A Review of Sub-lethal Systemic Neonicotinoid Insecticides Exposure and Effects on Sensitive Receptors: Pollinators

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KEYWORDS: Neonicotinoids, Honeybee, Pollinator, Sub-lethal exposure.

Funding: This study was supported financially by the Department of Energy and Environmental Protection, State of Connecticut (Contract No. 042103580).

Financial Disclosure: Author has declared no competing financial interest.

ABSTRACT

Background and objective: Beekeepers around the world have been reporting the ongoing weakening of honeybees (*Apis mellifera*) health and subsequently the increasing colony losses since 1990. However, it was not until the abrupt emergence of colony collapse disorder (CCD) in the United States in 2006 that has raised the concern of losing this important perennial pollinator. In this report, we provide a summary of the sub-lethal effects of pesticides, in particular of neonicotinoids, on pollinators' health from papers published in peer-review journals.

Method: We have identified 30 papers from a literature search on PubMed and Google Scholar using the following combined key words of "pollinators", "honeybee", "bees", "pesticides", or "neonocitinoids", as of September 30, 2014, and from a cross-reference check of a report made available by European Parliament in preparation to fulfill their regulatory mandate on the issue of protecting pollinators among their membership nations. Those paper are relevant to examine the effects of sub-lethal pesticide exposures on the health of honeybees (*Apis mellifera*), bumblebees (*Bombus terrestris*), and other bees.

Results: The weight-of-evidence of this review clearly demonstrated bees' susceptibility to insecticides, in particular to neonicotinoids, and the synergistic effects to diseases that are commonly present in bee colonies. One important aspect of assessing and managing the risks posed by neonicotinoids to bees is the chronic effects induced by exposures at the sub-lethal levels. More than 90% of literature published after 2009 directly or indirectly demonstrated the adverse health effects associated with sub-lethal exposure to neonicotinoids, including abnormal foraging activities, impaired brood development, neurological or cognitive effects, and colony collapse disorder.

Conclusions: The rising awareness of protecting honeybees and other pollinators worldwide is

directly related to the emergence of honeybee colony collapse disorder (CCD). Considering neonicotinoids are the most widely used insecticides, it is a conceivable challenging task to protect honeybees and other pollinators from foraging in the environment where the sub-lethal levels of neonicotinoids are ubiquitous.

INTRODUCTION

Pollinators, in particular bees, are critically important in sustaining biodiversity by providing essential pollination for a wide range of crops and nature plants. They contribute to human health and wellbeing directly through the production of nutritious food, honey and other feed supplies such as: pollen, wax, propolis, and royal jelly. United Nations (FAO) estimated that bees pollinated 70% of crop species that provide 90% of food supplies worldwide.

Beekeepers around the world have been reporting the ongoing weakening of honeybees (*Apis mellifera*) health and subsequently the increasing colony losses since 1990. However, it was not until the abrupt emergence of colony collapse disorder (CCD) in the United States in 2006 [1,2] that has raised the concern of losing this important perennial pollinator. A recent United Nations report highlighted the persistence of CCD worldwide [3] and called for changes in honeybee colony management in order to save this important pollinating insect. CCD is a symptomatic disease and commonly characterized by the sudden disappearance of adult honeybees in winter from hives containing adequate store food (e.g. honey, nectar, and pollen). It is generally agreed that some losses of bee colonies during winter is common in apiculture, however, never in the history of beekeeping has the losses of honeybee hives occurred in such a magnitude, over a widely distributed geographic area, and lasting for many years.

While the prevailing opinions in U.S. suggest the linkage of CCD to multi-factorial causes including pathogen infestation, beekeeping practices (including malnutrition), and pesticide exposure in general [4-14], recent scientific findings linking declines of bee colonies with exposure to pesticides, in particular to the systemic neonicotinoid insecticides, appear to be gaining traction [15-20], and have led to new regulatory control in the European Union [21]. In light of the important ecological and economic values of pollinators, there is a need to take immediate

action to identify anthropogenic factors associated with the declining numbers of pollinators in order to sustain crop production and environmental conservation.

In this report, we provide a summary of the sub-lethal effects of pesticides, in particular of neonicotinoids, on pollinators' health from papers published in peer-review scientific journals. We first conducted a literature search on PubMed and Google Scholar using the combined key words of "pollinators or honeybee or bees and pesticides", which yields more than 200 papers, as of September 30, 2014. We then supplemented the literature search by a cross-reference check with the Report titled *"Existing Scientific Evidence of the Effects of Neonicotinoid Pesticides on Bees"* [22]. This report was a result of a request made by European Parliament in preparation to fulfill their regulatory mandate on the issue of protecting pollinators among their membership nations. This cross-reference check yielded additional 47 papers to the final list. We then excluded papers from this summary review if; a) papers do not contain either pesticide exposure or toxicological endpoint data in associated with bees or pollinators, b) papers only included flies or beetles as the study insects, c) papers reported the use of pesticides that are not registered to be used in the United States, or d) papers were not written in English. At the end, we have identified 30 papers, as listed in Tables 1-4 that are relevant to examine the effects of pesticide exposures on the health of honeybees (*Apis mellifera*) and bumblebees (*Bombus terrestris*).

PESTICIDE EXPOSURE ASSESSMENT IN BEES

It is well documented in the literature that bees are constantly being exposed to a long list of pesticides. Those pesticides that are either brought back by bees from the outside foraging environment or applied by the beekeepers for treating infectious diseases in which could inadvertently harm individual bees, as well as the whole colony. A 2010 study has

demonstrated the magnitude of pesticide contamination in bee hives by analyzing hundreds of pollen, wax, foundation, brood, and adult bee samples for approximately 120 pesticides.[23] Those convenient samples were collected from different studies and epidemiological surveys aiming to investigate possible threats of pesticides to colony health, specifically CCD. Unfortunately, none of the above study reported data showing the comparison of pesticide residues in hives exhibiting with and without CCD symptoms.

Regardless, Mullin et al.[23] have shown that hives treated with common miticides are often detected with much higher levels of residues of fluvalinate, coumaphos, or amitraz inside the hives. The finding of 98% of comb and foundation wax samples contained up to 204 and 94 ppm of fluvalinate and coumaphos, respectively, is very alarming comparing to the national average of up to 12ppb of coumaphos and fluvalinate in the survey of US honey samples. Accordingly, the persistent exposure to those miticides has led to the development of resistance to *Varroa* mites in bees. The huge concentration gap of fluvalinate and coumaphos between honey and comb/wax samples has three implications. First, it indicates the excessive use of both pesticides by beekeepers over the years, probably for battling the worsening *Varroa* mite infestation. Second, because of the resistant development to those pathogens in bees, the intention of applying more miticides to control or prevent pathogen infestation in hives is not only counter-effective but could lead to a more serious mite infestation problem in the future as well. Lastly, the high levels of fluvalinate and coumaphos residues found in the hives could no doubt put additional pressure on bees' health. Mullin et al.[23] stated that fluvalinate has long been considered a relatively "safe" pesticide for honeybees if applied at the concentration below 65.85 µg/bee. However, US EPA (1995) reported the LD_{50} of fluvalinate as 0.2 µg/bee, a 330-fold lower than the common application concentration.[24] Those findings highlight the extreme challenge for the survivals of bees because of the extensive exposure to various agrochemicals

and the worsening mite infestation problem. Chauzat et al.[25] also reported coumaphos and fluvalinate residues as the most commonly detected pesticide residues inside the hives with average concentrations of 925 and 487 ppb, respectively.

Pesticide residues measured in pollen samples might be a more realistic matrix for assessing pesticide exposure in bees during foraging activities. Also, data from pollen samples could help us to establish the field-realistic pesticide exposure levels encountered by bees. Besides high levels of fluvalinate and coumaphos, Mullin et al.[23] found approximately 100 pesticides in the stored pollen samples, including systemic pesticides, such as azoxystrobin (1-107 ppb), trifloxystrobin (1-264 ppb), propiconazole (3-361 ppb), thiacloprid (2-115 ppb), acetamiprid (14-134 ppb), and imidacloprid (6-206 ppb). Bernal et al.[26] reported more than 30% of stored pollen samples contained multiple pesticides with concentrations ranging from 1 ppb to 2,930 ppb. A comparable study published by Krupke et al.[27] also demonstrated that bees living and foraging near cornfields in Indiana are being exposed to pesticides in several ways throughout the foraging seasons. During spring, extremely high levels of clothianidin and thiamethoxam were found in planter exhaust material produced during the planting of neonicotinoids-treated maize seeds. When maize plants reached anthesis, maize pollen from treated seed was found to contain clothianidin and other pesticides that are readily available for bees to collect. They showed that 3 and 10 of 20 pollen samples collected directly from bees using pollen trap contained thiamethoxam and clothianidin, respectively. Fungicides were also frequently detected: azoxystrobin and propiconazole were found in all pollen samples while trifloxystrobin was found in 12 of the 20 pollen samples analyzed. Concentrations (µg/g) of thiamethoxam, clothianidin, trifloxystrobin, azoxystrobin and propiconazole in pollen collected from returning bees of hives placed adjacent to maize fields planted with treated seeds ranged from non-detected to 7.4, non-detected to 88, non-detected to 9.8, 4.3 to 66, and 3.2 to 23.8,

respectively.

The concern of pesticide contamination is not limited to pollen or nectar that bees have access to. During foraging, bees often look for water on the ground puddles, or leaf guttation drops, an accessible and alternative source of water for bees. Girolami et al.[28] showed that by growing corns from seeds coated with 4 different neonicotinoids at the rage of 0.5-1.25 mg/seed, leaf guttation drops germinated from those seeds could contain neonicotinoids at the ppm levels, with maximum concentrations of up to 100 ppm for thiamethoxam and clothianidin, and up to 200 ppm for imidacloprid. Those levels were approximately 5-6 orders of magnitude higher than those found in pollen or nectar, and therefore posed extreme acute toxicity to bees in which dead bees were found after minutes of consuming those guttation drops. By taking into account the persistence of those dangerously high levels of neonicotinoids and the wide planting of neonicotinoids-coated corn seeds, Girolami et al. concluded that this is a threatening scenario for bees and other pollinators, and does not comply with an ecologically acceptable situation.

Since it is conceivably difficult to compare pesticide levels in samples collected from bees and their hives across studies because many factors would affect the final concentrations in those samples, the attempt to quantitatively assess the “field-realistic” pesticide exposures in bees is a foreseeable challenging task. If the field-realistic levels for a certain pesticide that bees would encounter in the environment were existed, it is likely to encompass a very wide range of concentrations. The data presented in the above studies would support this conclusion. While the interest of this work is to identify the risk of the declining bee population associated with pesticide exposures, regardless of the levels, this review will focus on sub-lethal exposure to neonicotinoids that are commonly present in bees’ foraging environment and relevant to the

causation of CCD.

THE ASSOCIATION OF PESTICIDE EXPSOURE AND ADVERSE HEALTH OUTCOMES IN HONEYBEES (*Apis mellifera*)

The majority of literature demonstrating adverse health effects of sub-lethal pesticides to honeybee did not exist until 2011, several years after the reported emergence of CCD, and no studies linking sub-lethal pesticide exposure to adverse chronic health effects in honeybees were published in peer-review scientific journal until 2009. This might signal the omission of the roles of pesticides at the sub-lethal levels in the deteriorating of honeybees' health in the research and regulatory communities. An earlier study has shown that spinosad, an insecticide derived from the bacterial species *Saccharopolyspora spinosa* has low risk to adult honeybees but has little or no effect on brood development. Spinosad residues that have been allowed to dry in all experimental conditions for 3 hr were not acutely harmful to honeybees when low-volume and ultralow-volume sprays are used.[29]

Brood development, adult bee longevity, and metabolic responses

Wu et al.[30] have shown that worker bees reared in brood comb containing high levels of many pesticides experienced multiple health effects including reduced adult longevity, increased brood mortality, delayed larval development, and higher fecundity of *Varroa* mites. Delayed development was observed in the early stages (day 4 and 8) of worker bee that leads to reduced adult longevity by 4 days in bees exposed to pesticides during development. As observed by the authors that pesticide residue migrated from comb containing high pesticide residues to the control combs after multiple brood cycles causing higher brood mortality and delayed adult emergence in bees reared in those control comb. Subsequently, survivability increased in bees reared in treatment comb after multiple brood cycles when pesticide residues

had been reduced in treatment combs due to the migration into untreated control combs. Medrzycki et al.[31] demonstrated a relationship between the quality of the brood rearing environment and the reduction in both longevity and susceptibility to insecticides in adult honeybees emerging from their larvae. They reported that by lowering the brood rearing temperature 2°C from the optimal 35°C, it strongly affected adult honeybees' mortality and their susceptibility to dimethoate, an organophosphate insecticide. Since it is well known that the physiology of adult honeybees can be affected by the health of their larvae and/or pupae, it implies that less than optimal brood rearing environment, such as temperature inside the hive and exposure to pesticide at the sub-lethal levels, could deteriorate the health of adult bees starting in the larval stage.

In addition to the property as an insecticide, neonicotinoids can also act as an environmental stressor, which can influence the metabolic and developmental buffering systems of organisms causing abnormities or death during development. Derecka et al.[32] analyzed molecular profiles of worker-honeybee larvae collected from hives that were given access to syrup tainted with sub-lethal level (2 µg/L) of imidacloprid in the field over 15 days. They found significant enrichment of genes functioning in lipid-carbohydrate-mitochondrial metabolic networks, suggesting diminished buffering and stability of the developmental program and likely causing an increased rate of developmental failure.

Foraging Difficulty

Decourtye et al.[33] had demonstrated that by feeding honeybee workers with sugar solution with 24 µg/kg of imidacloprid can decrease their foraging activity and also have negative effects of olfactory learnt discrimination task. Yang et al.[34] also investigated the foraging behavioral changes in honeybee workers with sub-lethal dosages of imidacloprid by

measuring the time interval between two visits at the same feeding site. The normal foraging interval of honeybee workers was within 300 seconds after training to fly to an artificial feeder 35 meters away from the hives. However, they found those honeybee workers delayed their return visit for more than 300 seconds when they were treated orally with sugar water containing imidacloprid, and the delayed percentage is imidacloprid concentration dependant. When bees were treated with imidacloprid higher than 1,200 μg/L, they all showed abnormalities in revisiting the feeding site, and the lowest effective concentration of imidacloprid is 50 μg/L.

Honeybee waggle dancing is an important cognitive behavior during foraging activities. The abnormal and decreased waggle dancing would negatively affect colony food source and reduce store honey weight gain in situations where recruitment is important, and will reduce colony fitness over the long term. Eiri et al.[35] tested the effect of sub-lethal doses of imidacloprid on bee sucrose responsiveness (SR) using the proboscis extension response assay. They found bees ingested sucrose solution contained imidacloprid (0.21 or 2.16 ng/bee) had higher SR thresholds 1 hour after treatment. Compared with controls, bees ingested imidacloprid (0.21 ng/bee) also produced significantly fewer waggle dancing circuits (10.5 and 4.5-fold fewer for 50% and 30% sucrose solutions, respectively) 24 hours post treatment.

In order to clarify the effects of sub-lethal exposure to pesticides on honeybee behaviors, Teeters et al.[36] used an automated video-tracking system (EthoVisionXT) to examine the distance that honeybees traveled in a 24-h period, the amount of time spent near a food source, and the amount of time a pair of worker bees spent interacting under sub-lethal dosage treatment. Worker bees were either treated with 0.3, 1.5, or 3 μg of tau-fluvalinate, or administered orally of sucrose agar containing 0.05, 0.5, 5.0, 50, or 500 ppb of imidacloprid. For distance traveled, bees treated with tau-fluvalinate moved significantly less than control bees at

all dose levels, as did 50 and 500 ppb of imidacloprid. Bees exposed to 50 and 500 ppb imidacloprid, but not tau-fluvalinate, spent significantly less time near the food source than control bees. The mean “interaction” times for bees also had a significant influence by both pesticides in which with the increase of dosage, the time of interaction decreased. In this study, a significant reduction in locomotor activity was also observed after exposure to both pesticides, suggesting an obvious behavioral effect.

Henry et al.[37] tested the hypothesis that sub-lethal exposure to thiamethoxam indirectly increases hive mortality rate because of homing failure in foraging honeybees. They simulated daily intoxication events that bees would have received by a field-realistic, sub-lethal dose of 0.07ppb of thiamethoxam (or 1.34 ng in a 20-ml sucrose solution). Bees were then released away from their hive with a microchip glued on their thorax so they can be monitoring by a radiofrequency identification (RFID) readers placed at the hive entrance. Mortality due to post-exposure homing failure was then derived from the proportion of non-returning foragers and corrected by data from non-treated bees for other causes of homing failure in treated foragers—such as natural mortality, predation, or handling stress. The results demonstrated substantial mortality due to post-exposure homing failure with the proportion of treated bees returning to the colony being significantly lower than that of control foragers ($p<0.05$). It is estimated that 10 to 32% of thiamethoxam treated bees would have failed to return to their colonies when foraging in treated crops on a daily basis. Schneider et al.[38] used the similar RFID technique to monitor the foraging behavior of honeybees after the treatment of sub-lethal doses of imidacloprid (0.15–6 ng/bee) and clothianidin (0.05–2 ng/bee) under field-like conditions. They found both imidacloprid and clothianidin could lead to a significant reduction of foraging activity and to longer foraging flights at doses of >0.5 ng/bee (0.02ppb assuming each bee weight 30mg) for clothianidin and >1.5 ng/bee (0.06ppb) for imidacloprid during the first three hours after

treatment. In the trials conducted with imidacloprid at 3ng and clothianidin at 2ng, only 25% and 21% of bees returned to the hives during a 3-hour observation period immediately after treatment, respectively. Conversely, almost all bees in the control groups and groups treated with lower doses returned. Among the bees that were not returned, they observed reduced mobility, followed by a phase of motionlessness with occasional trembling and cleaning movements, moving around with an awkwardly arched abdomen, or sometimes followed by a phase of turning upside down and lying on the back with paddling leg movements.

Tan et al.[39] demonstration that sub-lethal concentrations of imidacloprid can harm honeybee (*Apis cerana*) decision-making by significantly increasing the probability of a bee visiting a dangerous food source. They demonstrated that foraging on nectar containing 40 μg/L (34 ppb) of imidacloprid showed no aversion to a feeder with a hornet predator with 1.8 folds more bees chosen the dangerous feeder as compared to control bees, and 23% fewer foragers returned to collect the nectar. Bees that did return have collected 46% and 63% less nectar containing 20 μg/L and 40 μg/L of imidacloprid, respectively. Sandrock et al.[40] investigated the effects of sub-lethal dietary neonicotinoids exposure on honeybee colony. They used sub-lethal chronic exposure of thiamethoxam (5.0 ppb) and clothianidin (2.0 ppb) through feeding contaminated pollen and found significant decrease of colony performance and productivity, decelerated colony growth in the long-term (1 year) associated with higher queen supersedure rates, and a reduced tendency to swarm. Williamson et al.[41] illustrated that after 24 hr of exposure to sub-lethal doses of neonicotinoids ranging from 0.45 to 0.54 ng/bee of four neonicotinoids (imidacloprid, thiamethoxam, clothianidin, dinotefuran) foraging bees have experienced a subtle influence on their behavior, such as losing postural control during the motor function assay, failing to right themselves, or spending more time grooming. In a catch-and-release experiment, Fischer et al.[42] aimed to test the effects of neonicotinoids on honeybee

navigation. They found application at sub-lethal doses, 2.5 ng/bee (equivalent to 25 ppb) of clothianidin, 7.5 ng/bee (equivalent to 75 ppb), and 11.25 ng/bee (equivalent to 112.5 ppb) of imidacloprid, and 1.25 mg/bee (equivalent to 12.5 ppm) of thiacloprid, interfered with navigation of honeybees. Thiacloprid treatment slowed the flight speed of bees while the other neonicotinoids did not affect flight speed. Sub-lethal doses of the three neonicotinoids tested either block the retrieval of a remote memory or alter this form of navigation memory.

Results from above studies using similar technologies consistently demonstrated the abnormal foraging activities, or homing difficulties, in bees exposed to sub-lethal levels of thiamethosam, imidacloprid, or clothianidin, the 3 most commonly used neonicotinoids in the world. Since we can assume with a great confidence that bees that do not return to their hives within the three-hour period after leaving would not be able to survive, and are most likely died in the field, the sub-lethal effects of neonicotinoids in individual bees will subsequently lead to mortality and eventually the survival of the colonies.

Cognition/Neurological Impairment

In addition to affecting honeybee's foraging activities, sub-lethal exposure to neonicotinoids has also been shown to disrupt honeybees' behavior and learning abilities, which will subsequently impair their foraging and homing abilities. Decourtye et al.[43] demonstrated that imidacloprid at a sub-lethal dose (12 ng per animal) decreased the acquisition and the retention performances tested in the conditioned proboscis extension reflex (PER) paradigm. El Hassani et al.[44] observed a significant reduction of sucrose sensitivity in honeybee workers at the dose of 1 ng/bee of fipronil treated 1 hour after a thoracic application. They also indicated that fipronil at a sub-lethal dose of 0.5 ng/bee by topical application could impair the acquisition and retention performances of PER paradigm most likely due to the impairment of olfactory learning.

El Hassani et al.[45] postulated that acetamiprid at sub-lethal levels can affect gustatory, motor, and mnemonic functions in the honeybee. They showed that after oral ingestion of sucrose solution with acetamiprid at dose of 1 μg/bee increased bees' sensitivity to antennal stimulation, and impaired long-term retention of olfactory learning at doses below 0.1 μg/bee. Thoracic application of 0.1 and 0.5 μg/bee of acetamiprid induced no effect in behavioral assays but increased locomotive activity. The water-induced proboscis extension reflex also increased at 0.1, 0.5, and 1 μg/bee of acetamiprid.

Palmer et al.[46] have shown that using recordings from mushroom body Kenyon cells in acutely isolated honeybee brain, imidacloprid (50nM–10μM), clothianidin (200 nM), and the oxon metabolite of miticide coumaphos (50nM–1μM), can cause a depolarization-block of neuronal firing, and subsequently inhibit nicotinic responses. These effects are observed at the concentrations (50nM-10μM) that are encountered by honeybees in the foraging environment and within their hive, and are additive with repeated applications. Those new findings provided a neuronal mechanism that may account for the cognitive impairments caused by neonicotinoids and OP-based miticides commonly used in hives. It also demonstrated the cumulative effects on targeted cholinergic inhibition caused by multiple pesticides that bees are simultaneously exposed to, and therefore caused synergistic toxicity to bees. Similar finding and conclusion of exposure to field-realistic concentrations (10 and 100nM) of imidacloprid or/and coumaphos impaired olfactory learning and memory formation in honeybees have been made by Williamson and Wright[47]. In the experiment, they combined imidacloprid with coumaphos to simulate the situation where honeybees are exposed to pesticides in food and to miticides applied within the colony. They found that neither imidacloprid nor coumaphos has specific cholinergic effects on learning or memory. Bees exposed to imidacloprid were less likely to form a long-term memory, whereas bees exposed to coumaphos were only less likely to respond during the short-term

memory test. When bees exposed to these two pesticides simultaneously, the additive responses were observed. Williamson and Wright concluded that simultaneous exposure to sub-lethal doses of cholinergic imidacloprid and coumaphos significantly impairs foraging capabilities, implying that pollinator population decline could be the result of a failure of neural function of bees exposed to multiple pesticides in agricultural landscapes.

In a separate study, Williamson et al.[48] attempted to assess the acute effects of sub-lethal doses of imidacloprid (1.28 ng/bee) and coumaphos (1.18 ng/bee) on honeybees' learning and memory, but failed to reach any significant conclusions except for reporting a modest improvement in learning and memory when both pesticides were administered simultaneously. While the objective of assessing acute effects of sub-lethal doses is unknown, the data presented in Williamson et al.[48] were inconsistent to the majority of papers published. Boily et al.[49] first reported an increased AChE activity for both in-field and laboratory data. Sub-lethal doses of neonicotinoid insecticides (0.08, 0.16, 0.24, 0.30 ng/bee of imidacloprid, and 0.12, 0.24 ng/bee of clothianidin) increased AChE activity was found in caged bees, and after 2 weeks exposure in field experiments as well. The results suggest that the NOEL (no observable effects level) for imidacloprid alone is less than 0.08 ng per bee. They also found chronic exposure to imidacloprid significantly decreased survival at doses between 0.24 and 0.30 ng/bee. Although the increased AChE activities reported by Boily et al. are not consistent with the known mode of action of neonicotinoids, which is agonist acetylcholine and bind to the post-synaptic nicotinic acetylcholine receptors (nAChERs), Boily et al. presumed that because neonicotinoids occupy the binding-site of nAChERs, they tend to accumulate in the synapses and to stimulate the action of AChE, in a typical substrate enzyme cellular response.

Immune Suppression

It has been postulated that the increasing prevalence of *N. ceranae* in honeybee colonies combined with the ubiquitous presence of multiple pesticides in pollen and nectar that worker bees collected from their foraging environment contributes to the declining of honeybee colonies. Alaux et al.[8] demonstrated the interaction between imidacloprid (at the doses ranging from 0.7 to 70ppb) and the increase susceptibility of colony to microsporidia Nosema. By quantifying the strength of immunity at both the individual and social levels, they demonstrated that the activity of glucose oxidase, enabling bees to sterilize colony and brood food, was significantly decreased only by the combination of both factors compared with control, Nosema or imidacloprid-treated group. Vidau et al.[12] reported a synergistic effect of *Nosema ceranae* infection and sub-lethal insecticide exposure on honeybee mortality in a laboratory incubator setting. Honeybees were experimentally infected with spores of *N. ceranae* in the lab and then exposed to fipronil at 1ppb, thiacloprid at 5.1ppm, or untreated. They found exposures to fipronil and thiacloprid had no effect on the mortality of uninfected honeybees compared to the untreated control group over the duration of experiments. However, honeybees infected with *N. ceranae* prior to thiacloprid exposure died significantly earlier than bees only infected with *N. ceranae*. Wu et al.[50] also demonstrated higher proportion of bees reared from the high pesticide residue brood comb became infected with *N. ceranae*, and died at a younger age, compared to those reared in low residue brood combs. Although both Vidau et al.[12] and Wu et al.[50] suggested that developmental exposure to pesticides in brood comb could increase the susceptibility of bees to *N. ceranae* infection, it is unclear how *N. ceranae* infection would have played a role in the early death of bees exposed to pesticides since the differences of mortality outcomes are clearly determined by the levels of pesticide exposure.

The interaction of Nosema infection and sub-lethal neonicotinoids exposure on honeybee was further demonstrated by Pettis et al.[51] They exposed honeybee colonies during

three brood generations to imidacloprid at 5 and 20ppb mixed in the protein patties, and then subsequently challenged newly emerged bees with the gut parasite, *Nosema* spp. They found Nosema infections increased significantly in the bees from imidacloprid-treated hives when compared to bees from control hives demonstrating an indirect effect of neonicotinoids on pathogen growth in honeybees. The results reported by Pettis et al. (2012) suggested that other than the known nAChR inhibition, sub-lethal neonicotinoids exposure could promote Nosema infection in bees, a new findings of adverse health outcomes to bees caused by neonicotinoids,. In addition, Pettis et al.[52] found that fungicide exposure could also increase Nosema infection in bees consumed fungicide-contaminated pollen. However, this finding is not consistent to the prior knowledge among beekeepers and bee researchers that fungicides are typically seen as fairly safe for honeybees. Pettis et al.[52] used pollen traps to collect pollen pellets from foraging bee's corbiculae before entering their hives and detected 35 different pesticides in those pollen samples. Azoxystrobin, a systemic fungicide, is the most commonly detected fungicide with mean and the maximum concentrations of 60 and 332ppb, respectively. Esfenvalerate (216ppb) and phosmet (14,700ppb), both OP pesticides, were at the concentrations higher than their median lethal dose to bees in at least one pollen sample. Those pollen data are useful as the supplement to those reported by Mullin et al.[23] and Krupke et al.[27] . While the high levels of pesticide contamination and the numbers of pesticides found in pollen that bees collected is worrying, it should be cautious when interprets the results from Pettis et al.[52] because pesticide contamination in pollen collected by bees depends upon what pesticides have been applied in locations where bees are foraging.

Besides promoting Nosema infection, sub-lethal exposures to clothianidin or imidacloprid have also been shown to interact with other virus on honeybees resulting in negatively modulates nuclear factor-κB (NF-κB) immune signaling and therefore adversely affects

honeybee antiviral defenses. By enhancing the transcription of the gene encodingNF- κB, Di Prisco et al.[53] demonstrated that neonicotinoids at sub-lethal levels could reduce immune defenses and promote the replication of the deformed wing virus (DWV) in honeybees bearing covert infection. Doublet et al.[54] also demonstrated an additive interaction with black queen cell virus (BQCV) leading to increased larval mortality after administering sub-lethal thiacloprid at 0.1 mg/kg, or a total of 17 ng of thiacloprid per honeybee larva over 5 days of feeding. Similar trend of increased mortality in adult honeybees was observed by Doublet et al. due to the synergistic interactions between *N. ceranae*, BQCV, and thiacloprid.

Synergic Effects of Neonicotinoids with Fungicides

It was not until 2013 when reports showing other pesticides could also play a potential synergistic role with neonicotinoids to compromise honeybees' health when exposure occurred simultaneously. Biddinger et al.[55] demonstrated a synergism of neonicotinoids and fungicides to honeybee (*A. mellifera*) and Japanese orchard bee (*Osmia cornifrons*) when using in mixtures as they are commonly applied in apple orchards. The interaction of 1:1 mixture of fungicide fenbuconazole and acetamiprid was 5 and 2 times more toxic to *A. mellifera* and *O. cornifrons*, respectively, than acetamiprid alone. Thompson et al.[56] reported similar findings in which they exposed honeybees concurrently with ergosterol biosynthesis inhibitor (EBI) fungicides (with 0.161 µg/bee of myclobutanil, 0.224 µg/bee of propiconazole, 0.358 µg/bee of flusilazole, and 0.447 µg/bee of tebuconazole) and several neonicotinoids. They found that the scale of synergism of increase in toxicity of neonicotinoids was fungicide dose dependent. With increasing the dose of at the maximum contact doses of propiconazole (22.4 µg/bee), the sensitivity to an oral dose of thiamethoxam increased over 8.3-fold and sensitivity to a contact dose of thiamethoxam increased by 3.6-fold.

Colony Collapse Disorder (CCD)

Although numerous papers that are previous discussed in this review have claimed the link of sub-lethal neonicotinoids exposures to CCD, along with the synergistic effects with Nosema infection or other pesticides, none of the study was able to demonstrate (or replicate) the hallmark post-mortem observations of CCD that are the sudden disappearance of worker bees from hives containing adequate store honey in winter. Hives suffered from CCD are empty without dead bees in and around the hives. Lu et al.[17] was the first study to replicate CCD in an *in situ* study in which colonies set up in natural environment were treated with sub-lethal doses of imidacloprid and monitored over multiple brood generations, including winter bee generation. They used a replicated split-plot design consisting of 4 independent apiary sites, and each apiary consisted of 4 different imidacloprid-treated hives and a control hive. The dosages used in this study (20, 40, 200, and 400 µg/kg of imidacloprid in high fructose corn syrup, HFCS) were administered to the whole colony each week for 13 consecutive weeks. Both control and imidacloprid-treated hives were healthy, and had no symptoms of diseases of any kind during the 13-week dosing regime, and were stayed alive 10 weeks afterward. Fifteen of 16 imidacloprid-treated hives (94%) were found dead 23 weeks post-imidacloprid dosing. Dead hives were remarkably empty except for stores of food and some pollen left, a resemblance to CCD. The survival of control hives managed alongside with those imidacloprid-treated hives unequivocally augments the conclusion of which sub-lethal imidacloprid exposure via HFCS intake led to CCD after several brood generations. Lu et al.[18] continued to show that sub-lethal exposure of imidacloprid or clothianidin at a dose of 0.74 ng/bee/day for 13 consecutive weeks impairs bees' ability to over winter that subsequently leads to CCD. They found both control and neonicotinoid-treated hives progressed almost identically during the experimental period, and observed no acute morbidity or mortality in either group until the arrival of winter. As ambient

temperatures began to fall, a steady decrease of bee cluster size in both control and neonicotinoid-treated colonies was observed. While such decline was quickly reversed in control colonies when ambient temperature began to rise, the cluster size for both imidacloprid and clothianidin treated hives continued to decline. The diminishing cluster size in the neonicotinoid-treated colonies finally led to the losses of 6 of 12 hives (50%) with symptoms resembling CCD. By extrapolating the toxicity scaling for honeybees to the lifespan of winter bees, Rondeau et al.[57] suggested that imidacloprid in honey at 0.25 μg/kg would be lethal to a large proportion of bees nearing the end of their life. Even with healthy bees, exposure to modest residues of imidacloprid in pollen (ranging 0.5–30 ppb) and honey (ranging 0.7–13 ppb) could easily cause problems for summer bees and especially for longer-lived bees going through the winter. The findings reported in Rondeau et al.[57] supported the conclusions made by Lu et al.[17,18] , and indirectly provided an answer of why CCD often happened in winter season.

Regardless, several recent reports have discredited the causal relationship of neonicotinoids to honeybee CCD. Pilling et al.[58] reported a four-year (2005-2009) field program aiming to investigate the long-term effects of repeated exposure of honeybee colonies to flowering crops treated with neonicotinoids in France. By monitoring the colonies throughout the four-year period, they demonstrated the mortality, foraging behavior, colony strength, colony weight, brood development and food storage levels were similar between treatment and control colonies, and colonies exposed to the treated crop were able to successfully overwinter and had a similar health status to the control colonies in the following spring. They concluded that there is a low risk to honeybees from systemic residues in nectar and pollen following the use of thiamethoxam as a seed treatment on oilseed rape and maize. However, the methodological aspects of the study, as well as the conclusions made by Pilling et al. have being questioned by many readers that triggered a further review by the PLOS ONE editorial board

(http://www.plosone.org/annotation/listThread.action?root=82356). The concerns included small field size for 6 colonies suggesting bees might feed themselves elsewhere, lack of description of surrounding area, relatively short time of exposure, the separation of treated and untreated crops was much shorter than the distance of foraging, and no statistical analysis involved in the data analysis. While PLOS ONE later concluded that readers' concern of methodologies and conclusions are legitimate, they also believed that the contributions from Pilling et al. could stand without the needs of additional independent peer-review to evaluate the questions and concerns raised by the readers.

Another report from Cepero et al.[59] also discredited the role of neonicotinoids in honeybee CCD by screening collapsed colonies from three professional apiaries in Spain for the presence of neonicotinoids in store pollen. They reported negative results for neonicotinoids in pollen samples but made no mention of other pesticides that have reported to be commonly present inside hives. Although Cepero et al. concluded that drivers of colony collapse may differ between geographic regions with different environmental conditions, or with different beekeeping and agricultural practices, this conclusion was not supported by the data presented in this paper, and is mostly likely Cepero et al.'s opinions. More importantly, those general factors have long been co-existed with beekeeping practices and pose no biological plausibility to honeybee CCD.

Culter et al.[60] reported a significant number of honeybee incidents in Ontario Canada, where exposure to neonicotinoids dust during planting of corn was suspected to have caused 67 cases of a total of 110 honeybee incidents. They explained most of these incidents (61 cases) were classified as 'minor' (death or abnormal behavior is observed in ≤10% of bees in any one colony) by the Canadian Pest Management Regulatory Agency, and only 6 cases were

considered ‘moderate’ (1000–3000 bees from each of five or more colonies, or 10–30% of bees in any one colony die or display abnormal behavioral effects) or ‘major’ (at least 3000 bees from each of five or more colonies, or 30% of the bees in any one colony die or exhibit abnormal behavioral effects). Culter et al. showed that in the same year, there were over three times as many moderate or major incidents (20 cases) caused by non-neonicotinoid pesticides including carbofuran, chlorpyrifos, coumaphos, diazinon, dimethoate, fluvalinate, formic acid, permethrin, and phosmet, involving numbers of hives or bees far greater than the number of moderate or major incidents suspected to be caused by neonicotinoid poisoning. They concluded that, while exposure of honeybees to neonicotinoid-contaminated dust during corn planting needs to be mitigated, other pesticides also pose a risk, if not a higher risk. They argued that by de-registering neonicotinoids for crop protection would force growers to revert to increased use of older broad-spectrum chemistries that neonicotinoids have largely replaced, with increased risks to pollinators. The viewpoints of Culter et al. on how neonicotinoids could harm pollinators’ health appeared to be dramatically different to the Ontario government in which a proposal was announced in November 2014 to reduce the use of neonicotinoids by 80% in order to reverse the declining trend of honeybee colonies by 2017 (http://www.omafra.gov.on.ca/english/pollinator/discuss-paper.pdf).

THE ASSOCIATION OF PESTICIDE EXPSOURE AND ADVERSE HEALTH OUTCOMES IN BUMBLEBEES (*Bombus spp.*)

Acute Toxicity/Direct Contact

Because the widespread use of pesticides in agricultural fields, Scott-Dupree et al.[61] conducted a laboratory-based toxicological study to determine the acute contact toxicity of 5 common insecticides, imidacloprid, clothianidin, deltamethrin, spinosad, and novaluron on

bumblebees [*Bombus impatiens* (Cresson)], alfalfa leafcutting bees [*Megachile rotundata* (F.)], and *Osmia lignaria* Cresson. They found clothianidin and imidacloprid are highly toxic to all three species, followed by deltamethrin and spinosad, Novaluron was found non-toxic to those 3 bees. Although they found bumblebees were generally more tolerant to pesticide toxicity by direct contact, this result was not consistent. To establish whether imidacloprid, a systemic neonicotinoid and insect neurotoxin, harms individual bees when ingested at environmentally realistic levels, Cresswell et al.[62] exposed adult worker bumblebees to dietary imidacloprid in feeder syrup at dosages between 0.08 and 125ppb. They found bumblebees progressively developed over time a dose-dependent reduction in feeding rate with declines of 10-30% 10ppb, and reduced mean daily locomotory activity on dosed syrup 125 µg/L [63]. Data from Scholer and Krischik [64] indicated that feeding sugar syrup treated with imidacloprid or clothianidin at 20 ppb (actual residue imidacloprid 16 ppb; clothianidin 17 ppb) have significant reduction in queen survival (37% in imidacloprid and 56% in clothianidin) worker movement, colony consumption, and colony weight compared to no neonicotinoids treatments. Feeding on imidacloprid or clothianidin can cause changes in behavior (reduced worker movement, consumption, wax pot production, and nectar storage) that result in detrimental effects on colonies (queen survival and colony weight).

Colony Vitality/Brood Development

Gels et al.[65] reported the effects of imidacloprid, chlorpyrifos, carbaryl, and cyfluthrin on native pollinators, specifically bumblebees after the applications on turf where they forage on the weed flowering. This is the earliest study aiming to assess the toxicity of various types of pesticides in pollinators. They measured colony vitality including numbers of brood, workers, and weights of queens, workers, and whole colonies after a period of 14-30 days post-

application. They found non-irrigated, or dry residues for all the test pesticides were detrimental to colony vitality for bumblebees, however toxicity was abated when the field is irrigated followed by pesticide application. Regardless the methods of application, Gels et al. found that foraging workers did not avoid pesticide-treated field. Mayes et al.[29] and Morandin et al.[66] reported similar adverse health effects in bumblebees resulting from spinosad insecticide exposure ranging from 0.2 to 0.8 mg/kg. Those adverse effects included adult mortality, brood development, weights of emerging bees, and foraging efficiency of adult bees. In addition, they found adult worker bees exposed to spinosad during larval development at 0.8 mg/kg were slower foragers than bees from low or no spinosad treated colonies.

Whitehorn et al.[67] conducted a study to simulate the likely effects in wild bumblebee colony to imidacloprid present on the flowers of imidacloprid-treated rapeseed. Colonies received either control, low (0.7-6 µg/kg), or high (1.4-12 µg/kg) for 14 days before they were placed in the field, where they were left to forage freely for a period of 6 weeks. They found bumblebees in imidacloprid-treated colonies gained significantly less weights and produced less numbers of queens than those in the control colonies. Laycock and Cresswell [68], however, provided a somewhat conflict results of imidacloprid's effects on brood development in bumblebees. They assessed the amount of brood (number of eggs and larvae) using a pulsed exposure regime in which bees received imidacloprid doses up to 98 µg/kg in 14 days of "on dose" followed by 14 days of "off dose" in small experimental colonies consisting a queen and four adult workers. They found a dose-dependent repression of brood production with productivity decrease during the "on-dose" period, followed by a dose-dependent recuperation during the "off-dose" period. In continuing of this work, Laycock et al.[69] examined the effects of thiamethoxam on bumblebees to a range of dosages up to 98 µg/kg in syrup for 17 days. They showed that bumblebee workers survival was shortened by fewer days and the production of

brood (eggs and larvae) and consumption of syrup and pollen in microcolonies were significantly reduced by thiamethoxam at the two highest concentrations, 39 and 98 µg/kg, whereas no detectable effects of thiamethoxam were found at levels between 1 and 11 µg/kg. By comparison to previously published data, they concluded that brood production in worker bumblebees is more sensitive to imidacloprid than thiamethoxam. Bryden et al.[70] shown that bumblebee colonies failed when exposed to sub-lethal levels of pesticide due to decrease in colony functions. Throughout the 42-day study period on exposing to sustained sub-lethal level of 10 ppb of imidacloprid in sucrose solution in bumblebee nest, while all colonies grew at a similar rate during the first 3 weeks, only control colonies continued to grow throughout the whole study. Colonies treated with imidacloprid began to shrink with decreased birth rates and increased mortality rates. On the 33rd days, the average colony size of the imidacloprid-treated colonies was significantly smaller than control colonies ($p < 0.05$), and this trend continued beyond the 33rd days.

Lawn treated with neonicotinoids could also post a threat to the survival of pollinators. Larson et al.[71] applied at the labeling rates of 0.45 and 0.23 kg a.c./ha for clothianidin and chlorantraniliprole (a non-neonicotinoid insecticide), respectively, on turf with about 30% cover of flowering white clover (*Trifolium repens* L.). Colonies of bumblebee (*B. impatiens*) were introduced two days after neonicotinoids application and last for 6 days, and then moved to another farm without any pesticide exposure for 6 more weeks. They found colonies exposed to clothianidin-treated weedy turf showed reduced foraging activity, increased worker mortality, delayed weight gain, and produced no new queens, but not those treated with chlorantraniliprole. They also reported nectar from clover blooms contained 171ppb of clothianidin. This study showed that bumblebees foraging on flowering clover on the recently clothianidin-treated lawns for less than a week could have the potential to impair queen production in bumblebee colony.

Smagghe et al.[72] demonstrated an exposure-route dependent toxicity of chlorantraniliprole in bumblebee workers and their offspring. They showed that while a risk assessment test demonstrated that direct contact exposure at 0.4ppm level had no effect on bumblebee worker survival, oral exposure *via* sugar water caused both acute and chronic toxicity. The most significant sub-lethal effect was on reproduction in colonies orally exposed to pollen treated with chlorantraniliprole. Finally, Cutler and Scott-Dupree [73] examined the effects of exposure to neonicotinoid seed-treated corn on commercial bumblebee colonies with the clothianidin detected at 0.1–0.8 ng/g. They concluded that bumblebee hives appeared to be healthy and had no effect on any hive endpoints measured (storage ability, brood development, and body weight), except for the decreasing number of workers. Although Cutler and Scott-Dupree [73]suggested that exposure during pollen shed to corn grown from neonicotinoid-treated shed poses low risk to bumblebee, it should be noted that those observations were collected at the cross-sectional manner in summer. Considering bumblebees were exposing to sub-lethal levels of clothianidin in pollen collected from neonicotinoid-treated corn, it is unlikely that bumblebees would have developed any adverse health endpoints, such as brood development and body weight, within such a short period of time.

Foraging Impairment

Gill et al.[74] showed that chronic exposure of bumblebees to neonicotinoids at levels close to field-level exposure could impair natural foraging behavior and leading to significant reductions in brood development and colony success. They have demonstrated that sub-lethal exposure to imidacloprid at 10ppb level causes impairment to pollen foraging efficiency, leading to increased colony demand for food as shown by increased worker recruitment to forage. Consequently, it appeared to affect brood development due to a higher number of workers

undertaking foraging, and subsequently resulted in reduced worker production, which can only exacerbate the problem of having an impaired colony workforce. These findings showed a mechanistic explanation linking effects on individual worker behavior to colony queen production, as a result of neonicotinoid exposure. Moreover, exposure to a second pesticide λ-cyhalothrin (a pyrethroid insecticide) applied at label guideline for crop use caused additional worker mortality in this study, highlighting a synergistic risk with different pesticides. In this study, colonies exposed to combined imidacloprid and λ-cyhalothrin were consistently affected in all measures of worker behavior and suffered the highest overall worker bee losses. Gill and Raine [75] used the RFID technology to identify effects of imidacloprid on overall foraging activity. They found that bees exposed to 10ppb of imidacloprid have suffered chronic behavioral impairment. Foragers from control colonies improved their pollen foraging performance as they are gaining experience, but bumblebees exposed to imidacloprid have become worse with higher frequency and longer foraging flights. Their analysis also showed a decrease in pollen collection efficiency of imidacloprid-exposed foragers in which they made more than 5 times more unsuccessful pollen foraging bouts than control foragers. They concluded that this could be due to the fact that treated individual foragers were carrying out fewer foraging bouts, and subsequently colonies responded by recruiting more foragers to make up for this shortfall in food intake rate. Feltham et al.[76] reported a consistent finding as of Gill et al.[74] and Gill and Raine [75]on the impairment of pollen collection efficiency as a result of imidacloprid exposure in bumblebees. They also used the RFID technology to determine whether bumblebee workers' foraging efficiency could be reduced by exposure to imidacloprid at the field-realistic levels (0.7 ppb in sugar water and 6 ppb in pollen). They found imidacloprid-treated bees brought back pollen less often than control bees did (40% vs. 63 % of trips, respectively), and when pollen was collected, treated bees brought back 31% less pollen per hour than controls did. However,

the nectar foraging efficiency of bees treated with imidacloprid was not significantly different than that of control bees. The consistent findings reported by Gill et al.[74], Gill and Raine [75], and Feltham et al.[76] provided an unequivocal evidence of foraging impairment caused by sub-lethal levels of imidacloprid in bumblebees. The synergistic effects caused by neonicotinoids and other pesticides are not only common for bees foraging in the environment, but will increase the propensity of colonies to fail as well.

THE ASSOCIATION OF PESTICIDE EXPSOURE AND ADVERSE HEALTH OUTCOMES IN OTHER BEES (*Hymenoptera: Apidae: Meliponinae, Osmia lignaria*)

Abbott et al.[77] examined the lethal and sub-lethal effects of imidacloprid and clothianidin on *Osmia lignaria* (Cresson) and *Megachile rotundata* (Hymenoptera: Megachilidae), respectively, by exposing their larvae to control, low (3 or 6 ppb), intermediate (30 ppb), or high (300 ppb) doses in pollen. They found no lethal effects for imidacloprid or clothianidin on *O. lignaria* and *M. rotundata,* and minor sub-lethal effects on larval development for *O. lignaria*, with longer developmental time at the intermediate (30 ppb) and high doses (300 ppb) of imidacloprid. Tomé et al.[78] studied native stingless bees (Hymenoptera: Apidae Meliponinae), which are key pollinators in neotropical areas but threatened with extinction due to deforestation and pesticide uses. They assessed the effects of imidacloprid ingestion by stingless bee larvae on their survival, development, neuromorphology, and adult walking behavior. Survival rates above 50% were only observed at doses lower than 0.0056 µg (a.i.)/bee. Although no sub-lethal effect on body mass or developmental time was observed in the surviving insects, they found that imidacloprid negatively affects the development of mushroom bodies in the brain and impairs the walking behavior of newly emerged adult

workers. These findings demonstrated the lethal effects of imidacloprid on native stingless bees and provided evidence of novel serious sub-lethal effects that may compromise colony survival.

Both Rossi et al.[79] and Catae et al.[80] showed the effects of imidacloprid and thiamethoxam in the non-target organs of Africanized *Apis mellifera*. Catae et al. examined the midgut and Malpighian tubule cells of Africanized *A. mellifera* in the newly emerged workers in which they were exposed to a diet containing a sub-lethal dose of 0.0428 ng a.i./L until 8 days. They found thiamethoxam is cytotoxic to midgut in which the damage is more evident in bees on the first day. However the damage was repaired on the eighth day. On the other hand, the Malpighian tubules showed pronounced alterations on the eighth day of exposure. Rossi et al.[81] aimed to evaluate the effects of chronic exposure to sub-lethal doses of imidacloprid on the brain of Africanized *A. mellifera*. They exposed the mushroom bodies of bees at 8.09 ng/bee, and 0.809, 8.09, and 1.618 ng/bee of imidacloprid in optic lobes, a region more sensitive to insecticides than other regions of the brain in bees. They observed the presence of condensed cells and cell death, and therefore concluded that sub-lethal doses of imidacloprid have cytotoxic effects on exposed bee brain, including optic lobes region. Sandrock et al.[82] investigated the influence of thiamethoxam and clothianidin in nectar substitutes on the entire life-time fitness performance of the solitary bee *O. bicornis* (red mason bee). They found dietary neonicotinoid exposure (2.87 µg/kg of thiamethoxam and 0.45 µg/kg of clothianidin in sugar water) has severe detrimental effects on *O. bicornis's* reproductive output. Neonicotinoids did not affect adult bee mortality; however, the number of completed nests was 22% less in the treatment population than the controls. Within the completed nests, the treatment population contained 43.7% fewer total brood cells, and relative offspring mortality was almost two-fold higher than the controls. In addition, there is a significantly male-biased offspring sex ratio. Treatment populations have 8.5% lower proportion of daughters, compared to the control

populations. Those studies have demonstrated that the continuous exposure to a sub-lethal dose of either imidacloprid, thiamethoxam, or clothianidin can impair organs that are critical to the survivals of bees but often omitted due to the unknown toxicological actions of neonicotinoids.

Policy Implication Synthesis

Because of their ecological and economic importance, the causes of declining of honeybees and other pollinators deserve a thorough evaluation. We summarized the sub-lethal effects of pesticides to honeybees, bumblebees, and other bees in Table 1, 2, and 3. The weight-of-evidence of this review clearly demonstrated bees' susceptibility to insecticides, in particular to neonicotinoids, and the synergistic effects to diseases that are commonly present in bee colonies. One important aspect of assessing and managing the risks posed by neonicotinoids to bees is the chronic effects induced by exposures at the sub-lethal levels. More than 90% of literature published after 2009 directly or indirectly imply the adverse health effects associated with sub-lethal exposure to neonicotinoids, including abnormal foraging activities, impaired brood development, neurological or cognitive effects, and colony collapse disorder. Since sub-lethal levels of neonicotinoids are ubiquitous in the environment where bees forage, it is a conceivable challenging task to protect honeybees and other pollinators from sub-lethal effects of neonicotinoids and other pesticides.

While it is straightforward to define the sub-lethal exposure, it might be problematic to determine the exact field-realistic levels of pesticide exposure. As many investigators claimed the uses of field-realistic exposure levels in their experiments, there is no scientific evidence to support the assertion. Establishing the field-realistic exposure levels may not be possible because so many factors could modify the levels of pesticides in the foraging environment that

bees encounter. For instance, pollen and guttation drops collected from corn grown from imidacloprid-treated seeds would have imidacloprid concentrations several orders of magnitude higher than pollen collected from dandelion flowers in which the main source of imidacloprid residue is from soil uptake. In addition, the temporal and spatial variations associated with the timing and the source of pesticide application would significantly affect the levels of pesticides in the field where bees are present. Even if the field-realistic levels were existed, it would actually encompass a wide range of concentrations for individual pesticides. The repeated attempts to emphasize the importance of applying field-realistic pesticide levels that bees would expose to in many studies are deliberately to undermine the fact of the ubiquitousness and persistence of neonicotinoids in the environment once applied. Instead, the focus should be to understand the adverse health effects of exposures to sub-lethal levels of neonicotinoids in bees and other pollinators over a longer period of time than a simple cross-sectional assessment.

Although this review only included studies focused on bees, it should be mindful when interpreting the outcomes cross bee species. Honeybee (*Apis mellifera*) is very unique among the pollinator family mainly because they are perennial social insects. Due to this distinct biology of honeybees, research findings obtained from bumblebees or other bees may or may not be directly applied to honeybees, or vice verse. Social bee colonies depend on the collective performance of numerous individual bees, including queen, drones, and workers. So while pesticide at certain levels may not have immediate adverse health effects on individual bees, it is not known whether it results in a severe cumulative effect at the colony level.

The rising awareness of protecting honeybees and other pollinators worldwide is directly related to the emergence of honeybee colony collapse disorder (CCD). The deliberate omission of the recognition of causes to CCD may put additional pressure on the recovery of

honeybee colonies. It is very clear from the literature that the detrimental effects of neonicotinoids not only affect individual bees, but also the survival of honeybee colonies. More importantly, unlike other diseases, CCD could not be prevented or managed by beekeepers. Unfortunately, the actions that are needed to curb the uses of neonicotinoids in order to reverse the ongoing losing trend of honeybee colonies are facing a great resistance because of its financial implications to agrochemical industry. The recent regulatory control in the European Union, as well as the proposed action by the Ontario government of Canada, on limiting certain uses of neonicotinoids in agricultural crops is the first step toward protecting bees and other pollinators' populations. While the effectiveness of the regulatory restrictions on neonicotinoids uses and its impact to agriculture will be thoroughly assessed in the near future, similar regulatory actions should be taken in the State of Connecticut to prevent further losses of those important pollinators.

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Table 1. Summary of literature review on the sub-lethal effects of pesticides in honeybees (*Apis mellifera*).

Study	Pesticide (dose)	Outcome
Derecka et al.[32]	Imidacloprid (2 µg/L)	Abnormities or death during development.
Decourtye et al.[33]	Imidacloprid (24 µg/kg)	Decrease foraging activity and have negative effects of olfactory learnt discrimination task.
Yang et al.[34]	Imidacloprid (50 µg/L)	Affect foraging behavior.
Eiri et al.[35]	Imidacloprid (0.21 ng/bee)	Impair colony fitness.
Teeters et al.[36]	Imidacloprid (50 and 500 ppb)	Foraging difficulty and reduction in locomotor activity.
Henry et al.[37]	Thiamethoxam (0.07ppb or 0.067 µg/L)	Foraging difficulty
Schneider et al.[38]	Clothianidin (0.5 ng/bee or 0.02ppb)* Imidacloprid (1.5 ng/bee or 0.06ppb)*	Foraging difficulty
Tan et al.[39]	Imidacloprid (20 µg/L)	Foraging difficulty
Sandrock et al.[40]	Thiamethoxam (5.0 ppb) Clothianidin (2.0 ppb)	Decrease of colony performance and productivity, decelerated colony growth.
Williamson et al.[41]	Imidacloprid, thiamethoxam, clothianidin, dinotefuran (0.45 to 0.54 ng/bee)	Behavior and locomotive impairment.
Fischer et al.[42]	Clothianidin (2.5 ng/bee or 25 ppb) Imidacloprid (7.5 ng/bee or 75 ppb) Thiacloprid (1.25 mg/bee or 12.5 ppm)	Interfered with navigation of honeybees.
Decourtye et al.[43]	Imidacloprid (12 ng/bee)	Cognition/Neurological Impairment.
El Hassani et al.[44]	Fipronil (0.5 ng/bee)	Impairment of olfactory learning.
El Hassani et al.[45]	Acetamiprid (0.1 µg/bee)	Impaired long-term retention of olfactory learning.

Study	Pesticide (dose)	Outcome
Palmer et al.[46]	Imidacloprid (50nM–10μM) Clothianidin (200 nM) Coumaphos (50nM–1μM)	Cognition/Neurological Impairment.
Williamson and Wright [47]	Imidacloprid (10 and 100nM) Coumaphos (10 and 100nM)	Impaired olfactory learning and memory formation.
Boily et al.[49]	Imidacloprid (0.24 ng/bee)	Increased AChE activity and decreased survival.
Alaux et al.[8]	Imidacloprid (0.7 to 70ppb)	Increase susceptibility of colony to microsporidia Nosema.
Vidau et al.[12]	Thiacloprid (5.1ppm)	Immune Suppression with Nosema.
Pettis et al.[51]	Imidacloprid (5 and 20ppb)	Increased Nosema infections significantly.
Doublet et al.[54]	Thiacloprid (0.1 mg/kg)	Additive interaction with black queen cell virus (BQCV) leading to increased larval mortality.
Lu et al.[17]	Imidacloprid (20-400 μg/kg of HFCS)	Leading to colony collapse disorder (CCD).
Lu et al.[18]	Imidacloprid or clothianidin (0.74 ng/bee/day)	Leading to colony collapse disorder (CCD).
Rondeau et al.[57]	Imidacloprid in pollen (0.5–30 ppb) and honey (0.7–13 ppb)	Leading to colony collapse disorder (CCD).

Table 2. Summary of literature review on the sub-lethal effects of pesticides in bumblebees (*Bombus spp.*).

Study	Pesticide (dose)	Outcome
Cresswell et al.[62]	Imidacloprid (10ppb)	Reduction in feeding rate.
Cresswell et al.[63]	Imidacloprid (125 µg/L)	Reduced mean daily locomotory activity.
Scholer and Krischik [64]	Imidacloprid (16 ppb) Clothianidin (17 ppb)	Reduction in queen survival, worker movement, colony consumption, and colony weight.
Mayes et al.[29] Morandin et al.[66]	Spinosad (0.8 mg/kg)	Adverse health effects included adult mortality, brood development, weights of emerging bees, and foraging efficiency.
Whitehorn et al.[67]	Imidacloprid (0.7-6 µg/kg)	Gained significantly less weights and produced less numbers of queens.
Bryden et al.[70]	Imidacloprid (10 ppb)	Colonies fail with decreased birth rates and increased death rates.
Larson et al.[71]	Clothianidin (0.45 kg a.c./ha)	Reduced foraging activity, increased worker mortality, delayed weight gain, and produced no new queens.
Smagghe et al.[72]	Chlorantraniliprole (0.4ppm)	Effect was on reproduction in colonies.
Gill et al.[74]	Imidacloprid at (10ppb)	Impairment to pollen foraging efficiency.
Gill and Raine [75]	Imidacloprid at (10ppb)	Chronic behavioural impairment, decrease in pollen foraging efficiency.
Feltham et al.[76]	Imidacloprid (0.7 ppb in sugar water and 6 ppb in pollen)	Decrease the pollen collect ability.

Table 3. Summary of literature review on the sub-lethal effects of pesticides in other bees.

Study	Pesticide (dose)	Outcome
Abbott et al.[77]	Imidacloprid (30 ppb and 300 ppb)	Sub-lethal effects on larval development and longer developmental time for *Osmia lignaria*.
Tomé et al.[78]	Imidacloprid (0.0056 µg (a.i.)/bee)	Negatively affects the development of mushroom bodies in the brain and impairs the walking behavior of newly emerged adult workers for stingless bees (Hymenoptera: *Apidae: Meliponinae*)
Rossi et al.[79] Catae et al.[80]	Thiamethoxam (0.0428 ng a.i./L)	Malpighian tubules showed pronounced alterations for Africanized *Apis mellifera*.
de Almeida Rossi et al.[81]	Imidacloprid (0.809, 8.09, and 1.618 ng/bee)	Cytotoxic effects on exposed bee brain, including optic lobes region for Africanized *Apis mellifera*.
Sandrock et al.[82]	Thiamethoxam (2.87 µg/kg) Clothianidin (0.45 µg/kg)	Fewer total brood cells, hugher offspring mortality, and male-biased offspring sex ratio for solitary bee *O. bicornis* (red mason bee).