia.

It's not just a problem in Indonesia, either. In parts of India, farmers spray more than 60 insecticides on their eggplant—known to locals as brinjal—during the growing season, mainly to protect the purple fruit from burrowing bugs, says Ponnuswami Balasubramanian, a plant molecular biologist at Tamil Nadu Agricultural University in Coimbatore, India. To reduce the insecticide load without losing the harvest, Balasubramanian, together with public sector researchers and a private Indian seed company, developed Bt versions of four varieties of eggplant that are popular in southern states. Monsanto was not involved, but still public outcry from GMO opponents blocked the eggplants from federal approval.

Nigerian farmers

"It was madness to stop Bt brinjal" says Kulvinder

who rely on traditional farming techniques lose up to 30% of their crops.

Gill, an agricultural geneticist at Washington State University in Pullman, Washington, who grew up in India and was not involved with the project. "People should not even be eating this brinjal because it has so much insecticide on it," Gill says, "Anything to reduce that would be extremely beneficial."

A continent away, Nigerian farmers who rely on crop rotation and other traditional farming techniques lose up to 30% of their crops of cowpeas, cabbage, and pumpkin leaves to pests, says Sylvia Uzochukwu, a food microbiologist at the University of Agriculture in Abeokuta, Nigeria. As a result, she says many farmers spray their fields heavily during the growing season. Uzochukwu has been talking with farmers and the public about the introduction of GM crops as a way to help the country's economy, reduce pesticides, and improve health.

The City and the Country

I first encountered De Jong on April 4, when he sat on a panel about GMOs in New York City hosted by the advocacy groups GMO Free NY and the Wagner Food Policy Alliance. The modest awkwardness that endears him to farmers didn't charm the audience. As De Jong explained how scientists create GMOs, they began to murmur, lost amidst De Jong's scientific jargon and meandering delivery.

De Jong did, however, liven up during a discussion in which Jean Halloran, a member of the panel from the Consumer's Union, suggested that farmers in the developing world could ditch pesticides, not use GMOs, and increase yields. "We favor a knowledge-based approach rather than a chemical-based approach to increasing production," Halloran had said.

Organic potatoes for sale at a cooperative market in Ithaca, New York.

De Jong did not find this solution realistic and asked, “Do you want to be the African farmer who has to apply insecticide every week—really nasty stuff—without protective equipment?” The question hung in the air for a second, and the panelist beside him repeated the no-chemical mantra.

Weeks later, De Jong tells me the panel opened his eyes. He was shocked at how people who don’t live near farms feel entitled to advise farmers, especially on environmental matters. “There is a romantic notion of environmentalism, and then there is actual environmentalism,” De Jong says. “Farmers are very conscious of the environment. They want to hand off their operation to their kids and their kids’ kids, so they maintain the land the best they can while doing what they need to do in order to sell their harvest,” he says. “My guess is that the majority of people who are anti-GM live in cities and have no idea what stewardship of the land entails.”

“I find it so tragic that, by and large, crop biotechnologists and farmers want to reduce their pesticide use, and yet the method we think is most sustainable and environmentally friendly has been dismissed out of hand.” He pauses as he recalls the event and says, “There is no scientific justification for it—it is just as if there is a high priest who decided, ‘Thou shalt not be GMO.’ ”

Into An Uncertain Future

Despite the anti-GMO movement and the hesitation of many governments to approve of new GM crops, biotechnology moves forward. During his recent trip to Indonesia, De Jong volunteered to help public sector scientists create a GM potato that resists potato blight, the disease responsible for the Irish potato famine that killed more than a million people in the mid-1800s. De Jong hopes to endow the potato with several modifications that won’t be easily overcome by the fungus-like organism that causes blight, *Phytophthora infestans*.

The project has an uncertain future, though, one that has less to do with science and more to do with politics. “I worry that when the Indonesian government nears its approval, Greenpeace will invade with a lot of money and a lot of PR stunts,” De Jong says. “The question is, what happens then?” (Greenpeace was contacted for comment, but offered no comment prior to publication.)

Some scientists hope to abate the public’s fears of “Frankenfoods” with cisgenic engineering, in which the inserted genes belong to the same type

of organism. Anton Haverkort, an agroecologist at Wageningen University and Research Centre in the Netherlands, is banking on that idea. Every year, 99% of potato farmers in the Netherlands spray fungicides to combat blight at a cost of 110 million euros, Haverkort says. To reduce chemical use, he is leading an effort to make a cisgenic blight-resistant potato, which is supported by the Dutch government, despite the cool reception GMOs receive in Europe.

"Some of the regulations required for crop approval are not science-based."

In the U.S., a potato engineered for a different purpose entered into one of the last stages of the approval process in March. Its developer, food company J.R. Simplot, calls it the "innate potato" because it carries no foreign genes. Instead, its genome is manipulated so that a protein that causes browning fails to activate and a cancer-causing chemical, acrylamide, which can form naturally when potatoes are cooked at high temperatures, does not accumulate.

However, De Jong doubts that cisgenic or innate potatoes will defuse GM opponents. "I suspect that they will still see it as tinkering," he says. To him, a more important question for these other approaches would be whether such potatoes remain effective over time. In the case of blight-resistance, the best potato is the one that the pathogen cannot gradually overcome.

De Jong inspects furrows on a potato farm near Cornell University.

Evidence-based studies can determine the answer to that question, and these are just the sort of questions that De Jong would like to ask. Rather than protest every GMO, De Jong wishes society would confront various aspects of genetic engineering—some of which trouble him, including

laws on how modifications are patented and priced and the inconsistent way in which regulatory agencies assess GMOs.

At the moment, only large corporations have the financial resources to weather the approval process, which, by and large, isn't standardized and can drag on for several years. Multiple US federal agencies, driven in part by the public's fears, ask for proof on safety and efficacy beyond a point that feels reasonable to some scientists. For example, people worry that inserted genes will spread to wild crops, but reported occurrences have been exceedingly rare and their existence is debated among the scientific community. As agencies examine and re-examine all imaginable scenarios, public sector projects and small businesses crumble under the pressure of paying employees while taking in no revenue.

"Some of the regulations required for crop approval are not science-based, and they have crippled the ability of the public sector to deploy GMOs for public benefit," De Jong says, "I can make a transgenic potato for less than $50,000, but I cannot afford to pay five to ten million dollars to go through regulatory hoops." As a result of hurdles like these, Monsanto's engineered corn and soybeans monopolize the market for GMOs. By enforcing their patents on GM seeds, Monsanto and other large corporations with GMO products can dictate how much farmers pay for seeds—a precarious dependency to be in when an additional 2 billion people in developing nations need to be fed in 2050. De Jong is pushing for policy reform.

De Jong's vow to speak his mind on GMOs could be futile, at least for now. It takes potato breeders decades to see one of their varieties succeed on the market, and De Jong says he would not be surprised if it takes longer than his lifetime for the public to accept genetic modification. But for people who spend years watching plants grow, patience is a virtue. For De Jong, those roots extend far back in his family tree. He is descended from a long line of Mennonites, a denomination of Christians renowned for their ability to farm even the poorest soil.

De Jong recalls a lesson from his past, when at 15 he asked his father why farmers did not buy a pretty potato variety with pale skin and yellow flesh. His father replied that consumers didn't want it. But a few years later, just such a potato hit it big—the Yukon Gold. "This taught me that if I think a potato is interesting, and others aren't hot on it, I should just trust my gut." Eventually, the rest of the world might catch on.

Tell us what you think on Twitter #novanext, Facebook, or email.

Photos by Amy Maxmen and Walter De Jong

Amy Maxmen

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Amy Maxmen is a freelance journalist in New York City who covers biology, medicine, and science policy.

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[Science special section follows this article.]

The Washington Post Print

We've covered the world in pesticides. Is that a problem?

By Brad Plumer, Updated: August 18, 2013

Pesticides have become an enduring feature of modern life. In 2007, the world used more than 5.2 billion pounds of weed killers, insecticides, and fungicides to do everything from protecting crops to warding off malaria.

And that's led many researchers to wonder what sorts of broader impacts all these chemicals are having. They've helped feed the world, yes, but they may also be causing health problems elsewhere. To that end, the latest issue of *Science* has a fascinating special section on the world's pesticide use. Here are a few good charts and highlights:

1) Pesticide use is rising almost everywhere, with a few key exceptions:

Note that pesticide sales in North America haven't grown very much — and usage actually seems to be declining in the United States (more on that below). The growth in Europe, meanwhile, is largely driven by a big uptick in sales in Eastern Europe. Meanwhile, sales are more or less stagnant in the Middle East and Africa.

2) There's a surprisingly large variation in how farmers in different countries use pesticides:

Of the 2.4 billion kilograms of pesticides used in 2007, the United States accounted for about 20 percent of the total. But notice that American farmers are relatively sparing in their use of pesticides — using just 2.2 kilograms per hectare of arable land. Compare that with China, where farmers are "less trained" and the figure is more like 10.3 kilograms per hectare.

Of course, the skill level of farmers is just one variable here. The type of crops can matter too. Pesticide use is also particularly high in countries with "valuable crops where pest pressures are high, including Colombian coffee and Dutch tulips." Meanwhile, use is low in Africa largely because of the high cost of pesticides.

3) Chemical pesticides have been quite effective in boosting agricultural yields.

"Long term research plots have shown increases in wheat yield from controlling insects and disease," write David Malakoff and Erik Stokstad. "Gains from plowing fallow fields have been exceeded by the advent of chemical herbicides and fungicides."

4) And insecticides have been invaluable in controlling malaria.

One recent study estimated that the growing use of insecticide-treated mosquito nets prevented the deaths of some 842,800 children between 2001 and 2010. The catch? "Insecticide resistance in the mosquito threatens those gains," the *Science* issue notes.

5) Yet scientists are starting to discover other problems that might accompany heavy use of pesticides.

Three long-term cohort studies now suggest that certain chemical pesticides can interfere with brain development in young children. And some experts suspect that a class of

pesticides known as neonicotinoids are at least partly responsible for the recent collapse in bee populations (though this is still disputed).

There are other, lesser-known impacts as well. Australia's wheat farmers are now dealing with one of the worst weed infestations in the world — an issue caused in part by overuse of herbicides, which led to resistant weeds. And some 300,000 people kill themselves each year by ingesting pesticides, largely in Asia. That's one third of the world's suicides.

And those are just the effects scientists know about. A notable paper from Heinz-R. Köhler and Rita Triebskorn points out that researchers still don't understand the full impact of many chemicals on broader ecosystems. "Although we often know a pesticide's mode of action in the target species," they write, "we still largely do not understand the full impact of unintended side effects on wildlife."

6) Pesticides *are* getting safer in wealthy countries like the United States. But that's not as true in the developing world.

"Developed countries have phased out the more dangerous compounds, such as parathion and other organophosphates," write Malakoff and Stokstad. "After the U.S. Food Quality Protection Act of 1996, several more have been banned altogether, limited to farm use, or further restricted to protect workers or the environment."

But many of these toxic chemicals are still widely used in poorer countries — in part because the more dangerous pesticides tend to be cheaper. "Surveys of farm worker health are scarce," they write, "but it's clear that pesticides cause more harm in the developing word. More toxic chemicals are still used, and basic safety equipment is often lacking."

7) Scientists are developing all sorts of intricate methods to reduce the world's dependence on pesticides, though sometimes simpler solutions work pretty well.

The chart above shows how overall pesticide use in the United States has declined 0.6 percent each year between 1980 and 2007. And it's dropped even *faster* in corn fields — in part because of the widespread use of Bt corn, a genetically modified breed of corn that's engineered to be toxic to pests. One hitch? There's now some evidence that certain insects are becoming resistant to the Bt corn, especially in areas where it's used heavily. That could lead to a resurgence in pesticide use.

But perhaps researchers will come up with new strategies. The *Science* issue outlines on some of the other clever ideas in the works: "New synthetic chemicals to protect crops hold the promise of stronger and more specific protection with less collateral damage. And some crops won't need pesticides at all: Scientists are developing plants whose immune systems can ward off fungal, bacterial, or viral diseases, and they are using RNA interference to help plants fight insects—a new technology that could hit the market before the decade ends."

Then again, sometimes low-tech ideas work pretty well too. One piece points out that a soap-opera-centered campaign in Vietnam helped convince local rice farmers to stop overusing pesticides. And in Australia, farmers are now using non-chemical control techniques, such as burning seeds, to control a massive weed problem that was brought on, in part, by heavy herbicide use.

Related: There's also this free podcast from *Science* that covers this topic in more depth.

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From *Science*

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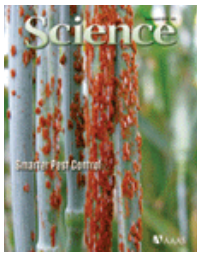

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E. Stokstad, "Field Research on Bees Raises Concern About Low-Dose Pesticides" *Science* **335**, 1555 (2012)

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M. Henry *et al.*, "A Common Pesticide Decreases Foraging Success and Survival in Honey Bees" *Science* **336**, 348-350 (2012)

P. Whitehorn *et al.*, "Neonicotinoid Pesticide Reduces Bumble Bee Colony Growth and Queen Production" *Science* **336**, 351-352 (2012)

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Science. ISSN 0036-8075 (print), 1095-9203 (online)