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The formulation makes the honey bee poison

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A B S T R A C T

Dr. Fumio Matsumura's legacy embraced a passion for exploring environmental impacts of agrochemicals on non-target species such as bees. Why most formulations are more toxic to bees than respective active ingredients and how pesticides interact to cause pollinator decline cannot be answered without understanding the prevailing environmental chemical background to which bees are exposed. Modern pesticide formulations and seed treatments, particularly when multiple active ingredients are blended, require proprietary adjuvants and inert ingredients to achieve high efficacy for targeted pests. Although we have found over 130 different pesticides and metabolites in beehive samples, no individual pesticide or amount correlates with recent bee declines. Recently we have shown that honey bees are sensitive to organosilicone surfactants, nonylphenol polyethoxylates and the solvent N-methyl-2-pyrrolidone (NMP), widespread co-formulants used in agrochemicals and frequent pollutants within the beehive. Effects include learning impairment for adult bees and chronic toxicity in larval feeding bioassays. Multi-billion pounds of formulation ingredients like NMP are used and released into US environments. These synthetic organic chemicals are generally recognized as safe, have no mandated tolerances, and residues remain largely unmonitored. In contrast to finding about 70% of the pesticide active ingredients searched for in our pesticide analysis of beehive samples, we have found 100% of the other formulation ingredients targeted for analysis. These 'inerts' overwhelm the chemical burden from active pesticide, drug and personal care ingredients with which they are formulated. Honey bees serve as an optimal terrestrial bioindicator to determine if 'the formulation and not just the dose makes the poison'.

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1. Introduction: agrochemicals contribute to bee decline

Colony Collapse Disorder (CCD) and the general decline of pollinators continues to be a major threat worldwide [1,2]. Honey bee (*Apis mellifera* L.) overwintering colony losses in the US have averaged one-third since 2006, and yearly losses are now approaching 50% [3]. It is thought that multiple factors such as pathogens, parasites, malnutrition, and pesticide exposure have a role in CCD. The global diminishing of bees [4,5] and the relative importance of pesticides have both received much attention recently. Foraging bees are exposed to pesticides in agro-ecosystems as they gather nectar and pollen from flowers. Honey bees constitute a terrestrial model *par excellence* for agrochemical sampling in the environment, since their foraging range from a single hive averages about 6 kilometers in radius [6,7]. A comparative study of CCD-affected hives and healthy hives revealed the presence of over 130 different pesticides and metabolites out of 200 analyzed in over 1300 wax, pollen, and bee samples taken from managed hives across the US and Canada, with an average of 6 detections per sample [7–9]. Over 150 different pesticides have been found in samples from apiaries worldwide [10], and hives uncontaminated by pesticides, whether beekeeper-applied or not, are very rare. Managed honey bee colonies are intentionally exposed to miticides in an effort by beekeepers to control *Varroa destructor* Anderson & Trueman [10]. Not surprisingly, coumaphos and fluvalinate (two widely used in-hive miticides) were the two most frequently detected pesticide residues in managed hives [9]. However, no correlation was found between any one pesticide and CCD [5,7–11], suggesting that other more generic formulation ingredients may be involved. The combined effects of insecticides, fungicides and other agrochemical residues on honey bee sociality, foraging dynamics and floral specializations may jeopardize future production of food [1]. The role of agrochemical 'inerts' in the ongoing investigation of CCD/pollinator decline, and their effects on the physiology/behavior of honey bees have only begun to be investigated.

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2. Modern formulation technologies and agrochemical safety

Modern pesticide formulations and seed treatments, particularly when multiple active ingredients are blended, require proprietary adjuvants and inert ingredients to achieve high efficacy for targeted pests. An adjuvant, inert (term used in the US) or co-formulant (EU) is an additive (2,000 + in the US) used to enhance the performance or aid in the stability of formulations (20,000+) of active ingredients (1,000+). An adjuvant can be termed a surfactant, penetrant enhancer, activator, spreader, sticker, wetting agent, buffer, antifoaming agent, drift retardant, etc., and usually more than one of these functional descriptors is associated with the same chemical. Adjuvants are much less expensive than the active ingredient, but can reduce the effective pesticide dose needed by as much as 10-fold [12]. A surfactant is a surface active-agent (= detergent, emulsifier, soap) that reduces the surface tension of water. This is achieved by being structurally composed of distinct hydrophilic and hydrophobic moieties, often referred to as head and tail groups, to mediate both the mixing of hydrophobic pesticides with water to form solutions or the dissolution of hydrophobic plant and insect cuticles and membranes to allow active ingredients to penetrate. The head groups, regardless if hydrophilic or hydrophobic, can be neutral, anionic, cationic or amphoteric. An opposite polarity tail moiety, such as a hydrophobic hydrocarbon chain or hydrophilic polyoxyethylene or sugar, completes the surfactant molecule and serves in its classification.

Modern nonionic surfactants include alkyl and aryl ethoxylates, organosiloxanes, sorbitans and fatty acid esters [13–15]. Common formulation anionics include sodium dodecylsulfate, sulfonates, lauric and other fatty acids, glycolic acid ethers and phosphates. Cationics are exemplified by tallow amines and trialkylammonium salts. Other inerts function as co-solvents, higher-boiling liquids (antifreezes) used to keep formulation components in solution such as butanol, diethoxol, methylcyclohexanone, N-methyl-2-pyrrolidone (NMP), propylene glycol, and xylene. Emulsifiable and soluble concentrates are the most commonly used agrochemical formulations and are often a dynamic blend of nonionic with either anionic or cationic surfactants to produce micro-emulsions or solutions of mutiple components in a tank mix. Typical formulations contain less than 50% active ingredients with the remainder surfactants and solvents. Adjuvant use has evolved (Fig. 1) from focus on alkylphenol, alcohol, fatty acid and sorbitan ethoxylates in combination with sulfonates to new technologies comprising fatty (tallow) amine and organosilicone ethoxylates and co-solvents like NMP [16–18].

3. Formulations inerts and impacts on non-target species

Adjuvants are largely assumed to be biologically inert and are usually not included in risk assessments required to register a pesticide in the U.S. [19–21]. Of the 20 toxicological tests required to register a new pesticide in the US, 13 are conducted with only the active ingredient(s); only 7 short-term acute mammalian and avian toxicity tests use the entire formulation [22,23]. Medium- and long-term toxicity tests only examine the active ingredient(s). 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 US EPA as part of the pesticide registration process in contrast to the EU where toxicity for representative formulations is mandatory [24]. Moreover, the specific ingredients that make up spray adjuvants are considered trade secrets of the chemical companies that manufacture them and are therefore usually not disclosed [12,23]. In response to public concerns, the US EPA Inert Ingredient Assessment Branch recently conducted an open commentary period that ended on April 23, 2010 (Docket ID: EPA-HQ-OPP-2009-0635; cf [25]) to consider the disclosure of inert ingredients on pesticide product labels. EPA action on hundreds of responses is still pending. More label disclosure would allow increased user and consumer awareness of all potentially toxic chemicals in pesticide formulations as well as more thorough testing of the potential biological impacts.

Co-formulants and supplemental adjuvants that can be used in tank mixes often enhance the pesticidal efficacy as well as inadvertently the non-target effects of the active ingredient after application [26,27]. Numerous studies have found that pesticide active ingredients elicit very different physiological effects on non-target organisms when combined with their formulation ingredients [28]. Indeed, systemic movement of the top pesticide used globally, glyphosate, is determined by its formulation inerts. Glyphosate has negligible ecotoxicity without tallow amines and other adjuvants [18,29], including its toxicity to humans [30–32]. Formulation inerts often increase pesticide toxicity to aquatic insects, fish and amphibians [33,34]. The nonionic surfactant R-11 synergized the acute toxicity of the insecticides spinosad [35] and imidacloprid [36] on aquatic crustaceans, and in the absence of an insecticide reduced the growth rate of *Daphnia pulex* at concentrations found after application near aquatic systems at recommended field rates [21]. Clearly the formulation components themselves have a lot to do with the potency of the poison.
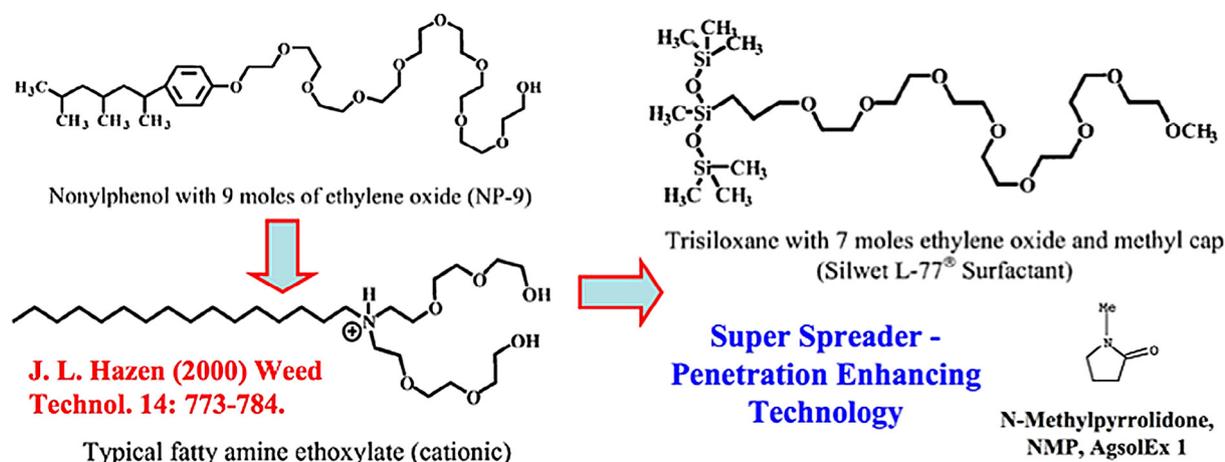

Fig. 1. Brief chemical history of modern adjuvant development.

Of increasing concern is a new generation of penetrating solvent/adjuvants in agrochemical formulations, which include NMP (CAS no. 872-50-4) and its higher homologs N-octyl and N-dodecyl pyrrolidones (CAS 2687-94-7 and 2687-96-9, respectively). These alkylpyrrolidones are used in synergistic combinations with themselves and other surfactants to create microemulsions that greatly enhance the ability of active ingredients, whether drugs or agrochemicals, to penetrate animal integuments and plant cuticles [37–39]. These penetrant enhancers have broad uses in addition to crop protection including human skin and hair care, and transdermal and oral drug delivery. The US EPA reassessed these inerts under FQPA in June, 2006 [40,41], and maintained their current exemptions from a requirement for a specific tolerance for human safety. Thus these alkylpyrrolidones are permitted for nonfood use in pesticide products at any level. Due to this inert status and their exemption from tolerance, environmental residues are not monitored though the use of this organic solvent is increasing [42,43] and currently at about 187,000,000 pounds per year in the US (Table 1). NMP has high toxicity for the aquatic invertebrate *Daphnia magna* with an LC_{50} of 1.2 mg/L, the most toxic solvent among a dozen tested, and 12 times more toxic than lindane, equitoxic to paraquat, although 4 and 22 times less toxic than malathion and disulfoton, respectively [44]. It has been documented that human acute poisoning to formulated imidacloprid was likely due to NMP, and not this neonicotinoid, based on the clinical symptoms [45]. Also, metabolites of the N-alklypyrrolidones can be directly neurotoxic [46] and are of concern because of documented substantial human exposure [47]. Multiple billions of pounds of synthetic organic chemicals are used and released into US environments as formulation ingredients like NMP, which are currently classified as generally recognized as safe (GRAS), and therefore have no mandated tolerances, and whose residues in the environment remain largely unmonitored by the EPA or others. These so called 'inerts' thus represent an overwhelming level of the total chemical burden from active pesticide, drug and personal care ingredient usage currently in the US.

4. Pesticide adjuvants and honey bees

Although agrochemical formulations usually contain inerts at higher amounts than the active ingredients, only rarely have these penetrating enhancers, surfactants and adjuvants been evaluated with regard to bee exposures and toxicities. Considerable recent progress has been made on surveying the prevalence of pesticide active ingredients within hives [10,48] and on improving the active-ingredient risk assessment for honey bees [49,50]. However, little work has been done to examine the safety to pollinators of pesticide adjuvants that are either included in pesticide formulations (formulation adjuvants) or tank-mixed and sprayed along with the formulated products (spray adjuvants). Given the fact that migratory honey bee colonies are exposed to so many pesticides, and the fact that these adjuvants are sprayed in conjunction with pesticide formulations, it follows that foragers are likely exposed to adjuvants as well. Particulate microencapsulated, slurry, suspension, water-dispersible granule and wettable powder formulations of pesticides can pose more risk to bees than sprayed solutions. This is due to the focused floral collection of pollen-sized particles and nectar resources by pollinators versus less extensive contact with foliar or other treated plant surfaces.

Insecticides, particularly the neonicotinoids used in systemic seed treatments and many other applications, have been associated with honey bee declines in France and more recently Germany, Italy, other European countries, US and Canada [51–53]. While probably independent of long-term declines, recent EU bee kills associated with a formulation error in a clothianidin seed treatment due to an inadequate sticker that led to toxic airborne dusts, have focused attention on neonicotinoids as putative causative agents for CCD. The result was a more classical pile of dead bees at the hive entrance, which is not consistent with CCD, where adult bee mortality is not usually observed. The impact of aerial dusts from neonicotinoid seed treatments on bees, including that from talcs used in planters, requires more evaluation [52,54], since formulations appear more acutely toxic than individual active ingredients [53,55]. New seed-planter lubricants are being developed to mitigate this problem.

Given the synergistic nature of certain chemicals, we are concerned that active ingredients may affect honey bees differently depending upon the inert ingredients in a particular product formulation or in certain combinations found in field use patterns [56–59]. A variety of nonionic surfactants and one organosilicone fluid at 500 ppm deterred honey bees from visiting pond water [56]. A simple detergent solution has been shown to kill swarms of unwanted feral or Africanized honey bees [58]. Goodwin and McBrydie [59] found that 4 out of 11 commercially available spray

Table 1
Some US pesticide active relative to inert ingredients: production and use patterns.

Chemical	Class	US Production (1000 pounds)[*]
Organosilicones (13 of > 560 chemicals)	Inert – defoaming	>496,080
Nonylphenol polyethoxylates[a]	Inert – surfactant	50,000–320,000
Glyphosate	Herbicide	250,000
Nonylphenols	Inert – surfactant	220,000
1-Methyl-2-pyrrolidinone (NMP)	Inert – solvent	184,703
Tallow amine and similar polyethoxylates	Inert – surfactant	>88,000
Organosilicone surfactants (>43 chemicals)	Inert – surfactant	withheld
Atrazine	Herbicide	67,000
2,4-D	Herbicide	35,000
Chlorothalonil	Fungicide	10,300
Mancozeb, Ziram, Maneb, Metiram, Thiram	Fungicide – dithiocarbamates	9,600
Chlorpyrifos	Insecticide – organophosphate	7,000
Pyraclo-,Azoxy-,Trifloxy-,Fluoxa- strobins	Fungicide	5,800
Captan	Fungicide	3,400
Clothianidin	Insecticide – neonicotinoid	1,800
Imidacloprid	Insecticide – neonicotinoid	1,700
Bifenthrin	Insecticide – pyrethroid	830
Cyhalothrin	Insecticide – pyrethroid	650
tau-Fluvalinate	Insecticide – pyrethroid	0.5

[*] Source: US EPA Chemical Data Reporting (2014, mostly 2010 production) http://epa.gov/cdr/ or USGS-NAWQA Pesticide Use Maps (2011) http://water.usgs.gov/nawqa/pnsp/usage/maps/.
[a] US EPA estimated total use, internal report 2007; cf. 857,000,000 lbs conventional pesticides.

adjuvants were toxic to honey bees at field rates; indeed Pulse® killed all adult honey bees both topically and orally at a 0.1% dose.

5. Toxicity and behavioral effects of adjuvants on honey bees

Formulations are generally more toxic than their respective active ingredients (a.i.) to bees. For example, Bravo Weather Stik® formulation of the fungicide chlorothalonil was at least four times more toxic orally to adult honey bees [60] and larvae [61] than the technical treatment. Taktic® E.C. (LD_{50} 28.5 μg/bee), in turn, was about 4 X more toxic orally than the a.i. amitraz (LD_{50} 103 μg/bee) [62]. Some tests with adjuvants have shown low or no toxicity to bees [63]. Other studies have established that inerts and spray adjuvants can directly control various pest insects [64,65]. The fact that some inert ingredients may indeed be more toxic than active ingredients [18,29,66] makes this issue especially important for the health of honey bees and other pollinators. Increased use of strong penetrants to promote systemic movement of new classes of pesticides such as lipid biosynthesis inhibitors [67,68] may be particularly detrimental to honey bees and other beneficial nectar and pollen feeders.

A larval rearing method was adapted to assess the chronic oral toxicity to honey bee larvae [69] of the four most common pesticides detected in pollen and wax [9] – in-hive miticides fluvalinate and coumaphos, and the fungicide chlorothalonil and insecticide chloropyrifos – and the common formulation solvent NMP. All pesticides at hive-residue levels triggered a significant increase in larval mortality compared to untreated larvae by over twofold, with a strong increase after 3 days of exposure. Among these four pesticides, honey bee larvae were most sensitive to chlorothalonil compared to adults. Although the penetration-enhancing solvent NMP was only moderately toxic to adult honey bees, it has much higher toxicity to larvae (Fig. 2). A 1% oral dose of NMP killed all the reared larvae during the first day of exposure [69]. Even for the lowest concentration of 0.01% (100 mg/L), the estimated time to cause 50% larval mortality was only 4 days (Fig. 2) during this 6-day chronic feeding bioassay. For adult bees, it took a 10% concentration of NMP to kill 50% within one day, so larvae are at least 20 times more susceptible to this formulation solvent than the adults. Studies are ongoing to determine by LC-ESI-MS the degradation pathway for NMP and its involvement in honey bee toxicity at field-relevant exposure levels and in interactions with disease factors (J. D. Fine, unpubl.).

While the adult honey bee is much more acutely susceptible than the adult Japanese hornfaced bee, *Osmia cornifrons* (Radoszkowski)

to a formulated neonicotinoid imidacloprid (Provado 1.6F), the opposite trend is found with another neonicotinoid acetamprid (Assail 30SG) [70]. For honey bees, the decreasing order of toxicity at LC_{50} was imidacloprid, λ-cyhalothrin, dimethoate, phosmet, and acetamprid. For *O. cornifrons,* the decreasing order of toxicity at LC_{50} was dimethoate, λ-cyhalothrin, imidacloprid, acetamiprid, and phosmet. By contrast, similar interactions of acetamprid (synergism) or imidacloprid (slight antagonism) with the fungicide fenbuconazole (Indar 2F) were noted [70]. While technical glyphosate has virtually no toxicity for honey bees, the Weathermax® formulation does [71]. Most arguments about how susceptible honey bees are to pesticides relative to other insect species [72] or related pollinators [73] have been based primarily on susceptibilities to the technical a.i. Bumble bees are highly susceptible to 1 ppm concentrations of strong emulsifiers such as perfluorooctylsulfonic acid [74]. Clearly differences in bee species susceptibility to common pesticides can challenge pesticide risk assessment. However, hazards cannot be predicted if the technical ingredients are tested alone and only on a single pollinator species.

Typical ecotoxicological testing for registering pesticides focuses on short-term (1–2 day) assays designed to determine the LD_{50} or LC_{50} of a particular chemical in a population of test organisms. Given the complex foraging, communicative, and navigational tasks honey bees must perform, sublethal and chronic effects of pesticides are especially important when compared to other, less sophisticated non-target species [75]. The proboscis extension reflex (PER) assay is a well-established associative learning assay that effectively simulates the feeding events that occur at a flower, but under a controlled laboratory setting. The neonicotinoid insecticide imidacloprid, in addition to being highly toxic to honey bees (average $LD_{50} = 40$ ng/bee), also impairs learning and memory at sublethal levels [75]. We used an improved, automated version of the PER assay to measure the olfactory learning ability of honey bees treated orally with sublethal doses of the most widely used spray adjuvants on almonds in the central valley of California [76]. California almond pollination is the single largest pollination event in the world. Spray adjuvants usage information is contained in the California Pesticide Information Portal database maintained by the California Department of Pesticide Regulations [77]. California is unique among all other US states in that spray adjuvants are considered pesticides and are reported similarly to pesticide active ingredients. Three different adjuvant classes (nonionic surfactants, crop oil concentrates, and organosilicone surfactants) were investigated. Classical conditioning PER tests (Table 2) were conducted on adult bees with adjuvant treatments in 50% sucrose [76].

Organosilicones (Dyne-Amic, Syl-Tac, and Silwet L-77) were more active than other nonionic adjuvants, while the crop oil concentrates (Penetrator, Agri-Dex, and Crop Oil Concentrate) were inactive (Table 2). Learning was impaired after ingestion of 20 μg of organosilicone surfactant equivalent to a 2 sec feeding at 1 μl/sec on a 1% adjuvant solution. Silwet L-77 was the most potent adjuvant tested, significantly reducing learning performance down to an oral dose of 5 μg/bee (5 sec feeding at 1 μl/sec on a 0.1% solution) beginning at the third acquisition (learning) trial (AT3). Honey bees treated with the nonionic surfactant Activator 90 experienced a similar reduction in learning beginning at AT5, while the other nonionic surfactants tested, R-11 and Induce, did not significantly impair learning. Ingestion was required for the tested adjuvant to have an effect on learning, suggesting the organosilicones are acting at a systemic level after entering the crop [76]. Generally, spray tank adjuvants are added to tank-mixes at concentrations of 0.1 to 5%. A forager visiting multiple flowers sprayed with an adjuvant/pesticide will receive a much larger overall dose than a 2 μl dose of a 1% adjuvant investigated here, since nectar crop loads can range up to 50 μl [78]. Thus organosilicone adjuvants at low concentrations independent of active ingredient(s) can impact the olfactory
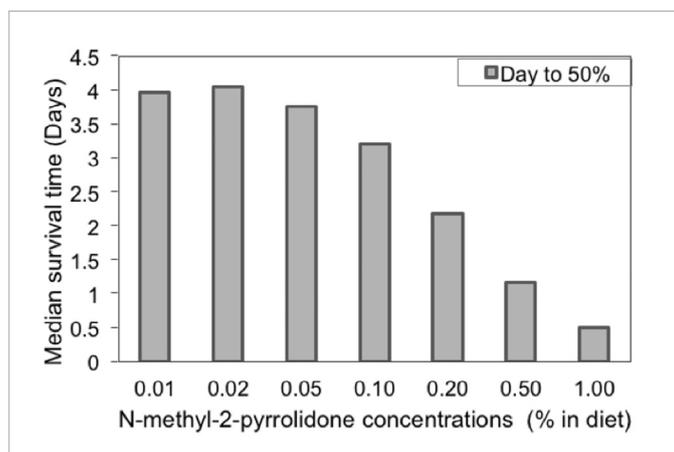

Fig. 2. Estimated time to 50% mortality for 7 concentrations of *N*-methyl-2-pyrrolidone (NMP) fed to honey bee larvae in royal jelly diet [69].

Table 2
Learning performance of honey bees during a sequence of acquisition trials (AT1-5) using odor and sugar stimuli, starting 5 minutes after ingestion of a spray adjuvant (1% v/v) or a sugar control.

Adjuvant	N^a	% Conditioned Response				
		AT1	AT2	AT3	AT4	AT5
Organosilicones						
Dyne-Amic	20	15.0^b	45.0	50.0*	55.0*	50.0*
Sylgard 309	20	0.0	40.0	55.0	60.0	50.0*
Syl-Tac	20	15.0	40.0	50.0*	45.0*	45.0*
Silwet L-77	20	10.0	35.0	50.0*	40.0*	50.0*
Non-ionics						
Activator 90	20	15.0	50.0	60.0	60.0	60.0*
R-11	20	15.0	45.0	65.0	65.0	70.0
Induce	20	5.0	50.0	65.0	70.0	70.0
Crop oils						
Penetrator	20	15.0	55.0	80.0	85.0	80.0
Agri-Dex	20	5.0	60.0	80.0	85.0	90.0
Crop Oil Concentrate	20	15.0	50.0	70.0	75.0	85.0
Aggregate Control	199	8.5	57.3	76.4	80.9	82.9

[a] N, number of bees per treatment group.
[b] Percent conditioned responses for each treatment group were compared to the aggregate control group for each treatment modality using the Fisher's exact test (* $P < 0.05$).

learning required for foraging, which could have potentially harmful effects on the colony and contribute to bee decline.

6. Organosilicone surfactants may impact bee health

Silicon, after oxygen, is the second most abundant element comprising 28% of the earth's crust. It forms with oxygen one of the strongest, most stable chemical bonds known, resulting in sand or silica and diverse silicate salts. Making use of this abundant resource to synthesize economic adjuvants and inert ingredients, including polysilicones and polysiloxane surfactants, is an expected outcome of the industrial age, although silicon itself is rare in biota, and generally considered a xenobiotic [79]. While the inert and highly lipophilic polysilicones have been used in commerce well over a half-century, the polysiloxane surfactants are a more recent discovery and innovation of the last 20 years [80].

The 'super-spreading' ability of organsilicones is thought to be due to the siloxane backbone of the hydrophobic group (Fig. 1), which allows the hydrophobe to be far more compact than that of conventional, carbon-based surfactants [19,20,81,82]. Thus organosilicones cause a greater reduction in surface tension than both nonionic surfactants and crop oil concentrates, making them the most potent surfactants and super-penetrants available to growers. The mechanism(s) of action within a honey bee that leads to learning impairment may be due to this extreme surfactant activity. Organosilicone adjuvants mediate both the mixing of pesticides of low to high polarity with water to form solutions and the dissolution of hydrophobic cuticles and membranes to allow active ingredients to penetrate. It is unknown whether a similar phenomenon is taking place within the crop or midgut of honey bees. Silicone surfactants are good insecticides and typically more toxic to insects and mites than other nonionics and crop seed oils. Aqueous solutions of the trisiloxane surfactant Silwet L-77 are toxic to aphids [83], tephritid fruit flies [84], citrus leafminers [85], two-spotted spider mites [86], Pacific spider mites, cotton aphids, western flower thrips, and grape mealybugs [87]. Silwet L-77 is known to synergize the neonicotinoid imidacloprid used in control of the Asian citrus psyllid [88]. For spider mites, leaf dip $LC_{50}s$ for all three trisiloxanes tested were less than 10 ppm [86], while for citrus psyllid a similar LC_{50} of 7.3 ppm was obtained [88]. Another organosilicone adjuvant Sylgard 309 sprayed at 0.025% was highly toxic to an aphid hymenopteran parasitoid [89].

Organosiloxane, nonyl- and octyl-phenol polyethoxylates are widely used as nonionic surfactants around honey bee hives or in their foraging areas as spray adjuvants or additives in agrochemical formulations (Table 1). There is one published method in the literature on the analysis of the prototype trisiloxane surfactant Silwet L-77 [90], and more recently a method developed to monitor this trisiloxane surfactant in the aqueous environment [91]. Essentially no monitoring and residue literature and has been published for the much more widely used silicone adjuvants Dyne-Amic, Kinetic, Sylgard, Syl-Tac, Widespread Max etc. This is in contrast to a large number of analytical monitoring methods available for the older classes of surfactants [92,93]. Having a selective monitoring method for the organosilicone surfactants that resolves them from the more ubiquitous polydimethysilicones [94] is key to understanding their fate and environmental impacts. Tank-mix adjuvants often increase pesticide residue amounts in the environment [95], and thus an analytical monitoring method is needed for all spray adjuvants, particularly these newer organosilicones which are notoriously difficult to identify and quantify using standard analytical methods [96]. The chemistry of polymeric organosilicone surfactants is complex, and many of the components have not been sufficiently isolated and chemical identities confirmed in order to assign an identifying CAS number. One can only see and begin to understand the consequence of an inert when you are able to analyze it.

To this end, we have developed a liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) method (Fig. 3) to resolve and sensitively analyze by selective ion monitoring the various trisiloxane and tetrasiloxane polymeric components with various methyl, hydroxy and acetyl end-capping [97] together with their synthetic impurities and degradates that are found in common organosilicone surfactants [90]. A parallel method for analysis of nonylphenol $(NP(EO)_n)$ and octylphenol polyethoxylate $(OP(EO)_n)$ surfactants in bee hive matrices was also developed [98]. For both the trisiloxane and $NP(EO)_{3-13}$ and $OP(EO)_{3-13}$ oligomers, mass ions for ammonium adducts were monitored. Trisiloxane surfactants with a single polyethoxylate (EO_n) chain and end-capped with methyl, acetyl or hydroxyl groups were extracted by the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach. Less than 2 grams of honey, pollen or wax were extracted. Identification and quantification were accomplished employing LC-ESI-MS.

Trisiloxane surfactants were detected in every wax sample and 60% of the pollen samples (Table 3), while no trisiloxane surfactants were found in honey [97]. Total trisiloxane surfactant concentrations were up to 390 ppb in wax and 38.8 ppb in pollen. Nonylphenol more than organosiloxane and octylphenol polyethoxylates were found in wax samples, while pollen and particularly honey residues were lower. $NP(EO)_n$ was detected in every sample with concentrations ranging from 26 to 10,239 ppb. Much higher $NP(EO)_n$ residues levels were found in wax followed by pollen than in honey [98]. $OP(EO)_n$ concentrations on average were more than 10 times lower in pollen and wax (Table 3). Others have recently detected trisiloxane surfactants in surface waters [99], and other adjuvants from agrochemical treatments including solvents and surfactants have been found in the environment using appropriate novel analytical methods [18,100,101]. These methods demonstrate a probable wide occurrence of substantial amounts of alkylphenol ethoxylates and organosiloxane surfactants in US beehives, and calls for a renewed effort to investigate the consequence of these adjuvants to bee health and the ongoing global bee decline.

There is recently great concern over the ubiquity of low MW cyclic methylsiloxanes as pollutants in the environment due to their wide use in personal care products, medicines, agrochemicals and cleaning formulations (Table 1), and in a wide diversity of industrial processes [102,103]. Much more toxicological information including their endocrine disrupting effects [104] is available on the cyclic
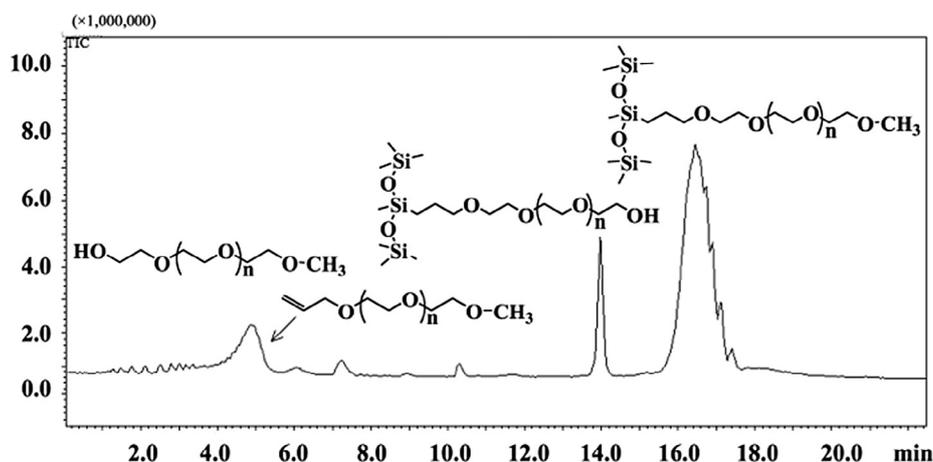
Fig. 3. LC-ESI-MS of 250 ppm Silwet L-77 on Shimadzu LCMS-2020. Positive Ion Chromatogram (total ion current) obtained after separation on a Shim-Pak XR-ODS 2 x 100 mm column. Structural identity of low retention time impurities based on MS data and comparison with literature values [90].

methylsiloxanes such as D4 and D5 and linear polydimethylsiloxanes [105], the older 'silicones' of commerce, particularly with regard to use in breast and other implants. However these 'silicones' are relatively inert, poor penetrants, and do not absorb well or enter efficiently into the transport systems of organisms due to their extreme non-polarity and lack of water solubility. The environmental fate of these compounds generally proceeds until their degradative mineralization down to silica [106,107].

The prevalence of organosilicone surfactants in pesticide formulations and their broad utility as tank adjuvants [108–110] suggests there is high potential for their producing harm to non-target species. Very little pertinent toxicology with regard to the tri- and tetrasiloxane surfactants is known. These super-surfactants should readily move across membranes and become systemic in plants and animals. Their biotransformation could eventually lead to mineralization and silicosis within sink tissues of the exposed organism. In spite of their great potential for environmental harm and their increasing high-volume use in agriculture and other commerce although their total production in the US is withheld (Table 1), peer-reviewed publications on their toxicology are extremely limited and documentation of their safety appears to be archived mostly within the confines of industry. The last reassessment publically disclosed by US EPA [111] with regard to setting a tolerance for organosilicone surfactants pooled all dimethylpolysiloxanes, fatty acid derivatives, waxes, mineral oils and even the polyethylene glycols into one category with the methylated silicones that chemically range from highly non-polar, water insoluble and high MW compounds that are truly poorly absorbed to super-penetrant organopolysiloxane surfactants that would readily be absorbed and are super-penetrating and super-spreading surfactants. Hundreds of thousands of pounds of these latter organosilicone surfactants are used every year on almonds in California alone [76,77]. Spraying of these adjuvants are allowed at almond bloom when pollinators are foraging [112]. The organosilicone adjuvant Break-Thru fed to nurse bees at 200 ppm in a pollen diet was found not to indirectly impact queen-rearing [113]. We have found much higher toxicity to honey bees fed directly on related organosilicone surfactants in 50% sucrose, with oral LC_{50}s for pure commercial trisiloxane surfactants ranging to below 10 ppm, and significant mortality down to 100 ppb ([114]; J. Chen et al. unpubl.). The environmental consequences of these potent non-ionic surfactants remain largely unknown.

7. Consequences of modern agrochemical formulation technologies to honey bees

Our focus is on the study of the role of pesticides and their co-formulants in honey bee and overall pollinator decline. Insufficient data exist to allow an extension beyond adult bee acute toxicity information for individual pesticide active ingredients into a reasonable holistic assessment of the consequence of total chemical loads and exposures that incorporate co-formulants and adjuvants, as well as the resulting agrochemical interactions. Our recent work indicates that honey bees are sensitive to common 'inert' ingredients used in agrochemical formulations and spray tank adjuvants. The vast majority of 'inerts' remain to be tested directly on honey bees and representative native pollinators. Our guiding principles include 'if you do not know what is used, you will never look for it' and 'if you do not look for it, you will never find it'. Data for total yearly

Table 3
Total concentrations (ppb) of trisiloxane capped with methyl (R = CH₃), hydroxyl (R = H), acetyl (R = C(O)CH₃), and of NP(EO)ₙ and OP(EO)ₙ in pollens, waxes and honeys.

Sample ID	Trisiloxanes			Alkylphenol polyethoxylates	
	R = H	R = C(O)CH3	R = CH3	OP(EO)n	NP(EO)n
Pollen					
#1				48	157
#2		9		37	347
#3			17	37	352
#4	21		19	47	463
#5			11	43	561
#6			22	43	215
#7			8		136
#8					59
#9					390
#10				71	86
Wax					
#1	14	206	129	159	273
#2			59	10	46
#3		66		31	187
#4			185		380
#5		386		21	117
#6		12			244
#7		11	11	16	92
#8	16			48	91
#9		14	23	53	124
#10	9	47		396	10123
Honey					
#1					32
#2					25
#3					28
#4					86
#5					44

use of co-formulants and adjuvants in plant protection products, pharmaceuticals, industrial sites, personal care products etc. in the US and other countries are hidden under the cloak of 'trade secrets' and 'proprietary information'. Organosilicone surfactants are increasingly being applied to agricultural agro-ecosystems as spray adjuvants, while nonylphenol and octylphenol ethoxylates are major toxicants in agrochemical formulations used around beehives (Table 1). Most adjuvants and inert ingredients are exempt from human safety tolerances, classified as GRAS, and thus no environmental monitoring is required. While some regulatory agencies test some formulations on honey bees, public access to this information is largely unavailable. Paired tests (formulation versus active ingredient) or tests with formulation blanks are not required to allow an independent assessment of co-formulants on honey bees or other non-target species. For the sustainability of honey bees, other pollinators and beneficial insects, it is vitally important that co-formulants be disclosed and their effects investigated.

There is a new tendency to market pesticide blends containing multiple classes of insecticides or fungicides, or their mixed combinations without any further ecotoxicology testing. Examples include across-class formulation of ryanodine-receptor modulators, neonicotinoids, pyrethroids, lipid-biosynthesis inhibitors, and chitin-synthesis inhibitors, and formulations of multiple classes of fungicides for tank mixes. Many insecticide and fungicide combinations are utilized for seed treatments and in crop pest control that can result in hive residues that synergistically combine by themselves or with miticides used to control *Varroa* to poison honey bees [115–117]. These blends usually require proprietary adjuvants to achieve high efficacy and broadly control many pests [118]. The impact of these synergistic blends on non-targets including bees and other beneficial species cannot be fully understood without knowing the identity of proprietary inerts and adequate testing on the appropriate species. The adjuvants themselves are largely assumed to be biologically inert and are therefore subject to minimal scrutiny and toxicological testing by regulatory agencies. A recent screening test using *E. coli* growth inhibition and a multiple end-point reporter assay to characterize the relative toxicity of different inert surfactants and solvents may be a useful approach in a high-throughput estimate of toxicity and mode of action of agricultural adjuvants [119]. Our results indicate that organosilicone surfactants are among the most toxic adjuvants, both sublethally and acutely, to adult honey bees [76,114]. Honey bees are exposed to a wide array of pesticides active ingredients [9,10,48,120,121] as they forage, meaning that they are undoubtedly exposed to many spray adjuvants as well. We have found 100% of other formulation ingredients targeted for analysis in beehive samples, while only 70% of the pesticide active ingredients searched for have been found.

The impact of systemic pesticides, seed treatments, formulation additives, and other pesticides and their combinations on non-target species, and their role in honey bee/pollinator health are of global consequence to food security. The effects of chronic exposure to pyrethroids, organophosphates, neonicotinoids, fungicides and their formulation ingredients can range from lethal and/or sublethal effects in brood (immature bees) and adults to reproductive effects on the queen [49,50,75] directly or via microbial symbionts [122]. Bee nutrition and physiological changes across seasons (summer versus winter bees) can have marked impacts on their pesticide susceptibility [123]. Attempts to correlate global bee declines or CCD with increased pesticide exposures alone [5,11,48,50] have not been successful to date. Two major complications with such attempts are that the time delay between collecting pollen contaminated with multiple pesticides and when it is actually consumed by bees or brood is not predictable in colonies, and the potential biotransformations of pesticides in beebread are completely undocumented. Pesticide interactions among various formulations as well as with other stressors including *Varroa* and

Nosema [5,124], viruses [4,125,126], beneficial hive microbes [122], and on bee immune systems [125] all require further study. We anticipate that if 'inerts' are influencing pesticide levels and general hive stress, formulation recommendations can be optimized for use in bee foraging areas. There is clearly a need to disclose more information on inerts in agrochemicals, since it appears that 'the formulation and not just the dose makes the poison'.

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