Maine Board of Pesticides Control

Miscellaneous Pesticides Articles February 2014

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BANGOR DAILY NEWS

Are there alien genes in your food?

By Nancy Oden, Special to the BDN
Posted Jan. 01, 2014, at 3:16 p.m.

"Alien genes" are genes from one species that are forced into a different species, creating a mutant life form that would never occur in nature. They're called genetically modified organisms, or GMOs, and are created by chemical and pharmaceutical corporations.

They include, for example:

— The bacterium Bacillus thuringiensis spliced into some potatoes and corn, causing the plants to produce a toxin to ward off insects;

— Fish genes into tomatoes (a product that didn't go on the market);

— Genes from antibiotics and pesticides inserted into corn, soy, canola, sugar beets — in your food today, unless you're eating organic.

Creating GMOs is not selective breeding within one species. Selective breeding cross-pollinates, for example, one tomato with another, leading to a somewhat different tomato. This happens in nature all the time. It would never lead to a tomato with fish genes in it.

Forcing potentially toxic material into the plants humans and animals eat is not meant to "improve" the plants; it's meant to sell more product — such as agriculture giant Monsanto's Roundup Ready soybeans, alfalfa, corn, cotton, canola and sugar beets, which contain tolerance to Roundup herbicides.

Don't wonder why the government allows these materials in your food: A former Monsanto executive is heading up the Food and Drug Administration. Surprised? Likely not.

When you eat GMO-containing food, you force your body to accept foreign, potentially toxic genes into every cell of your body as "food." Since few studies of GMOs have been done on humans, the bio-tech corporations can say, "GMOs aren't proven to harm humans." But there are good reasons to wonder.

Monsanto, Bayer, Syngenta, Dow and DuPont tell people food would cost more if they had to label GMO products. Nonsense. We need to know whether our foods contain GMOs. If there's nothing wrong with GMOs, why do they object so strenuously?

Truth: They're afraid that if you know what's in these GMO plants, you might not want to buy or eat them.

For instance:

— Pigs fed a GMO corn and soy diet contracted severe stomach inflammation. Animals fed GMOs also have shown disrupted liver, pancreas, kidneys and testes function.

— Residues of Roundup herbicide have been shown to remain within and on the corn, soy, canola, sugar beets, etc., that are heavily sprayed during their growth period. It cannot be washed off, so you're eating that herbicide in the GMOs.

— Agrobacterium tumefaciens and cauliflower mosaic virus are commonly used to breach plants ' cellular walls. Mutations of these foreign genes are taking place within these GMO plants; is this also happening to people who eat them?

Unfortunately, the so-called GMO labeling bill, LD 718, that passed the Legislature this year, and which Gov. Paul

LePage has said he'll sign, is a very weak bill. GMOs in Maine will not be labeled until other contiguous states decide to label.

The bill also excludes products from animals that have been fed GMOs — even though the GMOs the animals eat are in their cells. Thus, when you drink milk from GMO-fed cows, or eat butter, cheese or yogurt developed from these cows, you are eating the GMO from their bodies, too.

An honest label would tell you if the animals' flesh, eggs and milk are from GMO-fed animals.

But we can call it a beginning while we work toward a serious labeling bill, so people can know what's really in their food. Only then can we make good decisions about what to buy and feed our families.

Nancy Oden is an organic farmer who has lived in Washington County for 35 years. She may be reached at cleanearth@acadia.net.

http://bangordailynews.com/2014/01/01/opinion/are-there-alien-genes-in-your-food/ printed on January 2, 2014

Oregon home gardeners could face pesticide restrictions under proposed legislation

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on January 01, 2014 at 6:00 AM, updated January 01, 2014 at 4:06 PM

A Portland lawmaker plans to introduce legislation in February that would effectively ban home gardeners from using some pesticides implicated in **mass bee die-offs last summer** in Wilsonville, Portland and other cities.

Rep. **Jeff Reardon**, D-Portland, is crafting legislation to add four pesticides to a restricted use list, which would effectively force home gardeners to hire professional pesticide applicators or use less effective alternatives.

The legislation is necessary to protect bees that provide crucial pollination for flowers and crops, said Reardon, a home gardener. Bee populations have also declined in recent years from colony collapse disorder, which **some scientists say is linked to pesticide usage**.

"These are dangerous chemicals," Reardon said. "People who aren't willing to take the time and effort to become fully educated, then they should look for alternatives."

More

Continuing coverage of the deaths of an estimated 50,000 bumblebees.

Reardon seeks to restrict the use of dinotefuran, imidacloprid, clothianidin and thiamethoxam, which belong to a class of pesticides known as neonicotinoids. **The four pesticides are used in some Bayer, Ortho, and other garden products**. The pesticide Safari, which contains dinotefuran, was used in a **Wilsonville incident that killed 50,000 bees** last June after pesticide applicators **failed to follow label instructions**.

Environmental groups support the proposed legislation, but the bill's chances are unclear, particularly in the Senate, where environmental bills have faltered in the past year.

The bill will face opposition from groups like **Oregonians for Food & Shelter**. Scott Dahlman, the group's executive director, says there's no evidence that home gardeners' usage of neonicotinoids has caused mass bee deaths. The Wilsonville incident resulted from "blatant misapplications" from licensed pesticide applicators, he said.

"Pesticide regulation should be based on science," Dahlman said. "When we're not seeing a connection to a problem here, yet we want to restrict something, that raises a lot of red flags for us."

Neonicotinoids are also much safer for pesticide applicators than the alternatives, he said.

Gardeners like neonicotinoids because they're absorbed through the roots, protecting plants from the inside out, and can last for a year or more, said Jimmy Mack, a manager at **Portland Nursery**.

"It's a real easy application, and it lasts for a long time," he said. "That's why consumers love it, and growers, too."

Neonicotinoids like imidacloprid are the most effective pesticides against some bugs, such as the **lace bug that has infected azalea and rhododendron shrubs** since it arrived in Oregon in 2009. Alternatives are less effective and would require early protection, Mack said.

"Consumers are going to be upset they're losing their plants," Mack said. "The onus becomes: How do you educate people on what to do next? It'd be tough, but I think we'd adjust."

The **Oregon Department of Agriculture** in November **announced additional education and testing requirements** for licensed pesticide applicators, but Reardon wants home gardeners to meet similar requirements before they can use the pesticides.

Reardon wants the state to develop an online course that home gardeners would need to take before passing a test to obtain a pesticide license -- requirements that most hobby gardeners would be loath to meet. Computerized pesticide applicator tests cost about $58.

Oregon pesticide licenses are designed for commercial and agricultural uses, and the state currently doesn't require licenses for private backyard uses of neonicotinoids, said Dale Mitchell, manager of the pesticides program at the state Department of Agriculture.

The Washington State Department of Agriculture last year **declined a request to restrict neonicotinoids** and said there is no evidence that use of the pesticides is a significant contributor to the decline of bee colonies.

Meanwhile, the **European Union on Dec. 1 implemented a two-year ban** on three types of neonicotinoids, citing "high acute risks" to bees in certain circumstances. Authorized uses are restricted to professionals.

On a federal level, Congressman **Earl Blumenauer, D-Portland, has proposed legislation** to restrict four pesticides until the **Environmental Protection Agency finishes its ongoing review of all neonicotinoids**. Blumenauer acknowledged the **bill is unlikely to pass**.

-- Yuxing Zheng

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Morning Sentinel

Posted: 1:40 PM
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LePage signs Maine bill to label genetically modified food

Maine becomes second state to pass a law requiring food producers to label GMO food, but other states must follow before it goes into effect.

By Steve Mistlersmistler@pressherald.com
Staff Writer

Gov. Paul LePage has signed a bill that would require food producers to label foods that contain genetically modified ingredients. The law makes Maine the second state in the country to pass such a measure. However, other states must adopt similar legislation before Maine's labeling provision goes into effect.

click image to enlarge

A label on a snack item at a Portland supermarket indicates it is certified organic and does not contain any genetically modified ingredients.

2013 Staff File Photo/Gordon Chibroski/Staff Photographer

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The governor promised last year to sign the bill, which was sponsored by Rep. Lance Harvell, R-Farmington. His signature is symbolic because legislative rules don't allow the law to go into effect until the Legislature adjourns later this year. However, advocates of the bill hailed the law's eventual passage as a victory for advocates of laws to label genetically modified foods. Such proposals have been introduced in nearly 30 states as part of a national effort to compel Congress to enact a comprehensive labeling law.

Previous GMO labeling efforts have been staunchly opposed by agribusiness and the biotech food products industry, which have also spent millions to defeat ballot measures and state legislation.

Industry argues that labeling genetically engineered products unfairly stigmatizes modified foods despite a dearth of scientific research proving that they are any less healthful than those that are grown conventionally.

The U.S. Department of Agriculture estimates 70 percent of the products sold in American supermarkets contain genetically modified ingredients. The Food and Drug Administration regulates genetically modified foods, but regulators have left testing to the industry that is producing them.

Maine Conservation Voters' Executive Director Maureen Drouin said in a news statement that the new law "will give Maine people the information they need to make informed decisions about the food they and their families eat."

She added, "We thank Gov. LePage for recently signing the bill into law and thank Maine's Legislature for passing the bill with overwhelming support last year. We are thrilled that Gov. LePage has signed the GMO labeling bill," said Maine Organic Farmers and Gardeners Association Executive Director Ted Quaday. "MOFGA supporters have worked tirelessly, organizing five different legislative campaigns on this issue since the early 1990s. The time was right for a diverse and collaborative effort to take hold and move the discussion forward. People want and have the right to know what's in their food."

Still, the Maine GMO labeling law faces another challenge. The law doesn't go into effect unless five contiguous states, including New Hampshire, pass labeling laws. Late last year, the prospects of a New Hampshire law dimmed when a committee broke along party lines to oppose a labeling measure there.

The New Hampshire Legislature will take up the bill this winter.

The national battle over labeling laws has pitted activists in the organic food movement against a consortium led by the biotech industry and corporate food producers such as General Mills, Nestle USA and Monsanto.

It appeared that industry heavyweights were initially taken aback by activists who introduced labeling legislation this year in at least 30 states, according to the National Conference of State Legislatures. However, reports from New Hampshire indicate that the industry has rallied and become more effective.

The Maine bill brought together such factions as libertarian Republicans and liberal Democrats, creating strong support. So far, that hasn't happened in New Hampshire and the bill has become more partisan there.

The provision requiring passage in contiguous states was added to the Maine bill to help build broad support.

Proponents of the bill said the provision would quell concerns about an almost-certain lawsuit by industry groups and Monsanto, which vowed to challenge the laws in Maine and Connecticut on the basis that they violate the free speech and interstate commerce provisions of the U.S. Constitution.

Maine Attorney General Janet Mills told lawmakers last year that the bill was almost certain to face a legal challenge, and said she could not guarantee that her office could defend its constitutionality.

Already 64 countries around the world label foods that contain genetically modified ingredients, including all of Europe, Russia, China, Brazil, India and Saudi Arabia.

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Research for the Environment

Press Release, 10th December 2013:

Biodegradable or not?

Scientists are developing classifications in order to better differentiate readily-biodegradable from long-lasting pesticides

Leipzig. In order to improve the evaluation process for the long-term consequences of pesticides, scientists have developed a new detection method and a model that can enable determinations regarding whether and how readily biodegradable the residues of pesticides are. The study, conducted by scientists at the Helmholtz-Centre for Environmental Research (UFZ), the Rhine-Westphalian Technical University Aachen (RWTH) and the Technical University of Denmark has recently appeared in the scientific journal *"Critical Reviews in Environmental Science and Technology".*

Photo: Kara-Fotolia.de

Pesticides have a bad reputation: they harm the environment, have negative effects on the diversity of species and pollute the soil. "This is partially correct, but also partially incorrect. Pesticides are important for the efficacy of our modern agriculture methods. And pesticides are not necessarily pesticides – differentiation is necessary in this context. Generally speaking, biodegradability is supposed to be the top priority when deploying pesticides", says Prof. Dr. Matthias Kästner, Director of the Department Environmental Biotechnology at the Helmholtz-Centre for Environmental Research – UFZ in Leipzig.

Worldwide, today approximately 5,000 pesticides are utilized as substances for plant protection and for pest control. As varied as their respective effectiveness is, their effects on the environment are equally varied. Some pesticides are quickly biodegraded, while others take longer. And some of them create chemical bonds with components in the soil and form the so-called bound residues. One has always previously assumed that these residues were, per se, toxic. This is why pesticides that form more than 70% bound residues are no longer in compliance today. Kästner: "But what exactly is concealed behind these bound residues, i.e. whether or not they really are toxic or what chemical structures they have hidden, could not yet been evaluated."

By applying the so-called ^{13}C-method, Kästner and his team applied pesticides onto various reference soils and examined them thoroughly regarding their fate. For this purpose, they initially marked the pesticide to be examined with the non-radioactive, heavy carbon isotope ^{13}C – and tracked it in various bio-molecules with the aid of a mass spectrometer after completion of the experiment timeframe. In this manner the scientists were able to determine the residues, the changes in the pesticide, and its breakdown products in the soil.

The most significant result from the study states – there are various groups of bound residues. In the current issue of the technical journal "Critical Reviews in Environmental

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Science and Technology", the UFZ research scientists compile their results and introduce a classification system and a modelling approach for bound residues. As regards Type 1, the pesticide itself or its breakdown products of organic materials are deposited in the soil (humus) or trapped within, and can in principle be released at any time. If the pesticide has undergone a chemical bond with the humus, bound residues are allocated to the Type 2, which can only be released with difficulty. Residues from both Type 1 and Type 2 are to be categorised as toxicologically relevant. "At this juncture a precise examination must be carried out regarding whether or not approval of a pesticide that forms such residues in the soil is possible and defensible," says Matthias Kästner. As regards residues of the Type 3, the pesticide was decomposed by bacteria, and the carbon contained therein was transported into the microbial bio-mass. "For these kinds of residues, we can give the "all-clear" signal and confirm that there is no further risk", Kästner states. Pesticides, from which the bound residues in the soil are allocated to Type 3, could thus be approved without risk in the future. Conversely, pesticides, which heretofore were considered to be risk-free, could possibly be classified as critical using this method. Kästner says "Only when we are capable of differentiating between biodegradable and high-risk pesticide residues we can act accordingly. This is why we hope that the ^{13}C-method will be included in the dossiers of the approval procedure in the future. This is what we suggested to the German Federal Environmental Agency as well."

The initial findings from the UFZ study have already been accepted into the assessment processes of the officials involved in the approval procedure. Thus, for the residues of the approved pesticides 2.4 dichlorphenoxyacetic acid (2.4-D for short) and 2 methyl 4 chlorphenoxyacetic acid (MCPA for short), they were able to give the all-clear. "In order to better control the deployment of pesticides and their environmental consequences, we still have a lot of work to do", says Kästner. "The problems that we had with DDT (dichlorodiphenyltrichloroethane) and atrazine must not be repeated. Therefore, it is very important to understand what actually happens with pesticides after application." *Nicole Silbermann*

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Publication:

Matthias Kästner, Karolina M. Nowak, Anja Miltner, Stefan Trapp, Andreas Schäffer (2013): Classification and modelling of non-extractable residue (NER) formation of xenobiotics in soil – a synthesis. Critical Reviews in Environmental Science and Technology. DOI: 10.1080/10643389.2013.828270
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Links

Risk Assessment and Environmental Safety Affected by Compound Bioavailability in Multiphase Environments (RAISEBIO):
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At the **Helmholtz Centre for Environmental Research (UFZ)** scientists are interested in the wide-ranging causes and impacts of environmental change. They conduct research on water resources, biodiversity, the impacts of climate change and adaptation strategies, environmental and biotechnologies, bioenergy, the behaviour of chemicals in the environment and their effects on health, modelling and sociological issues. Their guiding motto: our research serves the sustainable use of natural resources and helps towards long-term food and livelihood security in the face of global change. The UFZ has over 1,100 employees working in Leipzig, Halle und Magdeburg. It is funded by the federal government, as well as by the State of Saxony and Saxony Anhalt.

The **Helmholtz Association** contributes to finding solutions for large and pressing issues in society, science and the economy through excellence in the following six areas of research: energy, earth and the environment, health, key technologies, structure of matter, transport and aerospace. With almost 35,000 employees and coworkers in 18 research centres and an annual budget of approx. 3.8 billion Euros the Helmholtz Association is the largest scientific organization in Germany. Work is conducted in the tradition of the renowned natural scientist Hermann von Helmholtz (1821-1894).

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Beekeepers call for pesticide ban

North Bay Nipissing News

Editor's note: The following is an open letter to Ontario Premier Kathleen Wynne, a copy of which was shared with our publication.

Dear Premier Wynne,

In the next 60 days we expect both the Federal and Ontario governments to announce actions on bee health. The choice is clear: take real action and protect Ontario's bees from neonicotinoid pesticides or allow another year of irreversible damage to our bee population and our beekeepers.

Health Canada, for whom we rely on for protection against harmful pesticides, has confirmed the linkage between neonicotinoid pesticides used to treat corn and soybeans and extensive bee-kills in both 2012 and 2013. With more than four million acres planted in soy and corn in Ontario, we have a situation that beekeepers cannot avoid.

Pesticide lobbyists have tried to create a divide between grain farmers and beekeepers. All farmers aim to be good stewards of their land. No farmer wants to put beekeepers - their fellow farmers - out of business. But if the status quo continues, pesticide companies will continue to profit at the cost of destroying our bees and our livelihoods.

Independent study after study shows the immediate and lasting effects of these pesticides on pollinators, birds and on our soil and water. A thorough scientific review convinced the European Union to ban neonicotinoids this year. Health Canada, itself, has concluded that, "current agricultural practices related to the use of neonicotinoid treated corn and soy are unsustainable."

In the words of Dr. Peter Kevan, professor of biology at the University of Guelph, "We have a situation with neonicotinoids now which is an exact parallel with the previous situation with DDTs ... where there was broad scale use over the entire landscape, and where this poison was everywhere."

When announcing your Local Food Act this year, you proclaimed: "Supporting local food does so much for Ontario." Clearly, without pollinators it will be impossible to realize the economic and health benefits of local Ontario foods.

Ontario must lead Canada. The public good of Ontarians is at stake. The OBA, its supporters and the 65,000 Ontarians who signed our petition, urge you to suspend the use of neonicotinoid pesticides now until we can determine if, or how they can be used safely.

Dan Davidson President, Ontario Beekeepers' Associatio

Environmental Leader

January 9, 2014

EPA Funds Aim to Reduce Pesticides, Improve Bee Health

The EPA has awarded almost half a million in funding to three universities — Louisiana State University, University of Vermont and Pennsylvania State University — for projects to reduce pesticide use and lower risks to honeybees.

The agricultural grants for Integrated Pest Management (IPM) practices also aim to control pests and save money, the agency says.

The Louisiana State University ($167,874) project aims to minimize impacts to bees from insecticides used in mosquito control. Practices and guidelines resulting from the project will be distributed to mosquito control districts and beekeepers throughout the US.

The University of Vermont project ($131,758) aims to reduce pesticide use and improve pest control while increasing crop yields on 75 acres of hops in the Northeast. The awardees will also develop and distribute outreach materials to help farmers adopt these practices. The project's goal is to reduce herbicide and fungicide applications by 50 percent while decreasing downy mildew, a plant disease.

The Pennsylvania State University project ($159,632) aims to protect bees and crops by reducing reliance on neonicotinoid pesticide seed treatments and exploring the benefits of growing crops without them. IPM in no-till grain fields will be used to control slugs and other pests that damage corn and soybeans. Researchers will share their findings with mid-Atlantic growers and agricultural professionals.

The EPA says protection of bee populations is among its top priorities. Some of the factors that contribute to the decline in pollinators include: loss of habitat, parasites and disease, genetics, poor nutrition and pesticide exposure. The agency is working with beekeepers, growers, pesticide manufacturers, the US Department of Agriculture and states to apply technologies to reduce pesticide exposure to bees.

Last month the Pollinator Stewardship Council, the National Honey Bee Advisory Board,

American Honey Producers Association, the American Beekeeping Federation and individual beekeepers sued the EPA over the agency's approval of sulfoxaflor, a neonicotinoid pesticide made by Dow Chemical.

Monsanto, Bayer and Sygenta are among the agrichemical companies funding honeybee research in the US as scientists investigate pesticides as a potential cause of honeybee decline. Bayer and Syngenta, which produce neonicotinoids, a class of pesticides linked to bee decline, are helping fund research at Iowa State University and Ohio State University to study the affect of insecticidal seed treatment dust on bee losses.

Monsanto, which uses the pesticides to coat its seeds, hosted a June 2013 summit to discuss potential bee solutions and says it's focusing its bee health research efforts on finding a way to control the varroa mite.

Energy Manager News

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Supreme Court hands Monsanto victory over farmers on GMO seed patents, ability to sue

Published time: January 13, 2014 21:51

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Reuters / Darren Hauck

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Tags
Ecology, Food, GMO, Law, Monsanto, USA

The US Supreme Court upheld biotech giant Monsanto's claims on genetically-engineered seed patents and the company's ability to sue farmers whose fields are inadvertently contaminated with Monsanto materials.

The high court left intact Monday a federal appeals court decision that threw out a 2011 lawsuit from the Organic Seed Growers and Trade Association and over 80 other plaintiffs against Monsanto that sought to challenge the agrochemical company's aggressive claims on patents of

genetically-modified seeds. The suit also aimed to curb Monsanto from suing anyone whose field is contaminated by such seeds.

The group of plaintiffs, which included many individual American and Canadian family farmers, independent seed companies and agricultural organizations, were seeking preemptive protections against Monsanto's patents. The biotech leviathan has filed over 140 lawsuits against farmers for planting the company's genetically-engineered seeds without permission, while settling around 700 other cases without suing.

None of the plaintiffs are customers of Monsanto and none have licensing agreements with the company. The group argued that they do not want Monsanto's genetically-modified organisms (GMOs) and want legal protection in case of inadvertent contact with the company's products.

The appeals court decision was based on Monsanto's supposed promise not to sue farmers whose crops - including corn, soybeans, cotton, canola and others - contained traces of the company's biotechnology products.

In a June 2013 ruling, the US Court of Appeals for the Federal Circuit in Washington, DC said it was inevitable, as the farmers' argued, that contamination from Monsanto's products would occur. Yet the appeals panel also said the plaintiffs do not have standing to prohibit Monsanto from suing them should the company's genetic traits end up on their holdings "*because Monsanto has made binding assurances that it will not 'take legal action against growers whose crops might inadvertently contain traces of Monsanto biotech genes (because, for example, some transgenic seed or pollen blew onto the grower's land).*'"

The panel's reference to *"traces"* of Monsanto's patented genes means farms that are affected by less than 1 percent.

The plaintiffs asked Monsanto to pledge not to sue, but the company rebuffed the request, saying, "*A blanket covenant not to sue any present or future member of petitioners' organizations would enable virtually anyone to commit intentional infringement.*"

Monsanto's GMO seeds are designed to withstand the company's own ubiquitous herbicide, Roundup. Recently, questions have begun to arise from the bioengineered seed's resistance to pestilence, which has caused some farmers to increase their use of traditional pesticides.

"*Monsanto never has and has committed it never will sue if our patented seed or traits are found in a farmer's field as a result of inadvertent means,*" said Kyle McClain, the Monsanto's chief litigation counsel, according to Reuters.

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US farmers challenging Monsanto patent claims appeal to Supreme Court 25

"The lower courts agreed there was no controversy between the parties," McClain added, *"and the Supreme Court's decision not to review the case brings closure on this matter."*

Organic Seed Growers and Trade Association President Jim Gerritsen expressed disappointment that the Supreme Court reaffirmed the previous ruling, refusing to hear the case.

"The Supreme Court failed to grasp the extreme predicament family farmers find themselves in," said Gerritsen, an organic seed farmer in Maine. *"The Court of Appeals agreed our case had merit. However ... safeguards they ordered are insufficient to protect our farms and our families."*

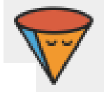
In addition to Monday's news and the appeals court decision against them, the plaintiffs - many of them non-GMO farmers and who make up over 25 percent of North America's certified organic farmers - also lost a district court case.

"If Monsanto can patent seeds for financial gain, they should be forced to pay for contaminating a farmer's field, not be allowed to sue them," said Dave Murphy, founder and executive director of Food Democracy Now!, in a statement *"Once again, America's farmers have been denied justice, while Monsanto's reign of intimidation is allowed to continue in rural America."*

"Monsanto has effectively gotten away with stealing the world's seed heritage and abusing farmers for the flawed nature of their patented seed technology," said Murphy. *"This is an outrage of historic proportions and will not stand."*

The case is *Organic Seed Growers and Trade Association, et al., v. Monsanto Company, et al*. Supreme Court Case No. 13-303.

Comments (83)



free-world-logic 14.01.2014 15:07

It would be interesting if the critics of GMO's actually took the time to learn the BASICS of bio-chemistry and genetic engineering as well as what the scientific method is, the difference in correlation and causation is and started with a mind open to the possibility that scientific proof could alter

EPA’s fast-track approval process for pesticides raises health concerns

Jan 15, 2014

-

  Katia Savchuk

  Reporting Intern

Topic(s):

- Business and Technology
- Health and Welfare

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The EPA, headquartered in the William Jefferson Clinton Federal Building, says it has enough data on pesticide Nanosilva to know that it’s safe while the manufacturer finishes testing.

Credit: c_nilsen/Flickr

Tiny particles of silver could appear soon in children’s toys and clothing, embedded inside plastics and fabrics to fight stains and odors.

No one knows how the germ-killing particles, part of a new pesticide called Nanosilva, affect human health or the environment in the long run. But regulators have proposed letting Nanosilva on the market for up to four years before the manufacturer has to submit studies on whether the particles pose certain dangers.

That's because the U.S. Environmental Protection Agency has backed approving Nanosilva through conditional registration, a fast-track process that recently has drawn criticism for oversight problems. Unlike regular registration, it allows a pesticide to be sold before all required safety studies are in. In this case, manufacturer Nanosilva LLC can move ahead even though it hasn't explored fully the potential health risks if the product were to seep out of plastic or be inhaled.

Nanosilva's approval, which could be finalized early this year, has renewed focus on the loophole, designed mainly to help the EPA speed up approvals of pesticides nearly identical to those already being sold.

Recent reviews have found vast problems with the EPA's oversight of conditional registration. An internal audit showed in 2011 that 70 percent of all active pesticides had been conditionally approved. The audit also concluded that the agency used the label too broadly. Since then, its use has increased. Figures the EPA provided in December put the number at 80 percent.

Thousands of pesticides kept conditional status for more than 20 years, the Natural Resources Defense Council, a nonprofit environmental advocacy group, found in 2010. The EPA says studies typically are due within four years.

And last year, federal auditors found the agency couldn't reliably track how many products were conditionally registered or whether safety studies were submitted. As a result, pesticides could linger on the market for years without critical tests, the Government Accountability Office warned in August.

These aren't new problems. At least seven independent reviews dating back to 1980 have noted flaws with the agency's systems for tracking pesticide registrations.

The EPA said it has enough data on Nanosilva to know that it's safe while the manufacturer finishes testing, as the law requires. But some scientists and environmentalists say the agency is taking a risk on products that are hardly essential, like sports clothing that doesn't stain or stink or toys that last longer.

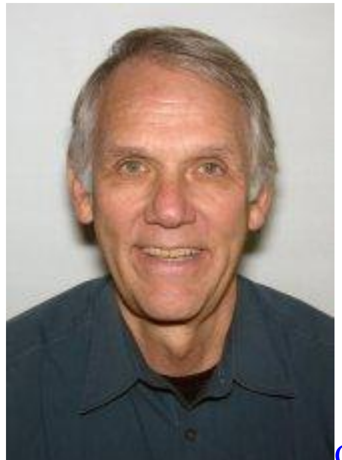
"You could allow some uses that are justified based on human well-being, such as medical implements, but to allow the possibility that nanosilver would be released in plastic on children's toys, and your kid could chew on it and ingest that material before we understand its toxicity – that's unnecessary risk," said Samuel Luoma, a research ecologist at the University of California, Davis. "It doesn't make any sense."

Conventional silver has been used as an antibacterial product for centuries. It releases ions that are deadly for many bacteria and fungi.

Recently, scientists have broken down silver into particles more than 1,000 times smaller than the width of a human hair – some not much wider than a DNA strand. They're called nanosilver. Nanosilva is just one brand that contains them.

Nanosilver can be embedded directly into plastics, fabrics and other materials. Companies say this helps products last longer. It also allows them to call items antibacterial and attract germ-conscious consumers. Nanosilver needs to be registered as a pesticide because it claims to kill bacteria and other live organisms.

Regular silver is highly toxic to fish and other aquatic life but isn't usually dangerous for humans. But scientists say nanosilver could pose unique hazards**,** and they know little about its long-term risks.

Click for larger image

Samuel Luoma, a research ecologist at the University of California, Davis, says the EPA is taking a risk by allowing nanosilver to be used in nonessential products before scientific testing is complete.

Credit: Courtesy of Samuel Luoma

Animal studies show that nanosilver can slip into cells and build up in the brain, heart and other organs. The EPA doesn't know whether nanosilver causes reproductive harm or cancer because there are no valid studies. Research on animals suggests that it can collect in the male reproductive system, potentially harming fertility, and may cause genetic mutations, which sometimes are linked to cancer.

Scientists have warned that nanosilver may be more toxic than regular silver and act as a carrier for other poisonous chemicals. Besides human health risks, researchers worry that nanosilver could kill fish and disrupt food chains if it makes its way into the environment.

The EPA argues that approving Nanosilva promotes innovation and lets consumers enjoy better products. The agency also says it didn't give the manufacturer enough time to do safety tests. The EPA didn't ask for those tests until nearly four years after an independent scientific advisory panel counseled the EPA on how it should evaluate nanosilver in 2009.

And, in evaluating Nanosilva, the EPA ignored some of that panel's advice.

The scientists told the agency to evaluate every nanosilver product separately. Just because one is safe doesn't mean others will be, they said. The agency instead figured out many health and environment risks based on studies on particles that were different from those in Nanosilva. The EPA said the tests were "scientifically appropriate."

Nanosilva officials couldn't be reached for comment.

This isn't the first time the EPA has conditionally approved pesticides containing nanosilver.

In November, a federal appeals court overturned the approval of two nanosilver products, ruling that the EPA had incorrectly found they posed no risks to toddlers. That decision didn't affect Nanosilva because the EPA used different calculations in each case.

Regulators still are grappling with how to deal with nanomaterials. While only two companies have asked for EPA approval, hundreds of products containing nanosilver already are on the market, according to an inventory by The Project on Emerging Nanotechnologies.

The EPA also has fast-tracked other controversial pesticides, including ones linked to the collapse of honeybee colonies and tree deaths.

All conditionally registered pesticides meet legal safety standards, the EPA said. The agency said it's taken steps to prevent staff from coding registrations incorrectly, which it said was the main reason numbers appeared high.

The EPA also has reviewed some conditionally approved products to look for missing data and other problems. But it hasn't traced the paper trail for all pesticides, as it told federal auditors it would do by last fall.

The EPA also doesn't have a concrete plan for the main fix auditors prescribed, an automated tracking system to guarantee that studies arrive and get reviewed. Currently, pesticide managers sometimes rely on handwritten notes and memory to keep track.

"Until they figure out the system, they shouldn't be using conditional registration," said Mae Wu, an attorney for the Natural Resources Defense Council.

The EPA first told federal auditors it would develop an automated tracking system more than 25 years ago.

This story was edited by Andrew Donohue and copy edited by Nikki Frick and Christine Lee.

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Environment
Bees

Scientists fit honey bees with tiny sensors to study decline of colonies

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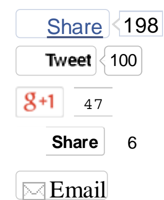
CSIRO-led team will use data they collect to investigate role of pesticides in collapse of 10m beehives worldwide



Oliver Milman
theguardian.com, Tuesday 14 January 2014 17.12 EST
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Thousands of Australian honey bees have been fitted with tiny sensors in a study to help understand what is causing the precipitous collapse of colonies around the world.

About 5,000 bees will carry the 2.5mm x 2.5mm sensors, like hi-tech backpacks, for the next two months at the study site in Hobart.

The CSIRO-led research will build data on the movements and habits of several generations of bees to

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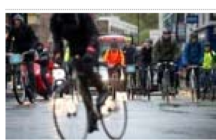

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shed light on the causes of colony collapse disorder, which causes the rapid loss of bees and has led to more than 10m beehives being wiped out worldwide in the past six years.

Australia is so far free of this phenomenon, as well as the deadly varroa mite, which has wreaked havoc on bee populations in almost every other country.

Researchers will place bees in a fridge set to 5C, which will send the insects to sleep. The sensors will then be carefully placed, under a microscope, onto the bees' backs before they are returned to the hive.

CSIRO will study four hives, each with about 50 tagged bees. Two of the hives will be provided a feeder with normal nectar and pollen while the other two will feed on nectar and pollen that contains a small amount of pesticide, which is thought to cause colony collapse.

Scientists will then be able to study the impact of pesticide on the bees' ability to complete their tasks and honey production. Bees are routine-based insects and any deviation will be observable.

CSIRO said the information would provide farmers and fruit growers with greater knowledge of bees. About a third of the food regularly eaten by humans requires pollination.

The results could also lead to government action on certain types of pesticides. Since 1 December farmers in the European Union have been banned from using three types of pesticide suspected of decimating bee populations.

"The sensors are basically a tag which lets us know how long the bees go for, how many follow them and so on," Paulo de Souza, lead scientist at CSIRO, told Guardian Australia. "We will monitor the hives for changes, such as whether the bees are slower to come back or go to other hives.

"This will be the largest study ever done of this kind, given that there will be 5,000 sensors. Two months is quite a long time to be studying them, too."

De Souza said that while Australian bees were in "pretty good shape" and are often exported overseas for pollination purposes, they still face looming threats.

"We don't know how long they will stay in this condition for – pesticides are one risk as well as monocultures of pollen that bees don't do well in," he said. "We are doing some things that might contribute to a future

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collapse, so it's important we study this area."

CSIRO will study other potential causes of colony collapse, such as hive management, after the pesticide research has finished.

It also plans to reduce the size of the sensors to fit onto flies, mosquitos and even smaller winged insects.

"We want to go smaller, maybe to 100 micrometres, although it can be hard to manipulate the sensors at that stage," de Souza said.

"The bees are amazing – they learn very quickly how to fly with the extra weight. They are very focused on their work. Fruit flies take a little longer."

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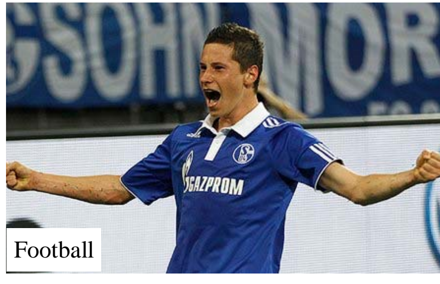

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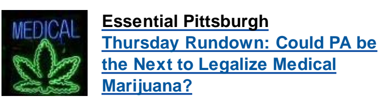

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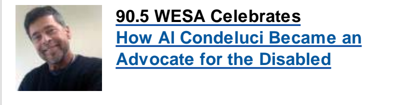

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Health **3:30** AM MON JANUARY 13, 2014

Pitt Researchers Make Discovery About Deadly Mosquito-Borne Virus

By JESSICA NATH

You might not have ever heard of eastern equine encephalitis virus (EEEV) — it only affects five to 30 people in the United States annually — but it kills about half the people it infects, doesn't have a cure and is becoming more common.

That's according to William Klimstra, who, along with other University of Pittsburgh researchers, has made a major discovery that could lead to possible treatment for and prevention against the mosquito-borne virus.

The researchers have discovered that EEEV uses a never-before-documented mechanism to "hijack" one of its host's cellular regulatory systems in an effort to suppress immunity.

Unlike other viruses, which continually replicate themselves, EEEV restricts its own replication using a microRNA-binding site, which can fool the human body.

Klimstra said the technique prevents the immune system from detecting and attacking the virus, so it travels to the brain without warning.

"The advantage that the virus gains from this is that when it gets into the brain, the brain hasn't had any signal from the peripheral tissues that they are infected," Klimstra said. "So the brain is essentially unprotected when the virus gets there and that's why the disease is so severe."

The researchers created a mutant version of the virus without the microRNA-binding site and found that the host's -- in this case mice -- immune system was able to attack the mutant version.

The virus might be rare now, but Klimstra said that could not be the case for long.

"Typically the virus is maintained in a cycle between birds and a swamp mosquito that people aren't very commonly exposed to, but in the last few years it's been found more and more in mosquitos that inhabit urban environments and prefer to feed on people," Klimstra said. "So there's concerns from a number of different areas in terms of the potential for increased cases."

EEEV causes inflammation of the brain, resulting in the sudden onset of headache, high fever, chills and vomiting. It can progress quickly to disorientation, seizures and coma.

The virus has a 30 to 70 percent fatality rate, but those who do survive usually suffer substantial brain damage.

Klimstra said they hope this discovery will help researchers develop vaccines and treatments for the virus.

"The virus where we've deleted the binding sites for the microRNA, that virus is actually a natural vaccine vector because it's naturally attenuated, it stimulates a much better immune response than the wild-type virus does, and something that we haven't mentioned yet is that it is also incapable of infecting a mosquito," Klimstra said.

He said this is important because it shows that the vaccine won't spread from an immunized person to someone else.

He said there is also potential to create a treatment by purifying high levels of the microRNA and administering them to infected people to block virus replication.

TAGS: University Of Pittsburgh (/term/university-pittsburgh) University of Pittsburgh Center for Vaccine Research (/term/university-pittsburgh-center-vaccine-research)

Exposure to pesticides results in smaller worker bees

A honey bee robs a comb. Photo by Lynn Ketchum

Exposure to a widely used pesticide causes worker bumblebees to grow less and then hatch out at a smaller size, according to a new study by Royal Holloway University of London.

The research, published today in the *Journal of Applied Ecology*, reveals that prolonged exposure to a pyrethroid pesticide, which is used on flowering crops to prevent insect damage, reduces the size of individual bees produced by a colony.

The researchers, Gemma Baron, Dr Nigel Raine and Professor Mark Brown from the School of Biological Sciences at Royal Holloway worked with colonies of bumblebees in their laboratory and exposed half of them to the pesticide.

The scientists tracked how the bee colonies grew over a four month period, recording their size and weighing bees on micro-scales, as well as monitoring the number of queens and male bees produced by the colony.

"We already know that larger bumblebees are more effective at foraging. Our result, revealing that this pesticide causes bees to hatch out at a smaller size, is of concern as the size of workers produced in the field is likely to be a key component of colony success, with smaller bees being less efficient at collecting nectar and pollen from flowers," says researcher Gemma Baron from Royal Holloway.

The study is the first to examine the impact of pyrethroid pesticides across the entire lifecycle of bumblebees. The topical research is at the heart of a national Bee Health Conference running in London from Wednesday to Friday this week (22-24 January 2014).

Professor Mark Brown said: "Bumblebees are essential to our food chain so it's critical we understand how wild bees might be impacted by the chemicals we are putting into the environment. We know we have to protect plants from insect damage but we need to find a balance and ensure we are not harming our bees in the process."

Given the current EU moratorium on the use of three neonicotinoid pesticides, the use of other classes of pesticide, including pyrethroids, is likely to increase.

Dr Nigel Raine, who is an Invited Speaker at this week's bee conference, said: "Our work provides a significant step forward in understanding the detrimental impact of pesticides other than neonicotinoids on wild bees. Further studies using colonies placed in the field are essential to understand the full impacts, and

conducting such studies needs to be a priority for scientists and governments."

Provided by Royal Holloway, University of London

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FRIDAY, JANUARY 17, 2014

NEWS

Genetically modified mosquito plans draw opposition

Posted by TERRENCE SMITH on Fri, Jan 17, 2014 at 3:51 PM

Jeffrey M. Smith

Genetically modified organisms were the topic Thursday night at the University of South Florida's Oval Theatre, but this time the focus wasn't only on what we eat.

The biotech company Oxitec has been pushing to release genetically modified mosquitoes in the Florida Keys, as part of an experiment aimed at curbing the spread of Dengue Fever. The hope is that the genetically modified mosquitoes, which would be sterilized males, breed and eventually kill off the species. In response, the GMO Free Florida, Food and Water Watch, and the Florida Keys Environmental Coalition have organized a statewide tour intended to inform the public and voice opposition.

Tampa served as the tour's first stop, drawing more than 100 people. The presentation, which was organized locally by Going Green Tampa, featured Dr. Carlos Garcia and prominent consumer advocate Jeffrey M. Smith, author of the worldwide bestseller *Seeds of Deception*.

Smith devoted the first half of his hour-long speech to the risks of consuming GMOs and familiar foe Monsanto, but pulled no punches in his criticisms of Oxitec's plan, citing the potential for risks and a lack of transparency by the company on the scientific side.

"The idea is to create mosquitoes, to release the males which don't bite, then they create sterile offspring. So you reduce the population of the type of mosquito that may carry Dengue Fever. They didn't tell us that actually 3% are not sterile. Millions will end up in the environment forever. And sometimes it's not actually the males, there's some female

that get in there too, but it's okay, they've done it before. And they didn't actually reduce Dengue Fever, but it's in the plans. Their numbers keep changing and most of it's hidden. ... The technology is leaky, i've talked to one of the top insect GMO scientists from Florida recently. She created the first genetically modified trial of insects in a lab and she had to talk to all these different agencies. They all showed up. And she said this is what we want. In science, we want transparency, thoroughness, care. What we're seeing with Oxitec and the FDA is hidden information and shoddy science."

Oxitec has previously released these mosquitoes in both the Cayman Islands and Malaysia in an attempt to curb Dengue Fever, which according to Smith prompted local outrage and ignores a potential solution that was actually found in Florida.

"In Malaysia where they released the genetically engineered mosquito, the people were very angry, but in another location they released a predatory mosquito that kills the type of mosquito that carries Dengue Fever. It was so successful, no outbreaks of Dengue Fever occurred afterwards, the community wanted more. Do you know where this was developed? In Florida! That's where this predatory mosquito is from. You can do this whole thing without genetically engineering anything new, without introducing a new mosquito that's never before been on this planet."

Smith even pointed out a possible risk to Florida's tourist economy, with even a rumor of "Frankenbugs" spreading diseases turning the state into a no-go area for international visitors.

"Imagine if someone was bit by a vampire mosquito in the keys and gets sick from something and dies. Imagine the person who thinks it's a genetically modified mosquito, whether it is or isn't, and imagine some paper of standing does a story about Frankenbugs in South Florida. Imagine if it gets picked up at a time of great anti-GMO sentiment or someone with a problem with GMOs dropped it somewhere else. Think about the risk to Florida tourism off a story. There was a rumor that genetically modified papaya was stolen from a field trial in Thailand and Europe cancelled all orders of papaya in Thailand. Imagine the risk that's being taken in Florida, so that a company can experiment on a population of mosquitos and Floridians."

Smith advised all in the audience to get in contact with the governor and tell him the idea was stupid. He ended his speech on a hopeful note, encouraging the audience and looking back on how far the anti-GMO movement has come. Major strides have been made recently, with both Cheerios and Post's Grape Nuts going GMO free.

"This is an amazing time, and we may take this information and feel burdened by it, but I'm going to give you another angle. Who else in history, what other generation, has had the opportunity to protect everyone who eats and all living beings and all future generations? This is unprecedented. This is more power to do good than our ancestors ever had in the history of the human race. ... It turns out this window is the most important window of opportunity in the history of GMO activism. In the history of any GMO opportunity, and because the GMO words are buzzing around the internet, it's happening. We're getting coverage for the first time. The other side is pulling out all the stops. They're trying to discredit me like crazy, so it must be working. This is the time now where a little energy and a little attention has such a leveraged, powerful response. ... But please, let us take this time together, on behalf of all living beings, that we can support and celebrate the nature of nature for all living beings and together we can claim a non-GMO food supply for all living beings and all future generations will celebrate us."

The tour continues, with a stop next week in Coconut Creek before ending in Key West.

Tags: GMOs, Oxitec, Mosquitos, Jeffrey M. Smith, Image

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Thank you for bringing awareness on these important issues so that we may make

informed consumer choices - At least with food! As-far-as these mosquitoes are concerned, if they get released, how does one avoid being bitten? What if you have a reaction? There are too many unanswered questions if we rush ahead with this experiment.

Thanks for covering some of the most important issues facing our State.

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Systemic Spread and Propagation of a Plant-Pathogenic Virus in European Honeybees, *Apis mellifera*

Ji Lian Li, R. Scott Cornman, Jay D. Evans, et al.
2014. Systemic Spread and Propagation of a Plant-Pathogenic Virus in European Honeybees, *Apis mellifera*. mBio 5(1): .
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RESEARCH ARTICLE

Systemic Spread and Propagation of a Plant-Pathogenic Virus in European Honeybees, *Apis mellifera*

Ji Lian Li,[a] R. Scott Cornman,[b] Jay D. Evans,[b] Jeffery S. Pettis,[b] Yan Zhao,[c] Charles Murphy,[d] Wen Jun Peng,[a] Jie Wu,[a] Michele Hamilton,[b] Humberto F. Boncristiani Jr.,[e] Liang Zhou,[f] John Hammond,[g] Yan Ping Chen[b]

Key Laboratory of Pollinating Insect Biology of the Ministry of Agriculture, Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing, China[a]; Department of Agriculture, ARS, Bee Research Laboratory, Beltsville, Maryland, USA[b]; Department of Agriculture, ARS Molecular Plant Pathology Laboratory, Beltsville, Maryland, USA[c]; Department of Agriculture, ARS, Soybean Genomic & Improvement Laboratory, Beltsville, Maryland, USA[d]; Department of Biology, University North Carolina at Greensboro, Greensboro, North Carolina, USA[e]; Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, USA[f]; Department of Agriculture, ARS, Floral and Nursery Plants Research Unit, Beltsville, Maryland, USA[g]

ABSTRACT Emerging and reemerging diseases that result from pathogen host shifts are a threat to the health of humans and their domesticates. RNA viruses have extremely high mutation rates and thus represent a significant source of these infectious diseases. In the present study, we showed that a plant-pathogenic RNA virus, tobacco ringspot virus (TRSV), could replicate and produce virions in honeybees, *Apis mellifera*, resulting in infections that were found throughout the entire body. Additionally, we showed that TRSV-infected individuals were continually present in some monitored colonies. While intracellular life cycle, species-level genetic variation, and pathogenesis of the virus in honeybee hosts remain to be determined, the increasing prevalence of TRSV in conjunction with other bee viruses from spring toward winter in infected colonies was associated with gradual decline of host populations and winter colony collapse, suggesting the negative impact of the virus on colony survival. Furthermore, we showed that TRSV was also found in ectoparasitic *Varroa* mites that feed on bee hemolymph, but in those instances the virus was restricted to the gastric cecum of *Varroa* mites, suggesting that *Varroa* mites may facilitate the spread of TRSV in bees but do not experience systemic invasion. Finally, our phylogenetic analysis revealed that TRSV isolates from bees, bee pollen, and *Varroa* mites clustered together, forming a monophyletic clade. The tree topology indicated that the TRSVs from arthropod hosts shared a common ancestor with those from plant hosts and subsequently evolved as a distinct lineage after transkingdom host alteration. This study represents a unique example of viruses with host ranges spanning both the plant and animal kingdoms.

IMPORTANCE Pathogen host shifts represent a major source of new infectious diseases. Here we provide evidence that a pollen-borne plant virus, tobacco ringspot virus (TRSV), also replicates in honeybees and that the virus systemically invades and replicates in different body parts. In addition, the virus was detected inside the body of parasitic *Varroa* mites, which consume bee hemolymph, suggesting that *Varroa* mites may play a role in facilitating the spread of the virus in bee colonies. This study represents the first evidence that honeybees exposed to virus-contaminated pollen could also be infected and raises awareness of potential risks of new viral disease emergence due to host shift events. About 5% of known plant viruses are pollen transmitted, and these are potential sources of future host-jumping viruses. The findings from this study showcase the need for increased surveillance for potential host-jumping events as an integrated part of insect pollinator management programs.

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Editor Anne Vidaver, University of Nebraska

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Address correspondence to Yan Ping Chen, Judy.Chen@ars.usda.gov.

The European honeybee (*Apis mellifera*) provides pollination services to 90 commercial crops worldwide. In the United States alone, honeybee pollination is valued at $14.6 billion annually (1). However, over the past several decades, there has been much concern throughout the world over the steep decline in populations of honeybees (2). Colony collapse disorder (CCD), a mysterious malady that abruptly wiped out entire hives of honeybees across the United States, was first reported in 2006 (3, 4) and has since spread around the world (5), exacerbating the already dire situation for honeybees. RNA viruses, alone or in conjunction with other pathogens, have frequently been implicated in colony losses (3, 6, 7).

Previous studies have shown that viruses that cause common infections in managed honeybees, *A. mellifera*, also infect other hymenopteran pollinators, including the bumblebee, which has also been declining worldwide (8–11). A study conducted by Singh et al. (11) reported that deformed wing virus (DWV), sacbrood virus (SBV), and black queen cell virus (BQCV), which are common in *A. mellifera*, were detected in eleven species of native bees and wasps as well as in pollen pellets collected directly from healthy foraging bees. Furthermore, the study by Singh et al. (11) showed that viruses in the pollen were infective, as illustrated by the fact that queens became infected and laid infected eggs after

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virus-negative colonies consumed virus-contaminated foods. This discovery raised concerns about a possible role of pollen in spreading viruses and suggested that viruses could possibly contribute to the observed pollinator decline around the world. In order to advance our understanding of the role of pollen in virus transmission of honeybees, we carried out a study to screen bees and pollen loads of bee colonies for the presence of frequent and rare viruses. Our study resulted in the serendipitous detection of a plant virus, tobacco ringspot virus (TRSV), in honeybees and prompted us to investigate whether this plant-infecting virus could cause systemic infection in exposed honeybees.

Generally, the majority of plant viruses are dependent upon herbivorous insects for their spread from one host plant to another in nature but cause infection only in plants that the insect vectors feed upon. To date, only a few plant viruses are known that also infect their insect vectors. *Rhabdoviridae*, a family of arboviruses carried by arthropods, has long been recognized to have a broad range of hosts throughout the animal and plant kingdoms (12). Flock house virus (FHV), a positive-stranded RNA virus of insect origin belonging to the family *Nodaviridae*, has been shown to replicate in plants as well as in yeast (*Saccharomyces cerevisiae*) and mammalian cells (13, 14). A recent study (15) showed that a plant-pathogenic virus, tomato spotted wilt virus (TSWV), which is a member of the family *Bunyaviridae*, could directly alter the behavior of thrips that vector it. The phenomenon of viral host range spanning the plant and animal kingdoms adds an additional layer to the already complex plant-pathogen-pollinator interactions and could have important epidemiological consequences.

TRSV is a type species of the genus *Nepovirus* within the family *Secoviridae* (16). TRSV infects a wide range of herbaceous crops and woody plants, some of considerable economic importance. The infected plants show discoloration, malformation, and stunted growth, accompanied by reduced seed yield or almost total seed loss due to flower and pod abortion. Of a number of plant diseases caused by TRSV, bud blight disease of soybean (*Glycine max* L.) is the most severe. It is characterized by necrotic ring spots on the foliage, curving of the terminal bud, and rapid wilting and eventual death of the entire plant, resulting in a yield loss of 25 to 100% (17). Like other members of the genus, TRSV has a bipartite genome of positive-sense, single-stranded polyadenylated RNA molecules, RNA-1 and RNA-2, which are encapsidated in separate virions of similar size. Both RNA molecules possess a genome-linked protein (Vpg) covalently bound at their 5′ ends. RNA-1 encodes a large polyprotein precursor that is proteolytically processed into protease cofactor (P1A), putative ATP-dependent helicase (Hel), picornain 3C-like protease (Pro), and RNA-directed RNA polymerase (Pol). RNA2 encodes a virion capsid protein (CP), a putative movement protein (MP), and an N-terminal domain involved in RNA-2 replication (P2A). Proteins encoded by RNA-1 are required for RNA replication, while proteins encoded by RNA-2 function in cell-to-cell movement and viral RNA encapsulation. RNA-1 is capable of replication independently of RNA-2, but both are required for systemic infection. Transmission of TRSV can occur in several ways. The numerous vectors include a dagger nematode (18), aphids, thrips, grasshoppers, and tobacco flea beetle (19–21); however, vertical transmission through seeds is important for long-distance dispersal of the virus (22). It has also been shown that honeybees transmit TRSV when they move between flowers and transfer virus-borne pollen from infected plants to healthy ones (23–26). It was, however, unknown prior to our study whether honeybees could become infected by plant viruses they physically encounter or consume.

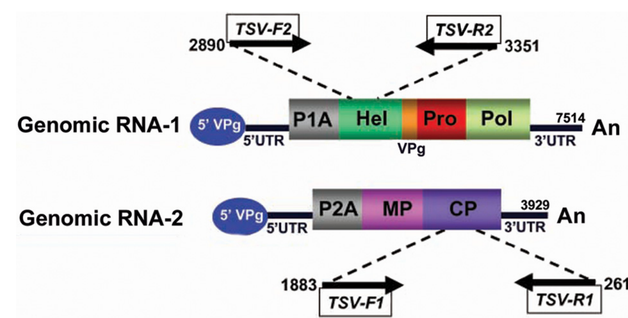

FIG 1 Schematic diagram showing the genome organization of TRSV and locations of primer sets used for virus distribution and replication studies. Open reading frames encoding proteins are boxed and labeled. Positions of primers utilized for amplification of the flanks are marked by black arrows for both RNA segments.

In the present study, we provide evidence that TRSV is present in honeybees and the infection can be widespread through the body of honeybees. TRSV in honeybees does not fit a circulative-propagative model of insect-vectored plant viruses, in which virions are ingested by an insect vector, replicate, and disperse to salivary glands for reinfection of the plant host. Instead, our data indicate that the replication of TRSV occurs widely in the honeybee body but not in the gut or salivary gland and that TRSV in conjunction with other bee viruses is correlated with winter colony level declines. Further, virus was found in a common ectoparasite mite of honeybees, *Varroa destructor*, but was restricted to the gastric cecum. This study presents a unique example of viruses that cause infection in both plants and animals.

RESULTS

Sequence identity of TRSV genomic segments and morphology of the virus isolates. Sequence analysis of cDNA libraries from purified virus preparation revealed overlapping and nonoverlapping clones of different lengths. About 75% of the clones ($n = 40$) matched the genome sequences of common honeybee viruses, including BQCV, DWV, and Israeli acute paralysis virus (IAPV). Unexpectedly, about 20% of the clones ($n = 10$) matched the sequences of TRSV for two genomic segments in the NCBI database. By assembling sequence fragments from different cDNA clones, we obtained a 1,545-bp length of nucleotide sequences encoding the RNA helicase and covering ~21% of the coding region of the polyprotein gene of genomic RNA-1. We also obtained a 2,024-bp long sequence encoding the complete capsid protein. A BLAST search of the helicase sequence showed highest identity with a TRSV strain isolated from bud blight disease of soybean (GenBank accession no. U50869), with 88% homology at the nucleotide level and 96% homology at the amino acid level. A BLAST search of the DNA fragment encoding the capsid protein showed strongest similarity to a TRSV strain from bean (GenBank accession no. L09205), with 96% homology at the nucleotide level and 99% homology at the amino acid level. The cDNA sequences were used to design two primer sets, TRSV-F1/R1 and TRSV-F2/R2 (Fig. 1), for the subsequent studies of TRSV replication and distribution in honeybees and *Varroa* mites.

Electron microscopy showed no obvious contamination from

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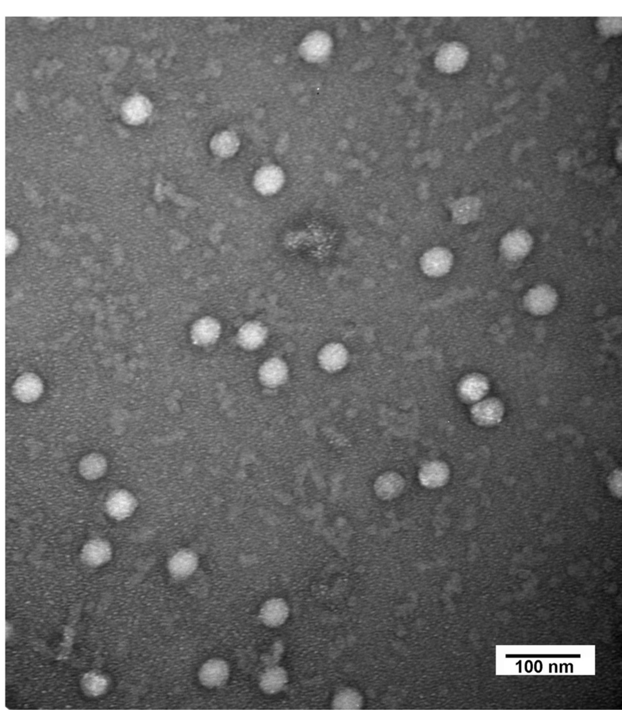

FIG 2 Electron microscopy of TRSV particles from infected honeybees. The presence of TRSV particles in viral preparation was confirmed by RT-PCR assay. Bar, 100 nm.

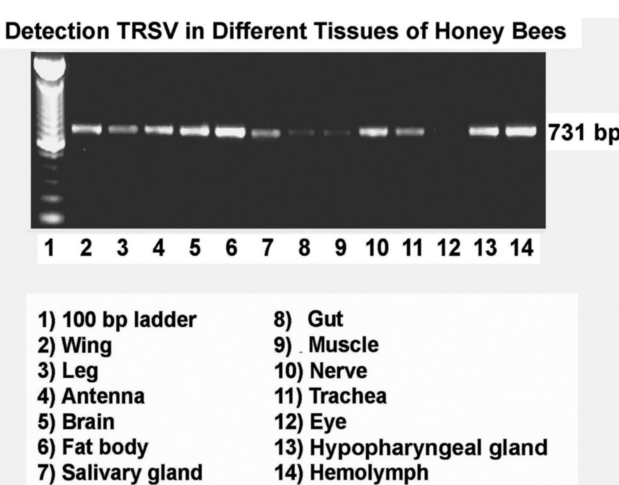

FIG 3 Detection of tobacco ringspot virus (TRSV) in different tissues of honeybees by conventional RT-PCR. The 731-bp bands on the right side of the gels indicate the presence of positive signal for TRSV.

host cellular material. Negatively stained viral particles had a diameter of 25 to 30 nm and an icosahedral shape, typical morphological features of secoviruses (Fig. 2), and RT-PCR assay confirmed the presence of TRSV in the viral preparation for EM analysis.

The purity of the virus preparation in our study was confirmed by electron microscopy. Electron microscopy showed no obvious contamination from host cellular material. Negatively stained viral particles had a diameter of 25 to 30 nm and an icosahedral shape, typical morphological features of secoviruses (Fig. 2). However, the viral preparation was determined by RT-PCR to contain not only TRSV but other bee viruses as well, including BQCV, DWV, and IAPV. It was not possible to definitely distinguish TRSV viral particles morphologically from these other bee viruses.

Distribution and replication of TRSV in infected honeybees. Although no apparent disease symptoms were observed in examined bees, TRSV was widespread in honeybee tissues, which was confirmed by the amplification of a 731-bp PCR fragment with the TRSV-F2/R2 primer set. Except for the compound eyes, TRSV was found in all tissues examined, including hemolymph, wings, legs, antennae, brain, fat bodies, salivary gland, gut, nerves, tracheae, and hypopharyngeal gland. Although there was the same amount of input cDNA, the intensity of the PCR signals varied between samples. Tissues of the gut and muscle had weaker PCR bands than other tissues, indicating a relatively lower level of TRSV infection (Fig. 3). It is unclear if the absence of PCR amplification in the compound eye was due to PCR inhibition previously reported for that tissue (27).

TRSV is a positive-stranded RNA virus replicating through the production of a negative-stranded intermediate; therefore, the presence of negative-stranded RNA constitutes proof of active viral replication. To investigate the replication of TRSV in bees, negative-stranded RT-qPCR was performed using a tagged primer system (28). Amplification and sequence analysis of a 462-bp negative-strand-specific product in different tissues showed that active replication of TRSV occurs in most tissues (Fig. 4). A single peak on the melting curve analysis corroborated the specificity of the amplicon. The lack of amplification following RT-qPCR of total RNA without primers in the reverse transcription reaction mixture ruled out any nonspecific effect from self priming due to the secondary structure of viral RNA or false priming by antigenomic viral RNA or cellular RNAs. Among tissues with detectable levels, the relative abundance of negative-stranded TRSV varied significantly ($P < 0.001$; one-way analysis of variance [ANOVA]). The brain had the lowest detectable level of negative-stranded TRSV and was chosen as the calibrator. The abundance of TRSV in other tissues relative to the brain ranged from 56-fold to 957-fold. The concentration of TRSV in additional body tissues showed the following ranking: muscle > hypopharyngeal gland > leg > fat body > trachea > hemolymph > antenna > nerve > wing. The replication of TRSV was not evident in the salivary gland, gut or compound eye (Fig. 5), although the presence of PCR inhibitors in the latter is a possibility (27).

Localization of TRSV in the ectoparasitic *Varroa* mite of honeybees. *In situ* hybridization showed that TRSV could also be detected in the ectoparasitic mite, *V. destructor*, collected from the same TRSV-infected bee colonies. Sections hybridized with a digoxigenin (DIG)-labeled TRSV RNA probe had strong staining within the storage organs of the mite, the upper and lower gastric ceca (Fig. 6A), although histopathological signs were not evident in these areas. No positive signal of TRSV was observed in other mite tissues, and no signal was observed with the negative-control probe (Fig. 6B).

Prevalence of TRSV infection in honeybee colonies. Of ten bee colonies included in this study, six were classified as described in Materials and Methods as strong colonies and four were classified as weak colonies. Both TRSV and IAPV were absent in bees from strong colonies in any month, but both were found in bees from weak colonies. As with other detected viruses, TRSV showed

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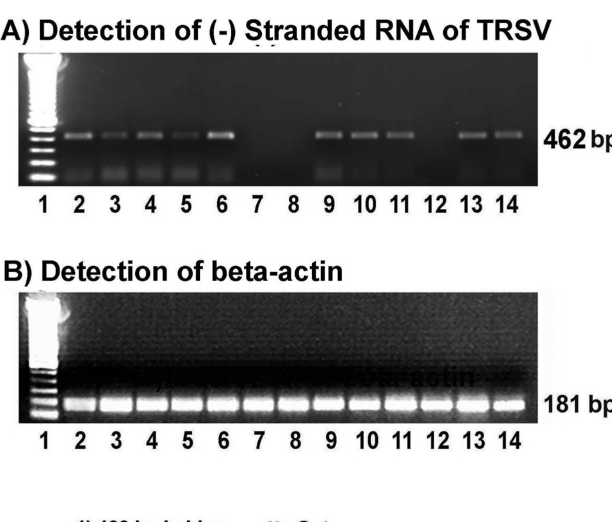

FIG 4 Detection of negative-stranded RNA of TRSV and housekeeping gene for β-actin in different tissues of honeybees by strand-specific RT-qPCR. The 462-bp bands on the right side of the gels indicate the presence of a positive signal for negative-stranded RNA of TRSV. The similar signal intensity of β-actin indicates the same amount of starting material in each tissue sample.

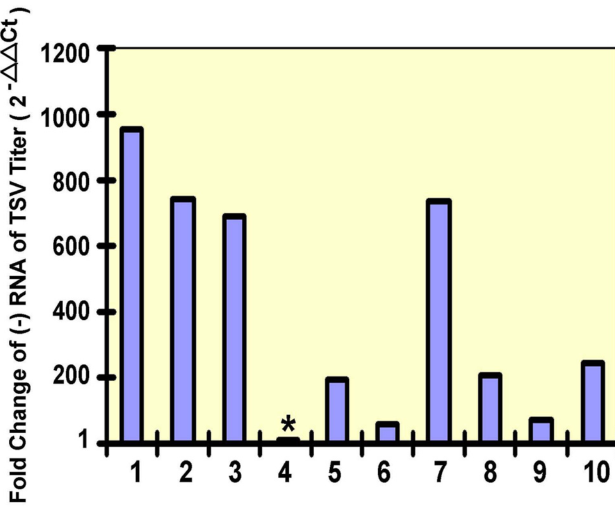

FIG 5 Relative abundance of negative-stranded RNA of TRSV in different tissues of honeybees. Brain tissue had the minimal level of TRSV and therefore was chosen as a calibrator. The concentration of negative-stranded RNA of TRSV in other tissues was compared with the calibrator and expressed as *n*-fold change. The *y* axis depicts fold change relative to the calibrator.

a significant seasonality. The infection rate of TRSV increased from spring (7%) to summer (16.3%) and autumn (18.3%) and peaked in winter (22.5%) before colony collapse. Of viruses detected in weak colonies, DWV was the most commonly detected, with an average annual infection rate of 44%, followed by BQCV, IAPV, and TRSV. Additionally, a low incidence of SBV and chronic bee paralysis virus (CBPV) infections was also detected in bees from weak colonies. While DWV and BQCV were detected in both healthy and weak colonies all year round, the prevalence of DWV and BQCV in weak colonies was significantly higher than that in strong colonies. The bee populations in weak colonies that had a high level of multiple virus infections began falling rapidly in late fall. All colonies that were classified as strong in this study survived through the cold winter months, while weak colonies perished before February. In Fig. 7A and B, the seasonal prevalence of TRSV along with other bee viruses in both weak and strong colonies is presented.

Phylogenetic characterization of TRSV isolates. Figure 8 illustrates the phylogenetic relationship among our TRSV isolates and viruses with existing GenBank TRSV sequence records, based on the partial capsid protein sequence amplified with primers. TRSV isolates infecting plants constitute the early lineages of the phylogenetic tree, and TRSV isolates from honeybees, bee pollen, and *Varroa* mites clustered together, branching next from the early lineage. There is no obvious sequence divergence among TRSV isolates from bees, mites, and bee pollen.

DISCUSSION

Among major pathogen groups, RNA viruses have the highest rate of mutation, because the virus-encoded RNA polymerases lack 3′→5′ exonuclease proofreading activity (29). The consequence of such high mutation rates is that populations of RNA viruses exist as "quasispecies," clouds of genetically related variants that might work cooperatively to determine pathological characteristics of the population (30). These sources of genetic diversity coupled with large population sizes facilitate the adaptation of RNA viruses to new selective conditions, such as those imposed by a novel host. RNA viruses therefore are the most likely source of emerging and reemerging infectious diseases, such as human immunodeficiency virus (HIV), severe acute respiratory syndrome (SARS), type A avian influenza A (H5N1), and swine origin influenza A (H1N1), that have engendered worldwide public health concern because of their invasiveness and ability to spread among different species (31–35).

Honeybees carry a strong electrostatic charge that ensures the adherence of pollen to their bodies, and they also actively store pollen in specialized pollen baskets on their hind legs. It is therefore not unexpected that the foraging behavior of honeybees could move virus-contaminated pollen to the flowers of healthy plants (26, 36). However, this study represents the first evidence that honeybees exposed to virus-contaminated pollen could also be subsequently infected and that the infection could be systemic and spread throughout the entire body of honeybees. About 5% of known plant viruses are pollen transmitted, and the genomes of the majority of plant viruses are made of RNA (37, 38), providing a large set of potential host-jumping viruses. The finding from this study illustrates the complexity of relationships between plant pathogens and the pollinating insects and emphasizes the need for surveillance for potential host-jumping events as an integrated part of insect pollinator conservation.

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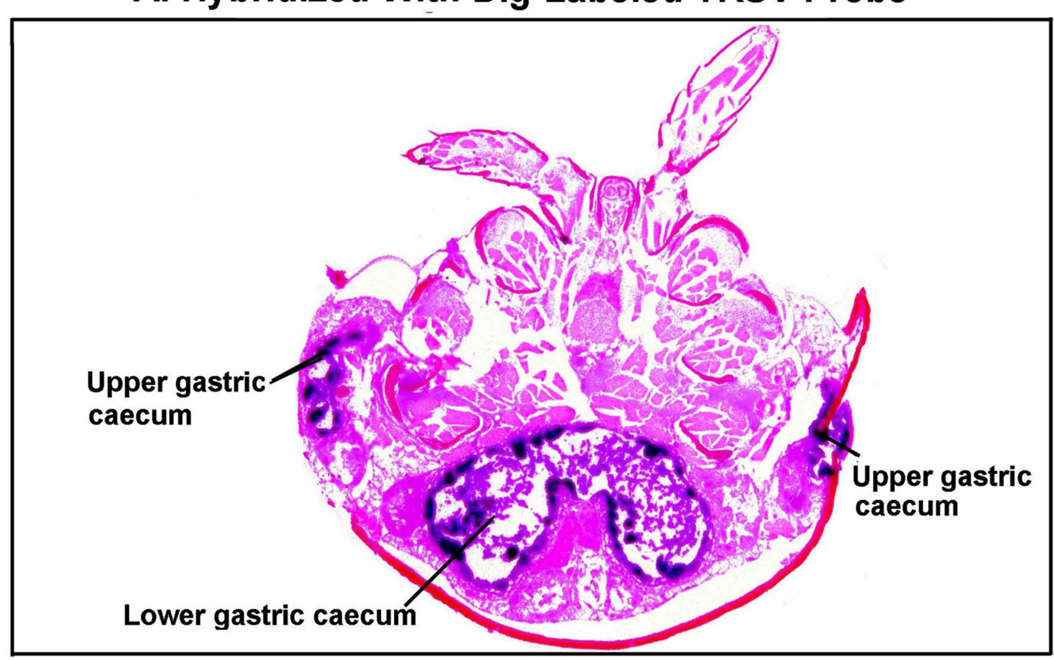

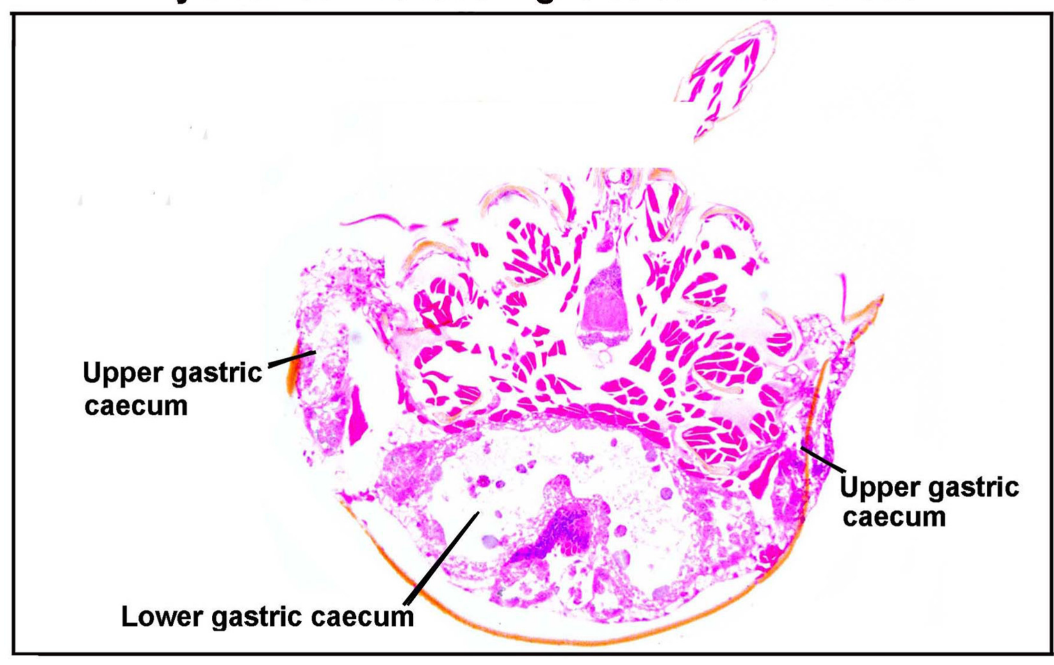

FIG 6 In situ hybridization analysis of *Varroa* mites. (A) The slides were hybridized with DIG-labeled TRSV probe. (B) The slides were not hybridized with DIG-labeled TRSV probe. The positive signal is dark blue, and the negative areas are pink. The infected tissues of the upper and lower gastric ceca are shown in dark blue.

For a virus to successfully establish infection in a novel host, the virus must overcome three major hurdles. First, it must have the opportunity to come into contact with a prospective host for the viral particles to gain entry into the host cells. Second, the virus must undergo genetic changes that mediate the entry of virus into host cells, typically through host receptors on the cell surface. The virus must also undergo genetic changes that can lead to the ability to bypass the host's immune defense and replicate its genome using the host's cellular machinery. Finally, the virus must gain the ability to spread horizontally between individuals of the same gen-

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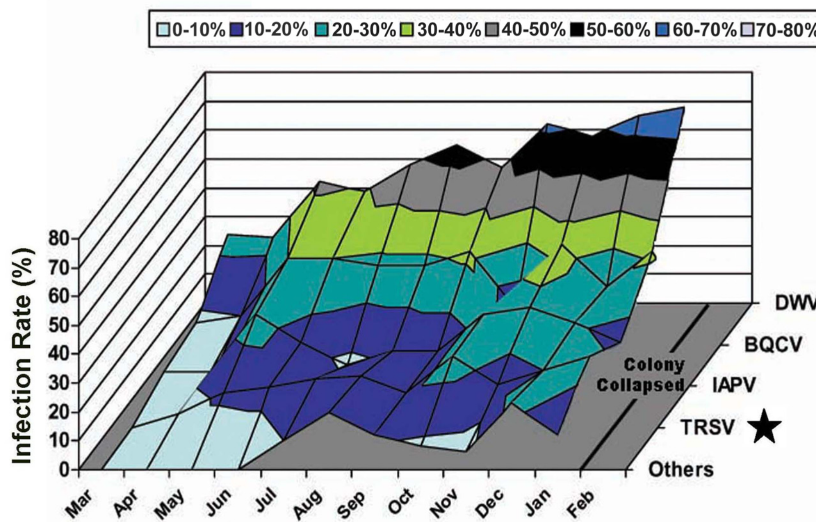

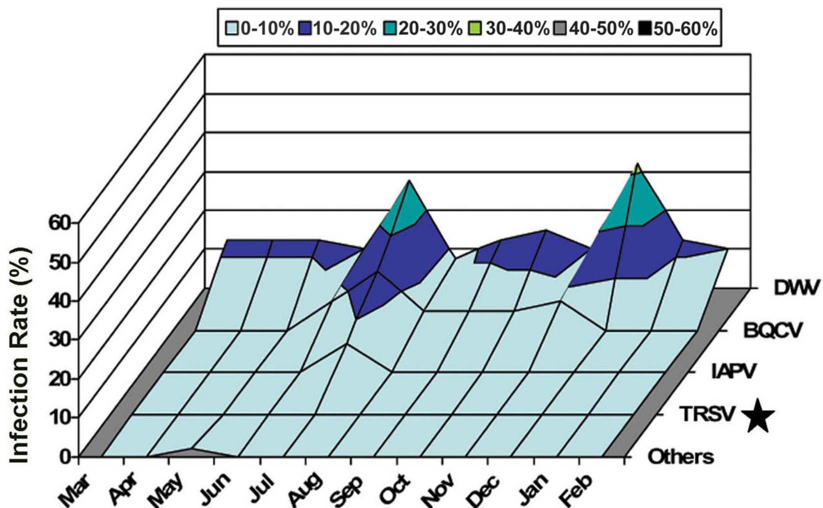

FIG 7 Seasonal prevalence of TRSV and other honeybee viruses in honeybee colonies. (A) Weak colonies. The prevalence of TRSV along with deformed wing virus (DWV), black queen cell virus (BQCV), Israeli acute paralysis virus (IAPV), and two rarely detected viruses, sacbrood virus (SBV) and chronic bee paralysis virus (CBPV) was found in all season. The viral infections reached their peaks in winter before the colony collapsed. Of viruses detected in weak colonies, DWV was the most prevalent, followed by BQCV, IAPV, TRSV, and others (SBV and CBPV). (B) Strong colonies. Only DWV and BQCV were detected in healthy colonies all year round, but the prevalence of the viruses in strong colonies was significantly lower in weak colonies. All strong colonies survived through the cold winter months.

eration within new host populations. The detection of replicate intermediates of TRSV in different tissues of honeybees and the prevalence of TRSV in bee populations provide strong evidence that TRSV has overcome these key hurdles. The presence of a TRSV-positive signal in parasitic *Varroa* mites suggests that *Varroa* could serve as a vector to facilitate the horizontal transmission of TRSV between bees in the colonies.

Food-borne transmission is one of the most important routes for virus transmission in honeybees. Infections of several honeybee viruses occur through ingestion of virus-contaminated food followed by dissemination of the viruses from the midgut into other tissues through the hemolymph (39). Since TRSV is a known pollen-borne plant virus, we initially believed that the presence of TRSV was restricted to the bees' digestive tract. However, titers of TRSV in our study were unexpectedly low in the gut. Viral replication was not detected in either the gut or the salivary gland. Instead, high titers of negative-stranded virus were found in the wing, nerve, antenna, trachea, hemolymph, and fat body, indicating replication in those tissues. The absence of virus replication in the tissues of the gut and salivary gland excludes the possibility of TRSV as a persistent-propagative virus which must first replicate in epithelial cells of the midgut and then migrate to the salivary glands to be ejected together with saliva. Our quantitative analysis suggests that TRSV is neurotropic in honeybees, with

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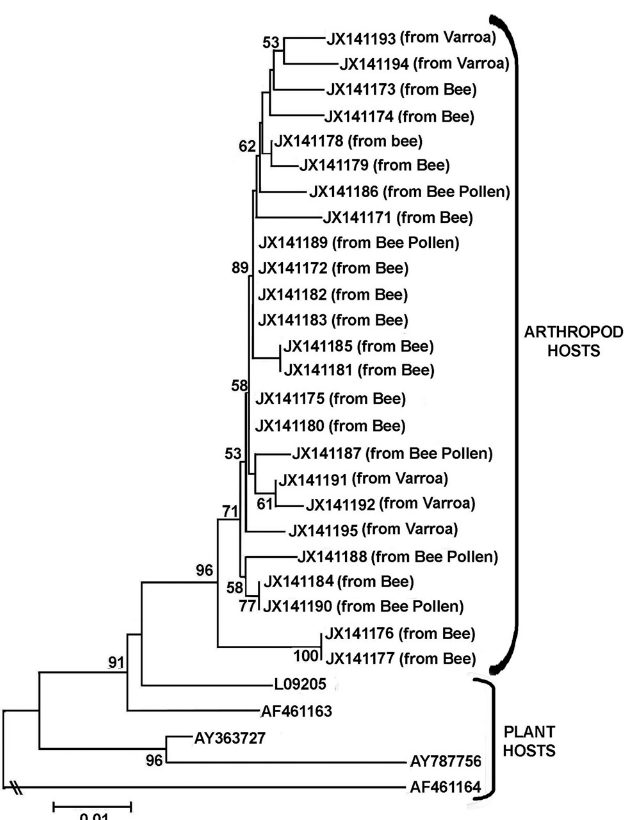

FIG 8 Phylogenetic tree showing the relationship of TRSV isolates from arthropod and plant hosts. The partial sequences of capsid protein of TRSV amplified from honeybees, *Varroa* mites, and bee pollen were compared with the corresponding regions of TRSV that were isolated from plants and retrieved from GenBank. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Numbers at each node represent bootstrap values as percentages of 500, and only bootstrap values of >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and in the units of the number of base substitutions per site. Individual sequences are labeled by their GenBank accession numbers.

more extensive infection detected in the nervous system than in other internal tissues, and therefore it is conceivable that severe TRSV infection can cause functional impairment of the nerve and muscle in honeybees. The low levels of TRSV in the gut suggests a possible result of sloughing off of infected epithelial cells from midgut as a host defensive mechanism or the possibility that TRSV might utilize some alternative invasion routes such as the neural or tracheal route. Further investigation of the virus transmission and pathogenicity is warranted.

The circulation of TRSV in bee hemolymph was further proven by the presence of TRSV in *Varroa* mites. *Varroa* is an obligate parasite of the honeybee and has been catastrophic for the beekeeping industry. Both adult mites and nymphs use their piercing mouth parts to penetrate the body wall of the bees and suck out the hemolymph. In addition to its direct detrimental effects on host life span and colony vigor (40–45), the feeding of mites on bees provides an entry for microbial pathogens (44). Indeed, the roles played by *Varroa* mites in acquiring and transmitting honeybee viruses have been experimentally demonstrated in several studies (46–49). The observation of the positive signal of TRSV within the storage organ of the mites suggests that the *Varroa* mite is not merely a mechanical vector that physically transports viruses from host to host with its mouthparts. More work is needed to confirm whether *Varroa* mites can act as a biological vector to support TRSV replication.

TRSV isolates from honeybees, *Varroa* mites, and bee pollen clustered together phylogenetically, indicating that they descended from a common ancestor. It is likely that *Varroa* mites obtained the virus from their hosts during the blood feeding and that the virus-infected bees contaminated the bee pollen when they mix plant pollen with their glandular secretions and honey to produce "bee bread." The finding that TRSV isolates from honeybees appeared to be derived more recently on the evolutionary timeline than TRSV from plants suggests that life cycles of the virus involving arthropod hosts evolved after host expansion. However, it remains to be determined whether TRSV possesses the ability to maintain persistent infection in honeybee colonies in the absence of newly inoculated viruses from visited plants or whether infected bees can subsequently inoculate healthy plants. It will similarly be helpful to screen other pollinator species for the presence of TRSV, since it is known that honeybees and other pollinators share some viral species (8–11, 50).

Sequence comparison of the TRSV isolates from this study with isolates with other accession numbers suggests that the capsid protein region is much more conserved than the RNA helicase region at the nucleotide level. The relatively high level of sequence similarity at the amino acid level for both capsid protein and helicase indicates a high level of structural and functional conservation. Nevertheless, substitution of a single or a few amino acids at the surface of virus particles can be sufficient to alter receptor recognition and thereby alter host range (51). Thus, the few amino acid polymorphisms observed in TRSV strains infecting honeybees may still be associated with cell tropism and host adaptation. It would be helpful to further characterize the complete genome of TRSV isolates from honeybees as well as from *Varroa* mites to deepen our understanding of genetic diversity of this virus. More work is needed to elucidate the molecular basis of cell tropism and host range modifications and to investigate the roles of the honeybee as a newly identified host in the epidemiology of TRSV.

The evidence of systemic spread and propagation of a plant-pathogenic virus in honeybees raises awareness of the potential impact of new viral disease emergence on bee health. While findings from this study have important implications for understanding TRSV transmission and pathogenesis, much remains to be learned about the intracellular life cycle, species-level genetic variation, and pathogenesis of the virus in honeybee hosts. Although the cause(s) of CCD and the decline in the worldwide bee population is not yet fully understood (52), a growing body of evidence has indicated that parasites and pathogens are key culprits involved in widespread disappearance/death and population declines of honeybees (3, 5, 53–57). The observation that increasing prevalence of TRSV in conjunction with other bee viruses in infected colonies is associated with gradual decline of host populations and winter colony collapse supports the argument that virus infections could have a significant negative impact on colony survival. While the simultaneous presence of multiple viruses and asymptomatic viral infections in honeybees as well as lack of a cell culture system for virus production (58, 59) makes Koch's postulates of disease causality difficult to fulfill, the observed negative

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correlation between the level of TRSV infections and size of host populations suggests that TRSV, in combination with other viruses, is likely a contributing factor to poor survivorship of honeybee colonies.

MATERIALS AND METHODS

Honeybee colonies and sample collection. Honeybee colonies used for this study were maintained in the research apiaries of the USDA-ARS Bee Research Laboratory in Beltsville, MD. For viral particle purification and tissue dissection, fifty adult worker bees were collected by removing a central frame filled with brood and covered with adult bees from a hive and gently scraping worker bees into a 50-ml conical tube. In addition, bee pollen that was processed by bees and stored in combs around the brood was collected using a spatula and transferred into 15-ml conical tubes. Individual *Varroa* mites that had crawled from brood cells onto the tops of brood frames were collected with forceps and transferred into 1.5-ml microcentrifuge tubes.

For assessing the effects of viruses on honeybees, the seasonal prevalence of virus infections was determined in ten colonies for a period of 1 year starting in March and finishing in February of the following year. Bee colonies were classified as strong or weak based on the size of adult populations, amount of sealed brood, and presence of food stores, as previously described (60). Bee colonies that had more than ten frames covered with adult workers and more than six frames filled with brood and food stores were defined as strong colonies, while bee colonies that had a small number of foraging bees flying in and out, fewer than ten frames of adult bees, fewer than six combs with brood, and small patches of food stores were defined as weak colonies. For each colony, samples of 20 adult workers were collected every month and stored at −80°C until subsequent RNA isolation for virus analysis.

Virus purification and electron microscopy. Thirty worker bees were frozen in liquid nitrogen, ground to a fine powder, and homogenized in 10-ml extraction buffer (0.1 M potassium phosphate buffer [pH 7.5], 0.2% diethyldithiocarbamate, 1/5 volume of diethyl ether). The mixture was emulsified with 5 ml carbon tetrachloride and centrifuged at 5,000 × *g* at 4°C for 30 min to remove tissue debris. Supernatant containing viruses was centrifuged once more at 5,000 × *g* at 4°C for 30 min and then filtered through a 45-μm filter to remove small tissue debris. The filtrate was then centrifuged at 10,187 × *g* for 6 h at 4°C to pellet the viral particles. The pellet was resuspended in 2 ml of 0.2 M phosphate-buffered saline (PBS) buffer. A 15-μl portion of viral solution was examined for the presence of virus particles in an electron microscope. The rest of the viral solution was saved for subsequent viral RNA isolation and cDNA library construction.

Virus particles were negatively stained with 2% uranyl acetate on a Formvar-coated Ni grid and viewed in a Hitachi H-7000 electron microscope at magnifications between ×33,000 and ×100,000.

cDNA library construction and virus-specific primer design. Total RNA was extracted by homogenizing the viral solution with TRIzol LS reagent (Invitrogen), a solution of phenol and guanidine isothiocyanate used for isolating total RNA from liquid samples according to the manufacturer's instructions. The resultant RNA pellets were resuspended in DNase- and RNase-free water (Invitrogen) in the presence of ribonuclease inhibitor (Invitrogen). The quantity and purity of RNA were measured with a NanoDrop spectrophotometer (NanoDrop Technologies). The cDNA library was constructed using a CloneMiner cDNA library construction kit (Invitrogen) per the manufacturer's protocol. First-strand cDNA was synthesized from extracted RNA using Superscript II reverse transcriptase with a biotin-conjugated *att*B2 oligo(dT) primer. After cDNA synthesis, the products were size fractionated by column chromatography to remove excess primers, adapters, and small cDNAs and cloned into an *att*P-containing donor vector, pDONR 222. The BP (recombination between *att*B and *att*P sites) reaction products were transformed into ElectroMAX DH10B T1 phage-resistant cells, and the transformed cells were plated onto LB agar medium supplemented with kanamycin (50 μg/ml). The positive clones were purified using the Wizard Plus miniprep DNA purification system (Promega). A total of 50 cDNA clones were randomly selected and sequence analyzed to confirm the presence of the insert.

Primers specific for TRSV RNA segments 1 and 2 were designed based on the nucleotide sequences obtained from cDNA clones of this study. The sequences of primers for amplifying a 462-bp region of helicase (Hel) of RNA segment 1 were TRSV-F1 (5′-CATGAATGTTGTTATCCAAT-3′) and TRSV-R1 (5′-TCCTCAGTAAATTTCATTTG-3′). The sequences of primers for amplifying a 731-bp region of capsid protein (CP) region of RNA segment 2 were TRSV-F2 (5′-GTGTGCTGTGACGGTTGTTCC-3') and TRSV-R2 (5′-TGCCAGACCACCCAAGATTCC-3′). Figure 1 illustrates the positions of primers.

Bee tissue dissection. Twenty adult worker bees were individually fixed on the wax top of a dissecting dish with steel insect pins. Under a dissecting microscope, about 10 μl of hemolymph was collected from each bee with a micropipette tip by making a small hole on the roof of the bee's thorax with a needle to make it bleed. Following hemolymph collection, the legs, wings, antennae, and compound eyes were cut off with a pair of fine scissors. The body was opened by cutting along the dorsal midline from the tip of the abdomen to the head with scissors. Tissues of the brain, fat body, salivary gland, gut, muscle, nerve, trachea, and hypopharyngeal gland were individually removed using a pair of fine forceps under a dissecting microscope. In total, thirteen tissues were collected from each bee, and a total of thirty bees were dissected. The scissors and forceps were wiped between tissues once with a cotton pad soaked with 10% bleach and once with a cotton pad soaked with 70% alcohol followed by a final rinse in sterile water. To prevent possible contamination with hemolymph, all tissues were rinsed once in 1× phosphate-buffered saline (PBS) and twice in nuclease-free water. The washing solution was changed every time for each tissue to prevent cross-contamination. All freshly dissected tissues were subjected to subsequent RNA extraction immediately.

Total RNA extraction and conventional RT-PCR. Total RNA was isolated from disserted tissues, adult bees, bee pollen, and *Varroa* mites using Invitrogen Trizol reagent according to the manufacturer's instructions. Conventional RT-PCR was performed on RNA samples extracted from adult bees, *Varroa* mites, different tissues, and bee bread collected from the same colony for the presence and distribution of TRSV. The Promega one-step Access RT-PCR system was used for virus detection as previously described (58). PCR products were purified and sequenced to confirm the specificity of the primers.

To determine the seasonal prevalence of TRSV in honeybee colonies, bee samples collected every month were subject to RT-PCR analysis individually for TRSV as well as other seven common honeybee viruses, including acute bee paralysis virus (ABPV), BQCV, chronic bee paralysis virus (CBPV), DWV, Israeli acute paralysis virus (IAPV), Kashmir bee virus (KBV), and SBV. The primer pair TRSV-F2/TRSV-R2 was used for RT-PCR amplification of TRSV. The primer sets used for RT-PCR amplification of common honeybee viruses have been reported previously (49, 58). Putative TRSV amplification products were purified and sequenced to confirm the specificity of the RT-PCR assay. The infection rate of each virus (20 workers) and strength of individual colonies were recorded every month throughout the year.

Strand-specific RT-qPCR. In order to determine the ability of TRSV to replicate in different tissues of honeybees, RNA samples were further analyzed for the presence and abundance of negative-stranded RNA, a replicative intermediate, using strand-specific reverse transcription coupled with quantitative PCR (RT-qPCR). For each tissue sample, the first-strand cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen) with Tag-TRSV-F1 (5′-AGCCTGCGCA CGTGGcatgaatgttgttatccaat-3′), where the capitalized sequence corresponding to Tag was published by Yue and Genersch (61). The synthesized cDNAs were then purified using a MinElute PCR purification kit (Qiagen) followed by a MinElute Reaction Clean kit (Qiagen) to remove short fragments of oligonucleotides and residue of enzymatic reagents to

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prevent amplification of non-strand-specific products (28). cDNA derived from negative-stranded RNA was amplified using the Brilliant SYBR green qPCR master mix (Stratagene) with a 0.4 μM concentration each of the Tag (3′-AGCCTGCGCACCGTGG-5′) and TRSV-R1 primers in a 25-μl volume according to the manufacturer's protocol. To normalize the qPCR result, amplification of a housekeeping gene, the β-actin gene, was performed for each sample with a previously reported primer set (62).

The amplification for both TRSV and β-actin was carried out following the manufacturer's recommended protocol for thermal profile parameters for three-step PCR. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. Each sample was run in triplicate, and the qPCR assay was repeated twice. The amplification efficiencies of the SYBR green real-time RT-qPCR assay for both TRSV and β-actin were proved to be approximately equal (data not shown). The output of RT-qPCR assays for TRSV in different tissues was interpreted by using the comparative cycle threshold method ($\Delta\Delta C_T$ method). The average C_T value (ΔC_T) of TRSV in each tissue was normalized using the C_T value corresponding to the endogenous control, β-actin, with the following formula: ΔC_T = average C_T(TRSV) − average C_T(β-actin). The tissue that had the lowest level of TRSV was chosen as a calibrator. The ΔC_T value of each tissue was subtracted from the ΔC_T value of the calibrator to yield $\Delta\Delta C_T$. The concentration of TRSV in each tissue was calculated using the formula $2^{-\Delta\Delta CT}$ and expressed as *n*-fold difference relative to the calibrator.

***In situ* hybridization.** Purified amplicons corresponding to the region flanked by the TRSV-F2 and TRSV-R2 primer set were incorporated into a pCR2.1 TA cloning vector upstream of a T7 promoter (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Recombinant plasmid DNAs with the TRSV insert were linearized by restriction enzyme BamHI (New England Biolabs, Ipswich, MA) at 37°C for 2 h. The linearized DNAs were extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), precipitated by ethanol, and dissolved in nuclease water. The DIG-labeled RNA probe complementary to TRSV genomic RNA was synthesized using a DIG-RNA labeling kit (T7) (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol.

Live *Varroa* mites were fixed in 4% paraformaldehyde in 100 mM PBS (pH 7.0) overnight at 4°C, rinsed in nuclease-free water three times, and then stored in 70% ethanol (200 proof) at 4°C until used. Tissue dehydration was carried out by successive incubations in ethanol (70%, 95%, and 100%) and xylol (twice for 5 min each) and embedded in paraffin. Paraffin sections were cut 2 to 5 micrometers thick and mounted on poly-L-lysinated slides and stored at 4°C overnight. The sections were then rehydrated through a descending concentration of ethanol (100%, 95%, and 70%), dewaxed in xylol, treated with proteinase K (10 μg/ml) for 30 min, and acetylated with 0.33% (vol/vol) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min prior to hybridization.

The sections were prehybridized in prehybridization solution (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 40 μg/ml salmon sperm) at 58°C for 2 h and incubated in hybridization buffer with DIG-labeled TRSV probe solution to a concentration of 100 to 200 ng/ml probe in prehybridization solution at 58°C overnight. After hybridization, the sections were washed twice in low-stringency wash solution (2× SSC, 0.1% SDS) at room temperature for 5 min and washed twice in high-stringency wash solution (0.1× SSC, 0.1% SDS) at 52°C for 15 min. The hybridization signals were detected with alkaline phosphatase (AP)-labeled sheep anti-DIG antibody conjugate (Roche Applied Science). The conjugate solution was added to the dry sections and incubated at 4°C for 2 h in a humid chamber. The slides were rinsed three times with washing buffers. The color development was performed by adding the buffer solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to the tissue sections and incubating for 3 to 6 h at room temperature with protection from light. The color reaction was stopped by a 5-min wash in Tris-EDTA (0.1 mM, pH 8.0). The nonspecific staining was removed in 95% ethanol overnight. The sections were rehydrated through successive incubation in ethanol (70%, 95%, and 100%) and xylol (twice for 15 min each) and mounted in Eukitt resin. Negative control reactions included regular dUTP instead of DIG-labeled TRSV probe. *In situ* hybridization slides were observed under a light microscope (Eclipse TE 300; Nikon) and photographed with a Nikon digital camera (DXM 1200). Dark blue coloring indicates where the DIG-labeled probe bound directly to the viral RNA. The section hybridized with the negative control showed pink staining only from the application of nuclear fast red.

Phylogenetic analysis. The sequences of the 731-bp TRSV fragment amplified from the region encoding the capsid protein by the primer pair TRSV-F2 and TRSV-R2 from honeybees, bee pollen, and *Varroa* mites were compared with existing GenBank sequences isolated from plants. Phylogenetic analysis was conducted in MEGA4 (63). The sequences were aligned using ClustalW, and the sequences that could not be aligned unambiguously at both 3′ and 5′ ends were truncated. A tree was built using the neighbor-joining method (64) with distances computed using the maximum composite likelihood method (65). The reliability of the phylogenies was assessed by bootstrap replication (500 replicates) (66). Node labels correspond to bootstrap support, and values of >50% were regarded as evidence for the phylogenetic grouping.

Nucleotide sequence accession numbers. The cDNA sequence data have been submitted to the GenBank sequence database and assigned the accession numbers JQ710729 and JQ710730 for the helicase and capsid protein coding regions, respectively.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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NEWS

Studies highlight differences between banned pesticides

21 January 2014, by Alex Peel

Two separate studies have added new weight to the idea that some varieties of controversial neonicotinoid pesticides are less harmful to bees than others.

The research tested the effects of feeding two different neonicotinoid compounds, thiamethoxam and imidacloprid, to bumblebees. The EU recently banned both pesticides over concerns about their effects on bee health.

Ian Laycock, from the University of Exeter, led the studies.

'We found that both thiamethoxam and imidacloprid were capable of repressing feeding and brood production in bumblebees,' he says. 'But with thiamethoxam this only happened with very large concentrations of the pesticide – concentrations that bees are unlikely to encounter in the environment.'

In contrast, imidacloprid reduced both egg production and pollen consumption by half even at the very low concentrations that bees are likely to encounter when they forage on treated crops.

> 'What our study shows is that we cannot simply apply the conclusions about the safety of imidacloprid to other neonicotinoids'
> **Ian Laycock, University of Exeter**

'We think this occurs because imidacloprid produces a stronger repression of feeding in bumble bees than thiamethoxam, and this imposes a greater limitation on the bees' ability to produce eggs,' says Laycock.

'Brood production is particularly important in bumble bee colonies because the number of eggs and larvae a colony produces can dictate how many workers it has - and colonies with more workers tend to be more successful.'

'In particular they produce more queens that go on to found new colonies and keep the colony cycle going the following year. So our results raise further concern about the threat of imidacloprid to bumblebees.'

But the research also had some better news for bees. After two weeks' exposure, the bees were allowed to recuperate, and seemed to bounce back well.

This kind of 'pulsed' exposure is designed to replicate the scenario in the wild, where bees feed on mass-flowering crops, like oil-seed rape, for a window of just a few weeks while they're in flower. When the bloom is over, wild bees often switch back to pesticide-free wildflowers.

While the scientists tested imidacloprid on regular colonies, the research on thiamethoxam was carried out on micro-colonies, each made up of four worker bees kept apart from the queen. This allowed the scientists to monitor responses such as feeding, egg-laying and brood survival in precise detail.

In natural colonies, the queen bee does most of the brood production herself, and Laycock acknowledges that the lack of queens in the micro-colonies does limit the study's ability to replicate the bees' real-world environment.

But he believes the research still gives a useful insight into the different effects of neonicotinoid compounds. And he maintains that lab studies have an important role to play in gathering evidence on pesticides.

'It's impossible to perfectly replicate the real world in the lab, and for that reason some people will always question the

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'But carefully designed lab studies are vital because, unlike field studies, we can precisely control variables such as the concentration or dose of the pesticide that the bee receives.'

In December last year, the EU introduced a two-year moratorium on neonicotinoids. The UK voted against the ban, citing a lack evidence of harm to bees in field studies.

Laycock believes that the evidence against imadacloprid is probably strong enough to justify a temporary ban, giving researchers more time to look into its effects. But he says we shouldn't tar all neonicotinoids with the same brush.

'What our study shows is that we cannot simply apply the conclusions about the safety of imidacloprid to other neonicotinoids,' he says.

He is also concerned that the moratorium could encourage farmers to use alternative pesticides on their crops, whose effects on bees are unstudied, and may prove to be even worse.

These thoughts are echoed by Dr Chris Connolly, from the University of Dundee, who was not involved with either study.

'Whether or not these pesticides are responsible for bee decline, there is clear evidence that they have an effect, and so the moratorium seems like a wise precaution.'

'But it would be totally unwise if they are just replaced with other compounds, which we know very little about - it's irrational to set the bar higher for one pesticide than all the others. The whole thing needs to be looked at a bit more scientifically.'

> 'It's irrational to set the bar higher for one pesticide than all the others'
> **Dr Chris Connolly, University of Dundee**

One potential alternative, belonging to a group of chemicals called pyrethroids, was the subject of a separate lab study at Royal Holloway, University of London. Colony exposure to the pesticide was shown to reduce the size of bumblebees' offspring, posing a potential risk to colony success.

Keywords: Biodiversity, Biology, Ecology, Europe, Farming, Insects, Natural resources, Pollinators, UK,

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Ian Laycock, Exeter
Tuesday, 21 January 2014 - 13:12

Common crop pesticides kill honeybee larvae in the hive

Honeybee. Credit: Adam Siegel

Four pesticides commonly used on crops to kill insects and fungi also kill honeybee larvae within their hives, according to Penn State and University of Florida researchers. The team also found that N-methyl-2-pyrrolidone (NMP)—an inert, or inactive, chemical commonly used as a pesticide additive—is highly toxic to honeybee larvae.

"We found that four of the pesticides most commonly found in beehives kill bee larvae," said Jim Frazier, professor of entomology, Penn State. "We also found that the negative effects of these pesticides are sometimes greater when the pesticides occur in combinations within the hive. Since pesticide safety is judged almost entirely on adult honeybee sensitivity to individual pesticides and also does not consider mixtures of pesticides, the risk assessment process that the Environmental Protection Agency uses should be changed."

According to Frazier, the team's previous research demonstrated that forager bees bring back to the hive an average of six different pesticides on the pollen they collect. Nurse bees use this pollen to make beebread, which they then feed to honeybee larvae.

To examine the effects of four common pesticides—fluvalinate, coumaphos, chlorothalonil and chlorpyrifos—on bee larvae, the researchers reared honeybee larvae in their laboratory. They then applied the pesticides alone and in all combinations to the beebread to determine whether these insecticides and fungicides act alone or in concert to create a toxic environment for honeybee growth and development.

The researchers also investigated the effects of NMP on honeybee larvae by adding seven concentrations of the chemical to a pollen-derived, royal jelly diet. NMP is used to dissolve pesticides into formulations that then allow the active ingredients to spread and penetrate the plant or animal surfaces onto which they are applied. The team fed their treated diet, containing various types and concentrations of chemicals, to the laboratory-raised bee larvae.

The team's results are reported in the current issue of *PLoS ONE*.

"We found that mixtures of pesticides can have greater consequences for larval toxicity than one would expect from individual pesticides," Frazier said.

Among the four pesticides, honeybee larvae were most sensitive to chlorothalonil. They also were

negatively affected by a mixture of chlorothalonil with fluvalinate. In addition, the larvae were sensitive to the combination of chlorothalonil with the miticide coumaphos. In contrast, the addition of coumaphos significantly reduced the toxicity of the fluvalinate and chlorothalonil mixture.

According to Chris Mullin, professor of entomology, Penn State, these pesticides may directly poison honeybee larvae or they may indirectly kill them by disrupting the beneficial fungi that are essential for nurse bees to process pollen into beebread.

"Chronic exposure to pesticides during the early life stage of honeybees may contribute to their inadequate nutrition or direct poisoning with a resulting impact on the survival and development of the entire bee brood," he said.

The researchers note that fluvalinate and coumaphos are commonly used by beekeepers on crops to control Varroa mites, and are found to persist within beehives for about five years. Chlorothalonil is a broad-spectrum agricultural fungicide that is often applied to crops in bloom when honeybees are present for pollination because it is currently deemed safe to bees. Chlorpyrifos is a widely used organophosphate in crop management.

"Our findings suggest that the common pesticides chlorothalonil, fluvalinate, coumaphos and chloropyrifos, individually or in mixtures, have statistically significant impacts on honeybee larval survivorship," Mullin said. "This is the first study to report serious toxic effects on developing honeybee larvae of dietary pesticides at concentrations that currently occur in hives."

The team also found that increasing amounts of NMP corresponded to increased larval mortality, even at the lowest concentration tested.

"There is a growing body of research that has reported a wide range of adverse effects of inactive ingredients to human health, including enhancing pesticide toxicities across the nervous, cardiovascular, respiratory and hormone systems," Mullin said. "The bulk of synthetic organic chemicals used and released into U.S. environments are formulation ingredients like NMP, which are generally recognized as safe. They have no mandated limits on their use and their residues remain unmonitored.

"Multi-billion pounds of these inactive ingredients overwhelm the total chemical burden from the active pesticide, drug and personal-care ingredients with which they are formulated. Among these co-formulants are surfactants and solvents of known high toxicity to fish, amphibians, honey bees and other non-target organisms. While we have found that NMP contributes to honeybee larvae mortality, the overall role of these inactive ingredients in pollinator decline remains to be determined."

Other authors on the paper include Wanyi Zhu, graduate research assistant in entomology, Penn State, and Daniel Schmehl, postdoctoral associate in entomology and nematology, University of Florida.

Provided by Pennsylvania State University

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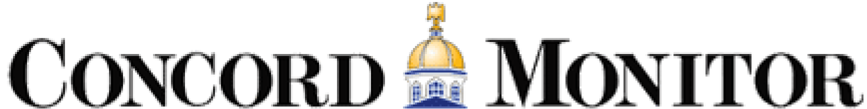

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New Hampshire House kills GMO labeling bill

By LAURA McCRYSTAL
Monitor staff
Thursday, January 23, 2014
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The House killed a bill yesterday that would have required labels on foods that are the product of genetic engineering.

Several House members said yesterday that they heard from many constituents who supported the bill because they fear that there are health risks associated with genetically modified organisms, or GMOs. But House members debated those health concerns yesterday, with the bill's opponents arguing that the risks have been exaggerated.

"There's a lot of hysterical momentum behind this anti-GMO movement," said Rep. Jim Parison, a New Ipswich Republican. "It's sort of like an angry mob seeking justice for a crime just by lynching the first possible suspect."

Parison encouraged the House to kill the bill, even if it would be unpopular with some constituents. He said the labeling would not necessarily protect consumers, and it would hurt business owners. The bill would hold retailers – not manufacturers or food processors – responsible for labeling products.

The House voted, 185-162, to kill the bill.

Supporters said the bill would have allowed consumers to know what is in their food and make their own decisions about the potential risks associated with GMOs.

"Our constituents have spoken about safety concerns," said Rep. Peter Bixby, a Dover Democrat. "Mandatory labeling would empower these individuals to make their own decisions."

Rep. Tim Smith, a Manchester Democrat, said he heard from many constituents who are in favor of GMO labeling. He read a letter from one woman who said, "I just want to know what goes into the food I give my

children, ages 3 and 8."

Other opponents of GMO labeling said the state did not have the ability to enforce it. Rep. Bob Haefner, a Hudson Republican, said New Hampshire cannot regulate food that comes from other states and suggested the bill could be challenged in court as unconstitutional.

"I will never argue about a right to know," Haefner said. "But . . . this is a federal issue. It is not a state issue."

(Laura McCrystal can be reached at 369-3312 or lmccrystal@cmonitor.com () or on Twitter @lmccrystal ().)

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Four Common Pesticides, Their Mixtures and a Formulation Solvent in the Hive Environment Have High Oral Toxicity to Honey Bee Larvae

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Abstract

Recently, the widespread distribution of pesticides detected in the hive has raised serious concerns about pesticide exposure on honey bee (*Apis mellifera* L.) health. A larval rearing method was adapted to assess the chronic oral toxicity to honey bee larvae of the four most common pesticides detected in pollen and wax - fluvalinate, coumaphos, chlorothalonil, and chloropyrifos - tested alone and in all combinations. All pesticides at hive-residue levels triggered a significant increase in larval mortality compared to untreated larvae by over two fold, with a strong increase after 3 days of exposure. Among these four pesticides, honey bee larvae were most sensitive to chlorothalonil compared to adults. Synergistic toxicity was observed in the binary mixture of chlorothalonil with fluvalinate at the concentrations of 34 mg/L and 3 mg/L, respectively; whereas, when diluted by 10 fold, the interaction switched to antagonism. Chlorothalonil at 34 mg/L was also found to synergize the miticide coumaphos at 8 mg/L. The addition of coumaphos significantly reduced the toxicity of the fluvalinate and chlorothalonil mixture, the only significant non-additive effect in all tested ternary mixtures. We also tested the common 'inert' ingredient N-methyl-2-pyrrolidone at seven concentrations, and documented its high toxicity to larval bees. We have shown that chronic dietary exposure to a fungicide, pesticide mixtures, and a formulation solvent have the potential to impact honey bee populations, and warrants further investigation. We suggest that pesticide mixtures in pollen be evaluated by adding their toxicities together, until complete data on interactions can be accumulated.

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Introduction

Recently, one hundred and twenty one different pesticides and metabolites were identified in the hive with an average of seven pesticides per pollen sample, including miticides, insecticides, fungicides, herbicides, and insect growth regulators [1,2]. Feeding on pollen and nectar in the larval diet directly exposes honey bee larvae transdermally, orally and internally [3]; therefore, the potential for chronic toxicity and synergistic interactions at the brood stage seems likely to occur, especially considering the fact that early life stages might be much more sensitive to certain contaminants relative to the adult stage. Several studies have demonstrated that insecticides ranging from insect growth regulators and encapsulated organophosphate formulations to systemic insecticides are more toxic to larvae than to adult bees [4–8]. Moreover, because beebread serves as an absolute requirement for developing bee larvae, pesticide disruption of the beneficial mycofloral community in the colony may thwart the processing of pollen into beebread and allow undesirable pathogens to thrive, therefore indirectly impacting the brood health [9,10]. Indeed, chronic exposure to pesticides during the early life stage of honey bees may thus contribute to inadequate nutrition and/or direct poisoning with a resulting impact on the survival and development of bee brood [11]. Conceivably, these impacts on the larval phase could lead to weakening of the colony structure over time. To date, only a few peer-reviewed pesticide toxicity studies assess the risks of oral toxicity of pesticides to honey bee larvae. Therefore, a goal of our study was to assess the chronic and mixture effects of common pesticides at realistic exposure concentrations on larval honey bee survival. In order to mimic realistic exposure scenarios of honey bee larvae to contaminated pollen food, we chose the four most frequently detected pesticides in the hive - fluvalinate, coumaphos, chlorothalonil, and chlorpyrifos, and tested them alone and in all combinations via chronic dietary exposure, at concentrations found in pollen and beebread.

The pyrethroid *tau*-fluvalinate and the organophosphate coumaphos have been used widely for Varroa mite control, and found highly persistent in the hive with an estimated half-life in beeswax of about 5 years [12]. These compounds have shown evidence of synergistic toxicity on adult honey bees at the level of cytochrome P450-mediated detoxification [13]. Chlorothalonil, a broad-spectrum agricultural fungicide with an unclear mode of action [14], is often applied to crops in bloom when honey bees are present for pollination, because it is currently deemed safe to bees. However, some fungicides have shown direct toxicity to honey bees or solitary bees at field use rates [15] and fungicides in stored

pollen are known to inhibit the growth of beneficial fungi thereby reducing the nutritional value of the pollen to bees [10]. Chlorpyrifos is a widely employed organophosphate in crop management [16] and its residues were frequently found in honey, propolis and dead bees. These in-hive (beekeeper applied) varroacides and out-of-hive (farmer applied) insecticides and fungicides may act alone or in concert, in ways currently unknown, to create a toxic environment for honey bee growth and development.

Another goal of this study was to examine the effect of an 'inert' ingredient on brood survival. Little data exist concerning the toxicity of 'inert' ingredients on honey bees, likely because bee toxicity information for pesticide formulations is not currently required by the U.S. Environmental Protection Agency as part of the pesticide registration process in contrast to the European Union where toxicity for representative formulations is mandatory [17]. Pesticide risk assessment is largely stymied by lack of public access to product-specific information of 'inerts' or co-formulants [18]. Some 'inert' ingredients such as those in formulations of the herbicide glyphosate are more toxic than active ingredients when tested on aquatic organisms [19]. That 'inert' more than active ingredients dominate pesticide formulations and spray tank adjuvants so to increase efficacy and stability of the pesticide makes it important to examine the role of 'inerts' on honey bee toxicity. Here, we studied the chronic toxicity of N-methyl-2-pyrrolidone (NMP, CAS 872-50-4) to bee brood development. The co-solvent NMP is used extensively in chemical processing and agricultural chemical formulations [20,21]. The NMP tested alone or in formulations has demonstrated developmental toxicity in rats by various routes of administration [22] and also has shown high toxicity potential for aquatic invertebrates [23]. There is presently no information in the published literature regarding toxic effects of NMP to honey bees. Our study will be the first to test if this common 'inert' ingredient is toxic to honey bee larvae by continuous dietary exposure, and will serve as a foundation for future studies exploring 'inert' toxicity.

Specific objectives of the present study using the standardized *in vitro* larval feeding method developed by Aupinel et al. [24] are to: (i) assess possible toxic effects of single pesticides on the survival of individual *A. mellifera* larva during a 6-d continuous feeding with contaminated diet; (ii) compare the sensitivity difference between larval and adult bees to the same pesticide exposure; (iii) determine whether the selected pesticides in all combinations at realistic concentrations have any synergistic effects; and (iv) examine the toxicity of environmentally realistic levels of the formulation ingredient NMP on larval survival. Measurable impacts on larvae should demonstrate the need to extend pesticide risk assessment for honey bees from primarily acute effects on adults to chronic impacts on brood survival and development, and of the need to consider both active and 'inert' ingredients in formulations, so that more informed decisions can be made by governments, beekeepers and growers about pesticide application inside and outside the hive.

Materials and Methods

Acquisition of 1st instar larvae

Honey bee (*A. mellifera*) 1st instar larvae were collected from two colonies of *A. m. ligustica* strain reared in our experimental apiary (GPS Coordinates: 40°49′20″N, 77°51′33″W). In order to collect newly emerged larvae, a honey bee queen was confined in the queen excluder cage and placed in the 2nd super from the bottom of the hive and positioned in the center of the super to allow for proper incubation of the newly laid eggs. After being caged for 30 h, the queen was released from the cage and eggs were incubated in the hive for 3.5 days. Frames of newly-hatched 1stinstar larvae were taken to the laboratory in a pre-warmed chamber (~35°C).

Diet preparation

Honey bee larval diet (adaptation of [24]) was prepared using 50% royal jelly (Beenatura.com), 12% D-glucose (Fischer Chemical, Fair Lawn, NJ, USA), 12% D-fructose (Fischer Chemical, Fair Lawn, NJ, USA), 2% yeast extract (Bacto™, Sparks, MD, USA), and distilled water (24%). Royal jelly was preserved at −80°C until use. Ingredients minus royal jelly were completely dissolved and filtered through a 0.2 μm membrane (Corning) to remove particulate matter and bacteria. This solution was poured onto royal jelly that was free of wax particles, and mixed thoroughly at room temperature using a spatula. Diet was stored at 4°C for a maximum of three days prior to use.

Pesticide application

The concentrations of applied pesticides were selected based on our previous laboratory findings of commonly found pesticides in pollen [1]. According to the survey of pesticide residues conducted on bee-related product samples from migratory and other beekeepers during the 2007–08 growing seasons, the most prevalent detections at 95th percentile values (levels at which only 5% of detections are higher) in trapped pollen samples were 0.3 mg/L (0.3 ppm) fluvalinate, 0.8 mg/L coumaphos, 0.15 mg/L chlorpyrifos, and 3.4 mg/L chlorothalonil (unpublished data up to 2009). Foraging bees may avoid and dilute contaminated pollen with that from alternative hosts; therefore, the level of contamination found in the trapped pollen pellets varies in relation to the foraging environment of the colony [1,2,25]. We have observed that apple pollen contributes approximately 10% of overall trapped pollen samples from hives placed in apple orchards during a 10-d pollination event (unpublished data). In addition, these pesticides have also been detected in other hive products at even higher levels including beebread, wax comb, foundation, and more rarely in bees. Developing bees are exposed to pesticide residues by contact with the wax, beebread and contaminated bees, so the level found in trapped pollen or royal jelly is not fully representative of actual exposure of larval bees to pesticides. For example, pollen residues of fluvalinate and coumaphos primarily originate by transfer from the contaminated comb wax, which contains much higher levels (e.g. 100-times) of these miticide residues [1,2]. Therefore, in the absence of exact measures of pollen residues in larval foods, we chose to test at 10 times the levels of these four pesticides found in pollen samples. We mixed fluvalinate (purity, 95%), coumaphos (purity, 99%), chlorpyrifos (purity, 99%), and chlorothalonil (purity, 98%) purchased from Chem Service (West Chester, PA, USA) in the larval diet at nominal concentrations of 3, 8, 1.5, and 34 mg/L, respectively. Our calculated concentrations are in accordance with the maximal levels of pesticides detected in both trapped pollen and beebread samples and within the range of 95 percentile values of four selected pesticides detected in hive samples [1]. Therefore, we believe that applying a factor of 10 can give a rough but realistic estimation of the actual exposure of larval bees through contaminated diet or direct transfer from much higher residues in the comb.

Pesticide treatments included four pesticides tested alone and in two, three, and four-component mixtures. To prepare stock solutions, each technical grade pesticide was individually dissolved in acetone and methanol, respectively. Each test solution was mixed thoroughly into the artificial diet at specific concentrations

and stored in 2 ml sterile glass vials (Corning, USA). We monitored three control groups in the study: untreated diet, one solvent-treated diet containing 1% methanol and another solvent control containing 1% acetone. We also tested the dietary toxicity of a range of N-methyl-2-pyrrolidone concentrations on larval survival. NMP can be used to 100% of the solvent in pesticide formulations [26]. Table S1 lists the percentage of the solvent NMP in some pesticide formulations that disclose it in MSDS. Here, we tested seven nominal concentrations including 0.01% (100 mg/L in diet), 0.02%, 0.05%, 0.1%, 0.2%, 0.5% and 1% (10,000 mg/L).

Each experiment was repeated twice including control (3 groups), single (6 treatment groups), mixture (binary mixtures: 6 treatment groups; ternary mixtures: 6 treatment groups; four-component mixtures: 2 treatment groups), and 'inert' toxicity tests (seven concentrations of NMP). Sample size for each treatment starting from the same experimental day is 3 replicates with 24 larvae per replicate.

In vitro larval rearing technique

Newly hatched 1st instar larvae were transferred from hive frames into sterile, 48-well culture plates (Corning, USA) for the *in vitro* rearing technique with 24 larvae per plate. Larval transfers were done in the lab without the use of a sterile hood. The sterile, push-in queen cups (B&B Honey Farm, USA) were placed in every other well. Diet was warmed to ~34°C in a heating block prior to larval transfer. Using an Eppendorf 10–100 µl variable volume pipette, 10 µl of each diet treatment was placed per queen cup. A 00 camel hair paintbrush was used to transfer each larva from the cell on the frame to the cup. The paintbrush was dipped into distilled water between each larval transfer to aid in a smooth transfer, and was sanitized by dipping in 95% ethanol after every four to five transferred larvae. Larvae were placed directly on top of the diet and inspected for mobility to ensure a quality transfer. Four additional queen cups were equally spaced in four of the remaining open wells before placing the lid on the culture plate, allowing for adequate ventilation of the larvae throughout the experiment. Each plate was placed in a humidity chamber and kept at 95% relative humidity with a 10% aqueous solution of sulfuric acid being used at the base of the chamber to maintain humidity. Humidity chambers were placed in an incubator at 34°C in the dark and were not disturbed throughout the experiment, except when replacing the diet for ~15 min/d.

For this study, only the survivorship of honey bees during the larval stage was monitored to evaluate the impacts of selected pesticides. Larval mortality was recorded daily by probing the larvae with sanitized forceps. The dead larvae were removed daily. Diet for each larval bee was replaced daily. Old diet was removed using a glass disposable pipette and new diet was immediately placed in each queen cup according to the following schedule to account for larval growth: day 1- 10 µl, day 2- 10 µl, day 3- 20 µl, day 4- 30 µl, day 5- 40 µl, and day 6- 50 µl.

Kaplan-Meier survival analysis

The 6-d larval survival data were segregated by pesticide treatment and analyzed using Kaplan-Meier survival analysis [27]. This estimate generally assumes independence among the individual death events and randomization within the treatment group. The hazard rate h(*t*) is the conditional probability of failure or death in a small time period given that the subject has survived up until a specified time *t*. The greater the value of the hazard rate, the greater the probability of impending death. The null hypothesis of no difference between survival curves of treatment and control groups was tested by the Log-rank test that weights each death by the square root of the total number of individuals at risk per time interval, placing less emphasis on deaths occurring later in the experiment. All the survival analyses were implemented in SAS survival program (SAS/STAT® 9.2 User's Guide).

Comparison between adult and larval sensitivity

The difference in sensitivity to the same pesticide between adult bees and larvae can be quantitatively evaluated by comparing the actual larval mortality per day from the *in vitro* test with the predicted mortality for adult bees if exposed to the same concentrations of pesticides. The larval mortality data were corrected with Abbott's formula beforehand. Here, the impacts of pesticide treatments on adult bees were estimated from the adult acute topical LD_{50} data converted to whole-bee LC_{50} values [1], because neither the chronic nor acute oral toxicity data of adult bees are currently available for all pesticides selected for this study. Predicted adult toxicity can be estimated as a function of the magnitude of toxicant exposure and the individual's sensitivity to a toxicant, which is generally characterized by the probit model [28].The predicted proportion of insects killed ($\hat{p}$), in probit transformed units, calculated as $\hat{p} = a + bx$ where a = intercept and b = slope from the regression of the transformed data and x is the log-transformed concentration or time. Results of probit analyses are reported typically as a concentration or time required to kill a certain proportion of the test insects (e.g., LC_{50}). Table 1 shows the average LC_{50} values from the literature [1] and probit slopes from other sources [28]. One exception is chlorothalonil, which is estimated using the default probit slope of 4.5 because its mortality levels under topical or oral applications to honey bees are found to be insufficient to establish a dose-response relationship. Therefore, the probit function for each pesticide to adult honey bees can be inferred from the LC_{50} values (x), probit mortality ($\hat{p} = 5$) and probit slope (b) [13,28]. Then, the probit model can be extrapolated to predict the probability of an impact of each pesticide on adult bee survival for a specified concentration. Using the Probit program in SAS 9.2 (SAS/STAT® 9.2 User's Guide), the predicted probit-type mortality can be transformed to the original percent units and compared with the actual larval percent mortality data. Using the compilation of acute data from different sources may complicate the accurate estimation of the adult toxicity because of the heterogeneity introduced by differences among the studies; however, given the limitations we felt this was a reasonable approach to obtain a first approximation of the differences in adult and larval sensitivity to the same pesticide exposure.

Pesticide interaction determination

We used significant departures from additive toxicity to define antagonistic and synergistic interactions between pesticides in mixtures [29]. The expected additive toxicity for the chemical mixture is the sum of each chemical's toxicity to larval survival, calculated as f chemical components in the pesticide mixture and h_i is the hazard rate for a specific component estimated from the laboratory bioassay data. The sum of the responses (Eh_n) to the individual components is estimated based on the assumption that the selected pesticide mixtures are the combination of substances with independent modes of action or similar modes of action. The mixture toxicity can be predicted as follows: *Additive interactions*–Simultaneous action of components in which the observed response of honey bee larvae to a mixture (h_n) is equal to the sum of the responses (Eh_n) to the individual components; *Synergistic interactions*–Simultaneous action of components in which h_n is significantly higher than Eh_n; *Antagonistic interactions*–Simultaneous action of components in which h_n is significantly less than Eh_n.

Table 1. Comparison between the predicted adult mortality rate (PM, %) for each tested concentration (Conc., mg/L) of four pesticides using a probabilistic toxicity model and the observed brood mortality rate (AOM, %) for bee larva from the 6-d *in-vitro* rearing experiments.

	Adult honey bee				Honey bee larva						
	Inverse probit prediction				*In-vitro* brood test						
Pesticide	β^a	LC_{50}^b	Conc.	PM^c	1-d^d	2-d^d	3-d^d	4-d^d	5-d^d	6-d^d	AOM^e
Fluvalinate	2.5	15.86	3	3.6	3.13*	8.06	12.28	10.00	11.11	68.85**	11.72
Coumaphos	2.9	46.3	8	1.4	6.25*	1.67	8.47	5.56	3.92	53.73**	8.60
Chlorothalonil	4.5	1110	34	4 E-10	0.00	8.93	7.84	12.77	7.32	56.60**	9.82
Chlorpyrifos	10	1.22	1.5	82	0.00	4.17	8.70	33.33**	32.14**	0.00	10.07

[a]β is the slope of the probit function for different pesticides [13,28].
[b]LC_{50} is the median lethal concentrations of each pesticide to adult honeybees [1].
[c]PM = predicted adult mortality rate (%) for each pesticide at the tested concentrations using inverse prediction of the probit function.
[d]1,2,3,4,5,6-d is the observed conditional mortality rate (%) for larval bees at each age (in day) in the *in vitro* rearing process.
[e]AOM = average daily mortality rate (%) for larval bees in the *in vitro* rearing process.
*Significant at $p<0.05$;
**significant at $p<0.001$. (Statistical differences in larval survival were assessed between pesticide-treated and solvent control groups.)
doi:10.1371/journal.pone.0077547.t001

We did not test different concentrations of each pesticide component and of the combinations to fit dose-response curves. Neither food intake nor concentrations of pesticides consumed by each larva were measured during the oral feeding. Therefore, this method does not allow exact quantification of the level of interaction but makes only an initial qualitative assessment of synergism or antagonism.

Results

Control toxicity

No significant differences in larval mortality were observed when larvae were reared on untreated artificial diet or diet mixed with 1% methanol or 1% acetone (Log-rank test, $p>0.05$) (data not shown). These three control groups showed an accumulative 6-d percent mortality of approximately 17.2% (Fig. 1), which is within the normal range observed for control mortality using the *in-vitro* larval rearing protocol [24,30]. Because control mortality exceeds 10%, the larval mortality data from treatment groups were corrected with Abbott's formula.

Single pesticide toxicity

Chronic exposure of bee larvae to each of the four pesticides at tested concentrations showed significant toxic effects on larval survival (Log-rank test, $p<0.0001$), resulting in an overall 2- to 4-fold reduction in the total 6-d percentage survival compared to the control mortality (Fig. 1A). Based on age-specific toxicity data, mortality rates for each pesticide were uneven across different larval stages (Fig. 1B). For 1-day-old larvae, 8 mg/L coumaphos and 3 mg/L fluvalinate were more toxic than the other two pesticides. The 2 and 3-day-old larvae showed similar sensitivity to different pesticide exposures, approximately 10% mortality per day. The 4 and 5-day-old larvae were most sensitive to 1.5 mg/L chlorpyrifos, causing more than 32% larval death each day (Table 1). A dramatic increase in larval mortality for 6–day-old larvae was observed in 34 mg/L chlorothalonil and the two miticide groups, ranging from 53.73% to 68.85%. Using the probit model, notable differences were found in pesticide sensitivity between the adult bee and larvae (Table 1). Among the four pesticides tested, 1.5 mg/L chlorpyrifos was the only treatment that adult bees were more susceptible to than the larvae. For the other pesticides, the larvae showed increased sensitivity over that of adult bees. Notably, chlorothalonil at the sublethal concentration of 34 mg/L was least toxic to adult bees, however most toxic to larvae followed by 8 mg/L coumaphos and 3 mg/L fluvalinate. On average, coumaphos was the least toxic to larval bees among the four pesticides.

Synergistic interactions

I. Chronic toxicity of chlorothalonil and coumaphos. The effects of chlorothalonil (34 mg/L), coumaphos (8 mg/L), and their mixture on larval survival through the 6-d development are shown in Fig. 2A. In the first 3 days of larval rearing, these three groups exhibited similar survival curves ($p = 0.1988$, Log-rank test). Subsequently, the larvae reared on the diet contaminated with the chlorothalonil/coumaphos mixture died most quickly. The risk of 4-day-old larvae being killed by the mixture was higher than for the other stages of larvae and the single pesticide groups. The hazard rate of the combination group ($hn(4) = 0.523$) was 9-times higher than the coumaphos group ($hCM(4) = 0.057$) and 3-times higher than the chlorothalonil group ($hCL(4) = 0.136$). The conditional probability of 4-day-old larvae being killed by the mixture treatment was 5-times higher than that of expected additive toxicity (Fig. 2B, $Ehn(4) = 0.0965$, $p<0.0001$, Mann–Whitney test). Therefore, the pairing of chlorothalonil and coumaphos produced a significant synergism on mortality of larvae older than 4 days.

II. Chronic toxicity of chlorothalonil and fluvalinate. For the 4-day-old larvae, the hazard rate of the mixture ($hn(4) = 0.78$) was the highest during the 6-d larval development, which was 7-times higher than the fluvalinate (3 mg/L) group ($hFlu(4) = 0.105$) and 5-times higher than the chlorothalonil (34 mg/L) group ($hCL(4) = 0.136$) (Fig. 2C). The chlorothalonil/fluvalinate mixture at the tested concentrations gave a synergistic interaction, which significantly magnified the hazard rate by 7 fold over the sum of the individual effects (Fig. 2D, $Ehn(4) = 0.121$, $p<0.0001$, Mann–Whitney test).

Additive interactions

I. Chronic toxicity of fluvalinate and chlorpyrifos. Larval survival on fluvalinate (3 mg/L) and chlorpyrifos (1.5 mg/L) declined the fastest among pesticide mixture treatments, ranging

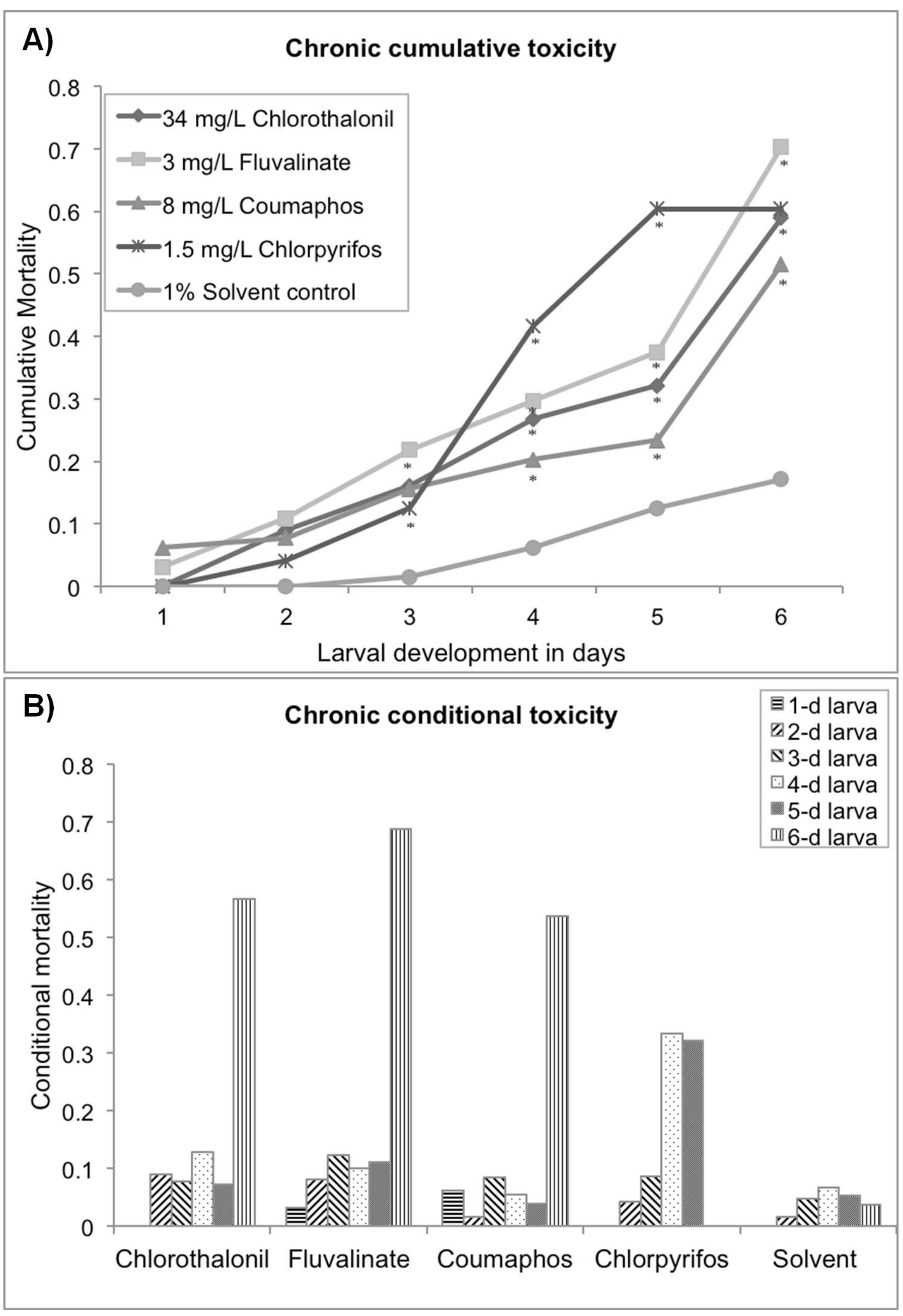

Figure 1. Larval survival during the 6-d development stage reared on artificial diet contaminated with four pesticides at the selected concentrations and a 1% solvent control. (A) shows the cumulative mortality of honey bee larvae through 6-d development continually exposed to 34 mg/L Chlorothalonil, 3 mg/L Fluvalinate, 8 mg/L Coumaphos, 1.5 mg/L Chlorpyrifos and 1% solvent; (B) illustrates the conditional mortality for different development stages of bee larva. Asterisks denote significant difference from the respective solvent controls (analysis of variance, Log-rank test, $p<0.0001$).
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from 4.17% to 70.83% (Fig. 3). No significant differences were found in larval survival between single component groups through the 6-d development (Fig. 3A, Log-rank test, p = 0.1711). This binary combination produced additive toxicity. The 6-d cumulative percent mortality caused by this mixture (hn = 71%) was slightly higher than the sum of the response to single components, but not at a significant level (Fig. 3B, Ehn = 48.96%, p = 0.171, Mann–Whitney test).

II. Chronic toxicity of chlorpyrifos and coumaphos. The larval chronic toxicity of this combination treatment was the highest among tested pesticide mixtures causing from 10.4% to 79.2% mortality during the 6 days. Survival was least affected by the diet with 8 mg/L coumaphos (Fig. 3C). The interaction between these pesticides showed an additive effect. The 6-d cumulative percent mortality of larvae reared on the mixture

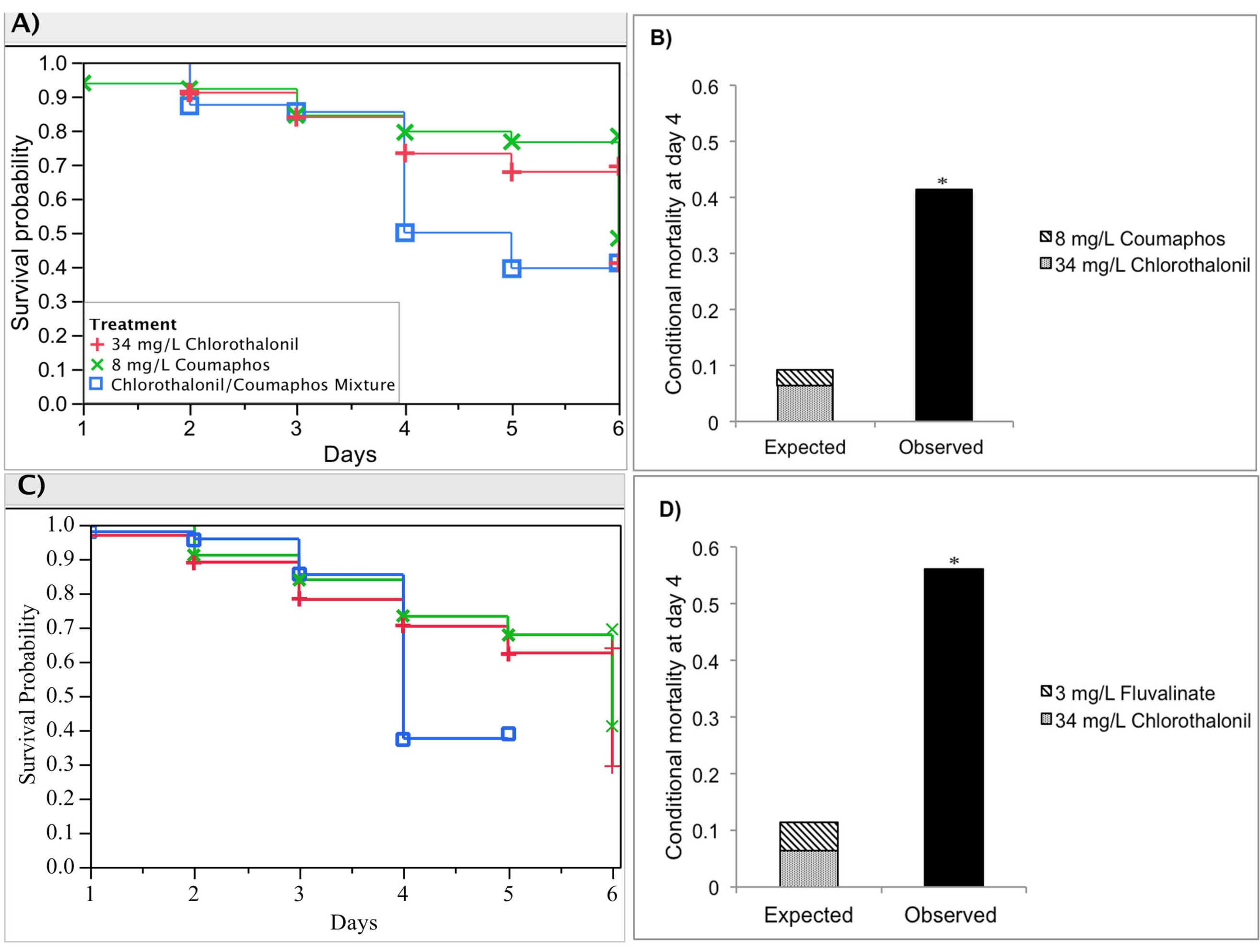

Figure 2. Synergistic interactions for two pairs of pesticide mixtures: 8 mg/L Coumaphos, 34 mg/L Chlorothalonil and the mixture; 3 mg/L Fluvalinate, 34 mg/L Chlorothalonil and the mixture. (A) and (C) show the respective Kaplan-Meier survival plots for honey bee larvae reared for each pair of pesticide mixture; (B) and (D) illustrate the interaction determination based on the deviation of observed mixture toxicity (black bar) from the expected additive toxicity (stacked bar). Asterisks denote significant difference from the expected additive toxicity (Mann–Whitney test, $p<0.0001$).
doi:10.1371/journal.pone.0077547.g002

(hn = 79.2%) did not differ significantly from expected additive toxicity (Fig. 3D, Ehn = 56%, p = 0.558, Mann–Whitney test).

III. Chronic toxicity of fluvalinate and coumaphos. The survivorship of larval bees on the combination and fluvalinate alone treatments exhibited a similar gradual declining trend, achieving the highest cumulative mortality at the end of the 6-d development (Fig. 3E). Both showed more toxicity to larval bees than coumaphos alone (Fig. 3E, p = 0.0425, Log-rank test). Fluvalinate and coumaphos, mixed at 3 mg/L and 8 mg/L respectively, showed an additive effect. The accumulative percent mortality in the mixture group (hn = 68.75%) did not vary significantly from the expected additive toxicity (Fig. 3F, Ehn = 60.94%, p = 0.052, Mann–Whitney test).

Antagonistic interactions

I. Chronic toxicity of fluvalinate and chlorothalonil at low concentrations. The 3.4 mg/L chlorothalonil and 0.3 mg/L fluvalinate mixture showed the least toxicity to larval development among pesticide combinations tested (Fig. 4A). Especially, for the 4-day-old larva, the hazard rate of individual component groups (hCL(4) = 0.214, hFlu(4) = 0.259) was greater than twice the mixture treatment (hn(4) = 0.088). This mixture showed antagonistic interaction, significantly reducing the hazard rate of 4-day-old larvae by three-fold from the expected additive toxicity (Fig. 4B, Ehn(4) = 0.2365, p<0.0001, Mann-Whitney Test).

Three-component mixture toxicity

All six possible pairings were selected to determine the toxicity for three-component mixtures including chlorothalonil/fluvalinate/coumaphos and fluvalinate/coumaphos/chlorpyrifos. The only significant difference found was when coumaphos (8 mg/L) was added to the two-component mixture of fluvalinate (3 mg/L) and chlorothalonil (34 mg/L), giving a 3% reduction in the 6-d accumulative larval mortality ($h_n = 38\%$) from the expected additive effect (Fig. 4C and 4D; $Eh_n = 41.41\%$, $p = 0.006$, Mann-Whitney Test). The other five pairings did not yield significant changes in larval survival when adding one component into the existing binary mixtures.

Four-component mixture toxicity

Two pairings of mixtures including chlorothalonil added to fluvalinate/coumaphos/chlorpyrifos and chlorpyrifos added to chlorothalonil/fluvalinate/coumaphos were tested at the same concentrations as before to determine toxicity interactions in going

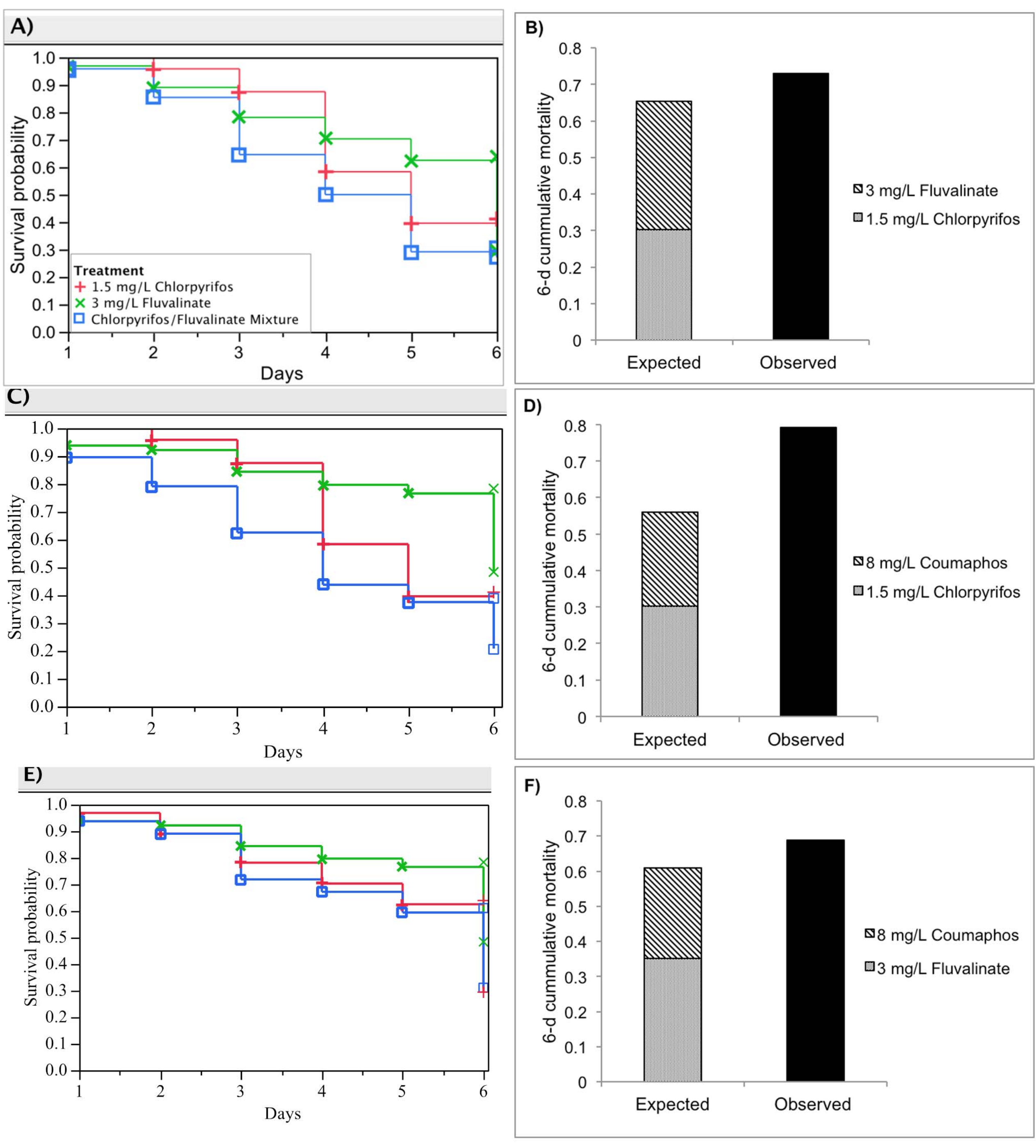

Figure 3. Additive effects for three pairs of pesticide mixtures: 3 mg/L Fluvalinate, 1.5 mg/L Chlorpyrifos and the mixture; 8 mg/L Coumaphos, 1.5 mg/L Chlorpyrifos and the mixture; 8 mg/L Coumaphos, 3 mg/L Fluvalinate and the mixture. (A), (C) and (E) show the respective Kaplan-Meier survival plots for honey bee larvae reared for each pair of pesticide mixture; (B), (D) and (F) illustrate the interaction determination based on the deviation of observed mixture toxicity (black bar) from the expected additive toxicity (stacked bar).
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from three- to four-component mixtures. There were no significant changes in larval survival when integrating a fourth component into these three-component mixtures. The four-component mixture caused 54.17% larval mortality at the end of the 6-d larval development.

'Inert' ingredient toxicity

Chronic exposure of bee larvae to the 'inert' ingredient NMP at seven different concentrations ranging from 0.01% to 1% greatly impacted larval survival (Fig. 5). Increasing amounts of NMP correspondingly increased larval mortality. A 1% concentration (10,000 mg/L) of NMP was the most acutely toxic, generating 100% mortality within 24 h after treatment. Even for the lowest

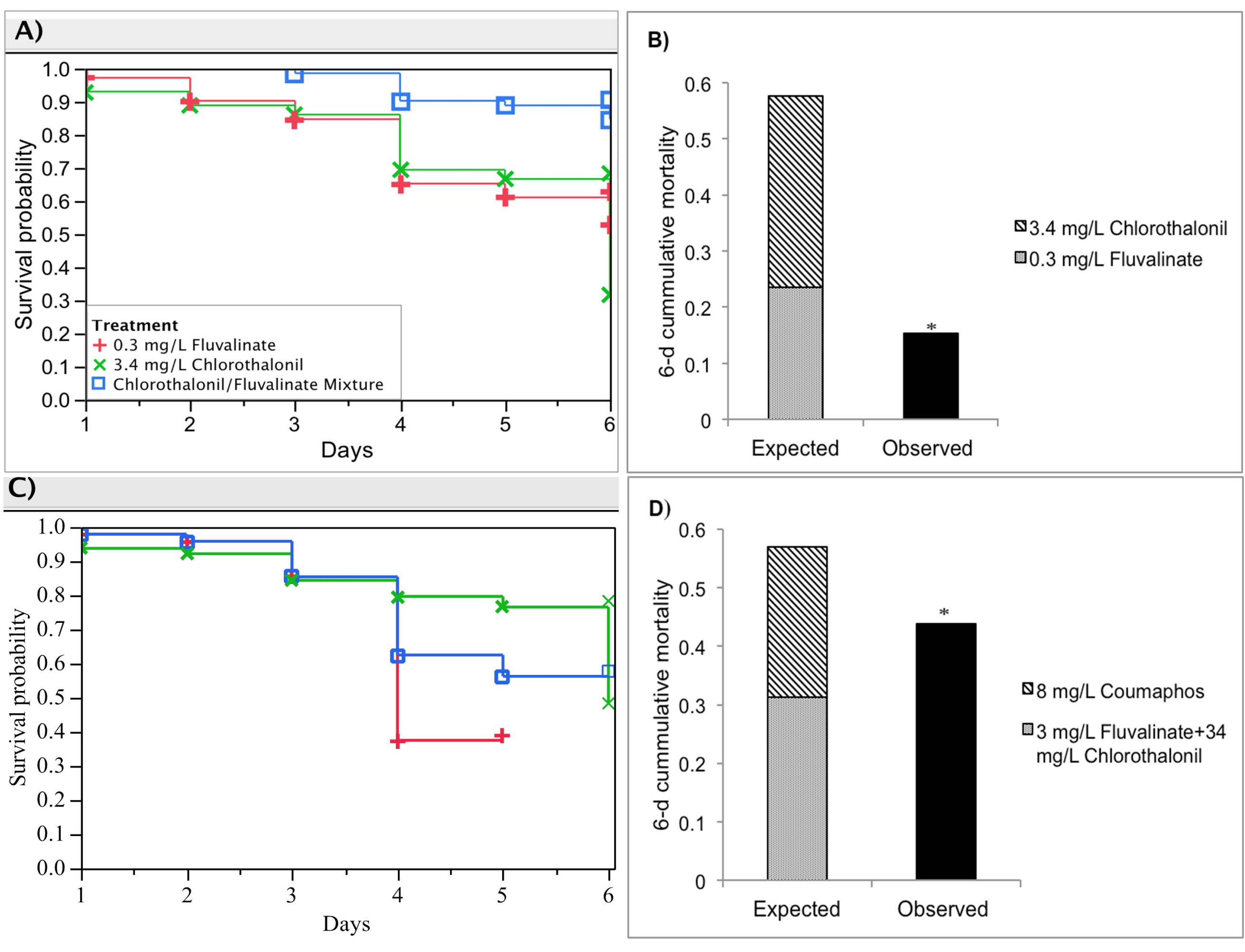

Figure 4. Antagonistic interactions for two pairs of pesticide mixtures: 0.3 mg/L Fluvalinate, 3.4 mg/L Chlorothalonil and the mixture; 3 mg/L Fluvalinate+34 mg/L Chlorothalonil mixture, 8 mg/L Coumaphos and the three-component mixture. (A) and (C) show the respective Kaplan-Meier survival plots for honey bee larvae reared for each pair of pesticide mixture; (B) and (D) illustrate the interaction determination based on the deviation of observed mixture toxicity (black bar) from the expected additive toxicity (stacked bar). Asterisks denote significant difference from the expected additive toxicity (Mann–Whitney test, $p<0.0001$).
doi:10.1371/journal.pone.0077547.g004

concentration of 0.01% (100 mg/L), the estimated time to cause 50% larval mortality was 4 days.

Discussion

Chronic toxicity

Our findings suggest that chronic dietary feeding at hive levels of common pesticide ingredients including the fungicide chlorothalonil, miticides fluvalinate and coumaphos, and insecticide chloropyrifos, individually or in mixtures, have statistically significant impacts on honey bee larval survivorship. A significant increase in larval mortality was found at or beyond 4-d of feeding. This is the first study to report serious toxic effects on developing honey bee larvae of dietary pesticides at measured hive residue concentrations. The maximum concentrations of fluvalinate, coumaphos, chlorothalonil, and chlorpyrifos found in our hive samples are 204 mg/L, 94.1 mg/L, 98.9 mg/L, and 0.9 mg/L, respectively (Table S2), which are much higher for the miticides and fungicide, or similar for the insecticide, to those levels tested here (Table 1). This chronic (6-d) toxicity is likely to be undetected in a conventional acute (24/48 h) toxicity study, resulting in potential underestimation of pesticidal effects. The lethal effects on honey bee larvae appearing after 4-d continuous exposure to pesticides at low concentrations are also observed in adult honey bees. The accumulated dose of the organophosphorus insecticides acephate, methamidophos or dimethoate resulting in 50% adult bee mortality was over 100-fold lower than the respective acute 24 h oral LD_{50} [31]. For these organophosphates and also the pyrethroids tested, their toxicity to worker bees was significantly increased by continuous versus single ingestion of the contaminated food. At low doses of imidacloprid, adult bee mortality was observed only 72 h after onset of feeding in contrast to immediate effects at much higher doses [32].

The causes for chronic larval bee toxicity for 6-d dietary subacute pesticide exposures remain unknown. It may be associated with the extended time needed to accumulate sufficient insecticide concentrations internally to exert nerve action at central target sites, which is consistent with the pharmacological receptor theory; or may reflect variation in honey bee detoxification capacities from the more peripheral to internal tissue sites. For instance, the results of high toxicity of low doses of all imidacloprid metabolites suggest the existence of binding sites with different

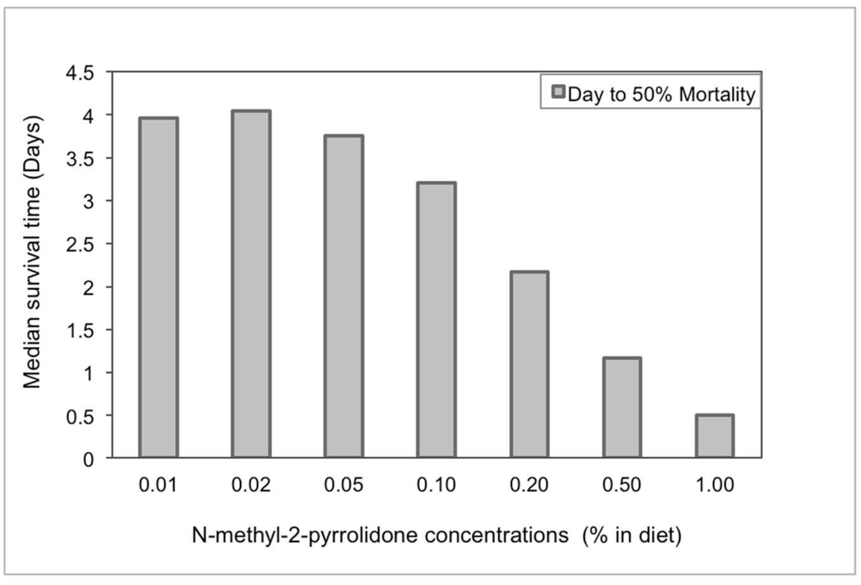

Figure 5. The estimated time to cause 50% larval mortality by seven nominal concentrations of N-methyl-2-pyrrolidone mixed in larval diet.
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affinities in honey bees [32]. Another explanation may be that honey bee detoxification mechanisms are not induced by chronic exposure of low concentrations of active substances, but require higher more acute concentrations to impact honey bee susceptibilities. In the former case, bee mortality would be latent due to the time needed for pesticide bioaccumulation, further favored by the more lipophilic pesticides fluvalinate, coumaphos, chlorpyrifos and chlorothalonil tested here. The latter case of acute higher concentrations driving induction of detoxification enzymes can result in both antagonistic and synergistic effects on the target-effective insecticide concentration depending on if the induced cytochrome P450 first activates (e.g., chlorpyrifos, coumaphos to respective oxons) or detoxifies (e.g., fluvalinate) the insecticide [33,34]. Other induced enzymes (e.g., hydrolases, glutathione transferases) will further degrade and detoxify the primary metabolites.

It is also plausible that more general stress mechanisms (e.g., altered feeding, suppressed growth) dominate the chronic response. For example, exposures of some repellent pesticides such as pyrethroids at sublethal levels have been demonstrated to impair feeding behaviors of honey bees and bumble bees [3,8]. In the case of honey bee larvae, they retain internally all metabolic wastes throughout the larval stage up to the pupal molt after which they defecate a waste pellet called the meconium [25]. Concentrations of pesticides and metabolites within brood tissues may result in continuous pesticide stress [35], which differs from the adult honey bee and most other insects where excretion of toxic wastes regularly occurs. Little information is available on the distribution of fluvalinate [36] and coumaphos [37] and their degradates in honey bee adults and brood. Further studies to examine the distribution and accumulation of fluvalinate, coumaphos, chlorpyrifos and chlorothalonil and their metabolites, in honey bees at different developmental stages are needed. Meanwhile, how honey bees at different life stages withstand chronic exposure need more detailed study of metabolic regulation in this social insect.

Remarkably, among the four pesticides tested in the present study, immature honey bees are highly vulnerable to the common fungicide chlorothalonil (Figs. 1 and 2). Dietary chlorothalonil killed more than 50% of larvae in 6 days at a level of 34 mg/L, a nontoxic dose to adult bees in acute bioassays (Table 1). This difference in larval to adult susceptibility was the largest among the four pesticides tested. It is unclear why, larval bees exhibited much greater sensitivity to chlorothalonil compared to adult bees; however, the present results demonstrate that investigating fungicide impacts on honey bees is particularly necessary for a realistic evaluation of pesticide impacts on colony health, given the frequent detections of chlorothalonil in pollen and wax samples. Hence, considering that honey bees are experiencing a diverse array of agrochemicals in the hive, the chronic toxicity test may better assess pesticide exposure for a honey bee colony.

Mixture toxicity

Currently, studies of mixture toxicity between different classes of pesticides at concentrations of environmental relevance are rarely available for honey bees [34]. The present study of four pesticides in all combinations is the first study to investigate the potential synergism of common pesticides at realistic exposure levels to larval bees. The present results showed interactions between binary combinations of synthetic pesticides tested were mostly additive, which can be attributed to the same or independent mode of actions of the pesticides involved [33,34]. For instance, additivity of the coumaphos/chloropyrifos mixture may be explained by their identical action as organophosphate inhibitors of acetylcholinesterase. The additive toxicity of the pyrethroid fluvalinate with either coumaphos or chloropyrifos is probably due to the independent primary action of the former on nerve sodium channels. Our result with larvae is not consistent with the adult honey bee study of Johnson et al., where the combination of fluvalinate and coumaphos was synergistic [13]. This discrepancy may be explained by the different life stage, lower insecticide concentration levels, and longer length of exposure used here.

The three and four component mixtures of tested pesticides have mostly demonstrated additive effects in larval bees. This finding is in general agreement with the Funnel Hypothesis [38], which states that the toxicity will tend towards concentration

additivity as the number of components in equitoxic mixtures increases. One exception was the significantly less than additive response when coumaphos was integrated into the fluvalinate and chlorothalonil mixture. That coumaphos antagonizes the synergistic effect of fluvalinate and chlorothalonil may be related to its possible induction of the detoxification of one or both of the other pesticides. This anomaly may be related to the observation that elevated coumaphos levels in brood had the highest discriminatory value with regard to healthy bee colonies whereas higher levels of this miticide in the pollen food correlated with colony collapse [39], again indicating that pesticide susceptibilities differ across honey bee developmental stages.

Remarkably, binary mixtures of chlorothalonil with the miticides fluvalinate or coumaphos were synergistically toxic to 4-day-old bee larvae. This is the first demonstration for honey bee brood of a synergistic interaction between dominant in-hive miticides and the frequently-encountered fungicide chlorothalonil at environmentally relevant concentrations. Synergism with chlorothalonil and fluvalinate but not coumaphos for adult honey bee toxicity has been noted previously [40,41].

Surprisingly, a significant antagonism was found for larval toxicity from the fluvalinate-chlorothalonil combination at one-tenth of the concentrations (Fig. 4) that otherwise exhibited a five-fold synergism (Fig. 2). One rationale behind this latter interaction, beyond the fact that the very diverging pyrethroid-multi-site chlorothalonil mechanisms of action may alone elicit synergistic effects, is that the high concentrations may directly inhibit detoxification enzymes. For example, the competitive inhibition of cytochrome P450 monooxygenase enzymes has been suggested to explain the synergistic interactions among pesticides for adult honey bees such as pyrethroid insecticides or mixtures of organophosphate insecticides and ergosterol biosynthesis inhibiting fungicides [42,43]. Also, synergism between chlorothalonil and the herbicide atrazine has been documented in aquatic species [44]. Modes of action for chlorothalonil range from inhibiting glutathione and other thiol-dependent enzymes or protein receptors, to disrupting or degrading cell membranes causing lysis that can enhance penetration of other pesticides [14]. The tendency toward antagonism of brood toxicity at the lower dietary chlorothalonil-fluvalinate concentration may be associated with alternative peripheral mechanisms such as gut microbial detoxification that may be overwhelmed at higher dosage where more internal neurotoxic effects of the pyrethroid can prevail. The consequence is that biphasic low and high dose response relationships may result depending on the extent of multiple peripheral and internal sites of action that diverge in sensitivity to the toxicants as well as to the available detoxification pathways that differ in a tissue-dependent manner to the concentrations required for their induction.

While the mechanisms of interactions among pesticides with diverse modes of action and their dynamics in the developing honey bee larvae are not known, application of the concentration-addition model combined with chronic feeding tests represents a starting point for investigation of mixture effects at realistic levels and their risks for this pollinator. Considering that the diverse arrays of chemicals [1,2,45] and general additivity exist in the hive environment, examining the toxicity of chemical mixtures in addition to single toxicants is critical for a realistic assessment of pesticide hazards experienced by honey bees and other non-target organisms. In today's agriculture dominated by mass monocultures, adults and larvae of *A. mellifera* are inevitably exposed to transgenic material via pollen consumption of GM-crops [46], which might be another confounding factor for bee health. Although minor evidence showed adverse effects of Bt-crops on *A. mellifera*, the risk assessment of combined effects of Bt-crops and pesticides are completely lacking [47–49]. Hence, the dose dependency of the synergy, the multitude of compounds, the differences in adult bees and larvae, the possibility of continuous exposures, and the interaction with GM pollen should be taken into account in the environmental risk assessment.

'Inert' toxicity

Another important health issue that involves pesticide formulations and bees is the consequence of the additives or so-called non-active ingredients. The commonly-used 'inert' solvent N-methyl-2-pyrrolidone was found here to be highly toxic to larval honey bees (Fig. 5). Unfortunately, despite the potential toxicity of 'inert' ingredients and their widespread use in pesticide products, their testing and risk assessment seems to be inadequate. There is a growing body of research that has reported a wide range of adverse effects of 'inert' ingredients to human health, including enhancing pesticide toxicities across the nervous, cardio-vascular, respiratory, and hormonal systems [18,50,51]. However, limited data exist on the potential impacts of 'inerts' on non-target pollinators, although recent studies implicate formulation additives or adjuvants as key risk factors [52]. As one example, the toxicity of the fungicide captan to honey bee brood development was attributed to formulation ingredients other than the active ingredient alone [53]. The lack of detailed information of the usage of formulation ingredients greatly impedes appropriate risk assessment of 'inert' ingredient toxicity; therefore, label disclosure of the composition of pesticide formulations would facilitate this much-needed evaluation.

Conclusions

The current study demonstrates the chronic oral and mixture toxicity of common pesticides at hive levels to honey bees at the larval stage. Most notable are the chronic larval toxicities of the fungicide chlorothalonil and its synergistic combinations with frequently used in-hive miticides, and the unexpected high toxicity of the formulation ingredient N-methyl-2-pyrrolidone. Considering the extensive detection of chlorothalonil and its coexistence with other pesticides in diverse combinations especially in hive pollen and wax, and its substantial larval toxicity alone and in mixtures shown here, the application of this and other fungicides during crop bloom cannot be presumed innocuous to pollinating honey bees. Given the critical sensitivity of larvae to chlorothalonil and its complex interactions with other pesticides, the potential impacts of fungicides on colony survival and development need further investigation. In the more complex milieu of this social insect and its aging hive environment, pesticides, formulation additives and their resulting mixtures may have greater long-term impacts on colony health than previously considered. Consequently, the scope of pesticide risk assessment for non-target honey bees should be expanded from the present emphasis on acute toxicity of individual pesticides to a priority for assessment of chronic and mixture toxicities that incorporate fungicides, other pesticide pollutants and their 'inert' ingredients.

Supporting Information

Table S1 Some pesticide formulations that disclose in msds the percentage of the solvent NMP.
(DOCX)

Table S2 Pesticide detections in 329 wax and 496 pollen samples collected 2007–12 from North American honey bee colonies.
(DOCX)

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Author Contributions

Conceived and designed the experiments: WYZ DRS CAM JLF. Performed the experiments: WYZ DRS. Analyzed the data: WYZ CAM. Contributed reagents/materials/analysis tools: WYZ DRS CAM JLF. Wrote the paper: WYZ. Provided suggestions and comments on the manuscript: CAM.

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Portland Press Herald

1:00 AM

Growing hemp nears legality in Maine, but just for research

The U.S. farm bill heading for final passage would allow it in Maine, but since industrial crops are still illegal, little study of it is expected.

By J. Craig Anderson canderson@pressherald.com
Staff Writer

Maine is one of a dozen states in which hemp could be grown for research purposes if the farm bill passed Wednesday in the U.S. House of Representatives becomes law.

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In this Oct. 5, 2013 photo, Jason Lauve, executive director of Hemp Cleans, looks at hemp seeds at a farm in Springfield, Colo. during the first known harvest of industrial hemp in the U.S. since the 1950s. Hemp and marijuana are the same species, Cannabis sativa, just cultivated differently to enhance or reduce marijuana's psychoactive chemical, THC. (AP Photo/Kristen Wyatt)

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But as long as commercial hemp production remains a violation of federal law, it's unlikely that research institutions in Maine would have much interest in studying it, said an official at one of the state's leading agricultural research centers.

John Rebar, executive director of the University of Maine Cooperative Extension, said the potential market for hemp remains unknown because it is illegal to grow commercially in the United States, and the farm bill wouldn't change that.

"Nobody has looked at hemp as a viable crop for Maine," he said. "Why would you commit to doing industrial hemp research?"

Industrial hemp, which contains only trace amounts of THC, the psychoactive compound found in its close cousin marijuana, has thousands of commercial uses including rope, fabrics, paper, wax, food-grade seeds and oil, and even fuel.

A state law passed in 2009 makes it legal to grow hemp commercially in Maine, if and when it becomes legal nationwide.

If the farm bill passes next week in the Senate and becomes law, as expected, hemp cultivation for research purposes will be allowed in states with their own laws permitting it.

Those states are Maine, California, Colorado, Hawaii, Kentucky, Maryland, Montana, North Dakota, Oregon, Vermont, Washington and West Virginia.

Advocates for the legalization of industrial hemp say it could become a hugely profitable crop for the United States, but Rebar said the point is irrelevant to farmers in Maine until hemp production is legalized.

Agricultural research in Maine is now focused on products with strong market demand, he said, such as hops for brewing beer, wheat for making bread, and cheeses.

"Agriculture is just as much a business as anything else," he said.

In 2003, the Maine Legislature commissioned the Maine Agricultural and Forest Experiment Station to assess the commercial viability of cultivating industrial hemp.

In response, the station's director produced a report that said Maine's soils and climate are adequate to produce it.

The report identified potential benefits to hemp production.

As a crop, hemp is highly pest-resistant and naturally suppresses weeds, which would reduce the cost to farmers and pollution associated with pesticides and weed killers, it said.

Paper made from hemp would reduce deforestation and make paper mills run cleaner, it said, because hemp requires fewer chemicals than wood for processing.

But those benefits are irrelevant as long as commercial hemp production remains a federal crime, the report said.

"As long as this is the case, new products and new uses for hemp will not develop," it said.

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Portland Press Herald

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Protecting Maine lobsters by banning pesticides gets tepid support

The Maine Lobstermen's Association says the state needs a comprehensive assessment of pesticide threat.

By Steve Mistler smistler@pressherald.com
Staff Writer

AUGUSTA — A bill designed to protect Maine's $340 million lobster industry by banning two pesticides that have been partially blamed for decimating lobster populations in New York and Connecticut is facing a headwind in the Legislature.

L.D. 1678 is sponsored by Rep. Walter Kumiega, D-Deer Isle. It would prohibit the use of methoprene and resmethrin, chemicals that were used during a massive mosquito spraying operation in 1999 to combat an outbreak of West Nile in areas along Long Island Sound.

Shortly thereafter, a severe die-off of lobsters wiped out the fishery there, although warming ocean temperatures and other factors are also believed to have played a role.

Rep. Michael Devin, D-Newcastle, told lawmakers on the Agriculture Conservation and Forestry Committee on Thursday that Maine should join Connecticut and ban the two chemicals, traces of which were found in dead lobsters studied in the sound.

"Whatever we apply in the terrestrial environment eventually makes its way to the coast and out to sea," Devin said.

"The cigarette butt you saw this morning on the sidewalk will end up in the Kennebec River and then flow down to the ocean. Insecticides ... all end up in our ocean."

The proposal, however, lacks the support of the LePage administration and the Maine Lobstermen's Association, a trade group representing the industry. Patrice McCarron, the association's executive director, told lawmakers Thursday that lobstermen are concerned about pesticides, but worry that banning methoprene and resmethrin could give a "false sense of security" while ignoring other chemicals that could be more harmful to lobsters.

McCarron said the association supports a more comprehensive analysis to determine which pesticides, if any, are affecting a fishery that pumps $1.7 billion into the state economy, according to estimates by the Lobster Institute at the University of Maine.

Such an analysis may be on the horizon.

The Department of Marine Resources, which oversees the lobster fishery, and other stakeholders are discussing whether to conduct a sediment survey of Casco Bay.

The study could be part of an assessment of the risk of all pesticides, not just methoprene and resmethrin.

The assessment would be overseen by the Maine Board of Pesticides Control, which regulates chemical use and helps set policy.

Henry Jennings, director of the pesticide control board, told lawmakers Thursday that the two chemicals have not been used by government agencies in Maine.

But the state would want the chemicals available for use if there is an outbreak of a mosquito-born disease such as West Nile, he said.

Jennings also said that recent studies have concluded that linking the two pesticides to the Long Island Sound lobster die-off was “fundamentally flawed.”

He warned that banning the chemicals in Maine could have unintended consequences.

“Banning chemicals without a careful assessment of what products will take their place is never sound publicly policy,” he said. “It generally leads to the use of higher-risk products in their place.”

He added that although Connecticut instituted a similar ban, the study lawmakers there used to justify it has since been invalidated.

No environmental groups testified Thursday.

Methoprene and resmethrin are commonly used in flea and tick control medicine for pets. The Maine Veterinary Medical Association opposes the bill.

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http://www.pressherald.com/politics/Tepid_support_for_pesticide_ban_bill_to_protect_Maine_lobsters_.html

Continued GAP certification

To schedule time for an audit to get GAP certification you may contact the Maine Department of Agriculture, Conservation and Forestry inspectors based in Aroostook County, at 800-292-6552 to schedule a GAP audit later in the spring or summer. Hourly travel costs along with inspection time expenses are included in the final bill for this service. In 2013 the costs were about $300 to $500. If the proposed increase goes through please keep in mind that this will almost double the cost for the certification. AgMatters, LLC can work with groups or one on one with Maine growers to help create their food safety plan in preparation for GAP and Good Handling Practice (GHP) Certification.

Growers who want to learn more about food safety practices for storage or processing facilities may want to attend a Sanitation Course offered by the University of Maine Cooperative Extension for a $65 fee (including course materials and lunch) on Wednesday, March 5th from 8 to 5 at 203 Hitchner Hall, the University of Maine, Orono. On March 12 and 13, Hazard Analysis Critical Control Point (HACCP) training is also available for food processors, including meat and poultry, for a $150 course fee. The training also held at 203 Hitchner Hall, the University of Maine in Orono from 8 to 5pm, includes course materials and lunch. At the end of this training you will have a full HACCP plan for your business.

For further questions, contact Theresa Tilton at 207.942.7396, or 800.287.1485 (toll free in Maine) or theresa.tilton@maine.edu. You may also contact Dr. Jason Bolton at Jason.Bolton@maine.edu or call 207.581.1366 if you have additional food safety questions or for consultation about setting up food processing facilities.

Researchers Conduct Important Spotted Wing Drosophila IPM Studies on Wild Blueberries

The Wild Blueberry Commission of Maine is collaborating with University of Maine Cooperative Extension faculty to develop studies in support of Integrated Pest Management for Spotted Wing Drosophila (SWD). The IPM research on the Spotted Wing Drosophila, an invasive species that infests small soft fruits such as wild blueberries, strawberries, and raspberries is known to reproduce exponentially while in soft fruit and berry crops. Drs. Frank Drummond, David Handley, and James Dill focused on evaluating preliminary trap and bait method for early SWD detection from which they determined that traps suspended above the crop with yeast and sugar mix seemed to have the best capture rate during August and September. Researchers want to determine what the relationship is between first and subsequent SWD trap captures and occurrence and severity of fruit damage in raspberries, strawberries; and highbush, and wild blueberry. Additional studies in the coming year will help determine:

Tractor demonstration at Field Day at Blueberry Hill Farm in Jonesboro, Maine.

- What are the most effective insecticides for SWD management and trap thresholds?
- Field edge or perimeter insecticide application strategies including, the most

continued on page 6

Continued SWD studies

effective crop sanitation tactics, and whether mass trapping of adult SWD and netting of small planting areas will reduce pest pressure

Look for more information about the results of 2013 studies by linking to the Extension website at http://umaine.edu/blueberries/factsheets/insects/ and review Spotted Wing Drosophila fact sheets. Also over the next year, the University of Maine SWD research studies are projected to determine: the overwintering success of the pest; the field movement inside of perimeter treatments that have occurred; and spring distribution in and around wild blueberry fields. Entomologists and IPM experts will also assess the possible use of biocontrol agents including parasitic insects. Extension presentations of results to growers are expected to occur at the late winter and spring education sessions around wild blueberry growing areas, as well as research education during Field Day at the Blueberry Hill Farm in July. As is the case with wild blueberry research, 4-6 years of IPM research is necessary to prove a practice works consistently. The Commission will continue to work with the UMaine wild blueberry research and extension team on conducting much needed research and ensuring IPM research funds.

Commissioner Walt Whitcomb (left) and Dr. David Yarborough, UMCE Wild Blueberry Specialist, at Field Day at Blueberry Hill Farm in Jonesboro, Maine.

Eastern States Exposition Wrap Up – The Big E 2013

With the good weather occurring over the course of the 17 day Big E in September 2013, the overall attendance record was broken again as the gate hit almost 1.5 million people during the fair! Maine Day at the Fair on the last Saturday was one of the record breaking attendance days during 2013. If you are interested in the vast entertainment offerings and exhibits at the Fair, visit their website at www.thebigE.com

The Wild Blueberry Commission of Maine wrapped up another successful Big E in the Maine building on the Avenue of States with 25 volunteer growers and their friends and family staffing the Public Education information booth at the Fair. As always we appreciate growers taking time out to come to the Fair, and talk to people about the special Maine wild blueberry. In the coming year the Wild Blueberry information and food booths will receive booth design upgrades to present a colorful professional exhibit in the Maine Building. We thank those who made 2013 such a successful year! We invite veteran and new volunteers to join us at the Big E in the Fall of 2014, a notice will be distributed in this spring!

In Spring of 2014, look for WBANA promotional materials that showcase a new brand identity!