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A Review of Sub-lethal Neonicotinoid Insecticides Exposure and Effects on Pollinators

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Abstract

Purpose of Review Beekeepers around the world have been reporting the ongoing weakening of honeybee health and subsequently the increasing colony losses since 1990. However, it was not until the abrupt emergence of colony collapse disorder (CCD) in the 2000s 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.

Recent Findings We have identified peer-review papers that are relevant to examine the effects of sub-lethal pesticide exposures on the health of honeybees (*Apis mellifera*), bumblebees (*Bombus terrestris*), and other bees from a literature search on PubMed and Google Scholar using the following combined keywords of "pollinators," "honeybee," "bees," "pesticides," or "neonicotinoids," and from a cross-reference check of a report made available by the European Parliament in preparation to fulfill their regulatory mandate on the issue of protecting pollinators among their membership nations.

Summary 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.

Keywords Neonicotinoids · Honeybee · Pollinator · Sub-lethal exposure

Introduction

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

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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 USA in 2006 [71, 72] that has raised the concern of losing this important perennial pollinator. A recent United Nations report highlighted the persistence of CCD worldwide [69] 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 disappearance of adult honeybees in winter from hives containing adequate store food (e.g., honey, nectar, and pollen). Although it is generally agreed that some losses of bee colonies during winter is common in apiculture, it never in the history of beekeeping has the losses of honeybee colonies due to CCD occurred in such a magnitude, over a widely distributed geographic area, and lasting for many years.

While the prevailing opinions suggest the linkage of CCD to multi-factorial causes including pathogen infestation,

beekeeping practices (such as malnutrition), and pesticide exposure in general [2, 3, 6, 13, 18, 22, 38, 68, 73, 74, 76], recent scientific findings linking declines of bee colonies with exposure to the systemic neonicotinoid insecticides appear to gain traction [29, 43–45, 51, 63], and have led to new regulatory control in the European Union [28]. 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 ecological conservation.

In this review, 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 keywords of "pollinators," or "honeybee," or "bees and pesticides," which yields more than 200 papers, published before September 30, 2014. We then supplemented the literature search by a cross-reference check with a Report titled "Existing Scientific Evidence of the Effects of Neonicotinoid Pesticides on Bees" [36]. This report was a result of a request made by the 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 review if (a) papers do not contain either pesticide exposure or toxicological endpoint data in associated with bees or pollinators, or papers contain exposure levels of neonicotinoids higher than the respective reference dose (RfD) or acceptable daily intake (ADI), (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 USA, or (d) papers were not written in English. At the end, we have identified 30 papers, as listed in Tables 1, 2, 3, and 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 variety of pesticides that are either brought back by bees from the outside foraging environment or applied by the beekeepers for treating infectious diseases. 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 reported data has shown the comparison of pesticide residues in hives exhibiting with and without CCD symptoms. A 2010 study has demonstrated the magnitude of pesticide contamination in beehives by analyzing hundreds of pollen, wax, foundation, brood, and adult bee samples for approximately 120 pesticides [49]. Mullin et al. [49] showed 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 with the national average of up to 12 ppb of coumaphos and fluvalinate in the survey of US honey samples. Chauzat et al. [11] 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. 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 development of resistance 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 also 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. [49] 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 established the LD_{50} of fluvalinate at 0.2 μg/bee, a 330-fold lower than the common application concentration [70]. 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.

Pesticide residues measured in pollen brought back to hives by bees might be a more realistic matrix for assessing pesticide exposure in bees. 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. [49] found approximately 100 pesticides in the stored pollen, 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. [4] reported more than 30% of stored pollen contained multiple pesticides with concentrations ranging from 1 to 2930 ppb. A comparable study published by Krupke et al. [39] also demonstrated that bees living and foraging near corn fields 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

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

Study	Pesticide (dose)	Outcome
Derecka et al.	Imidacloprid (2 μg/L)	Abnormities or death during development.
Decourtye et al.	Imidacloprid (24 μg/kg)	Decrease foraging activity and have negative effects of olfactory learnt discrimination task.
Yang et al.	Imidacloprid (50 μg/L)	Affect foraging behavior.
Eiri et al.	Imidacloprid (0.21 ng/bee)	Impair colony fitness.
Teeters et al.	Imidacloprid (50 and 500 ppb)	Foraging difficulty and reduction in locomotor activity.
Henry et al.	Thiamethoxam (0.07 ppb or 0.067 μg/L)	Foraging difficulty.
Schneider et al.	Clothianidin (0.5 ng/bee or 0.02 ppb) Imidacloprid (1.5 ng/bee or 0.06 ppb)	Foraging difficulty.
Tan et al.	Imidacloprid (20 μg/L)	Foraging difficulty.
Sandrock et al.	Thiamethoxam (5.0 ppb) Clothianidin (2.0 ppb)	Decrease of colony performance and productivity, decelerated colony growth.
Williamson et al.	Imidacloprid, thiamethoxam, clothianidin, dinotefuran (0.45 to 0.54 ng/bee)	Behavior and locomotive impairment.
Fischer et al.	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.	Imidacloprid (12 ng/bee)	Cognition/neurological impairment.
El Hassani et al.	Fipronil (0.5 ng/bee)	Impairment of olfactory learning.
El Hassani et al.	Acetamiprid (0.1 μg/bee)	Impaired long-term retention of olfactory learning.
Palmer et al.	Imidacloprid (50 nM–10 μM) Clothianidin (200 nM) Coumaphos (50 nM–1 μM)	Cognition/neurological impairment.
Williamson and Wright	Imidacloprid (10 and 100 nM) Coumaphos (10 and 100 nM)	Impaired olfactory learning and memory formation.
Boily et al.	Imidacloprid (0.24 ng/bee)	Increased AChE activity and decreased survival.
Alaux et al.	Imidacloprid (0.7 to 70 ppb)	Increase susceptibility of colony to microsporidia Nosema.
Vidau et al.	Thiacloprid (5.1 ppm)	Immune suppression with Nosema.
Pettis et al.	Imidacloprid (5 and 20 ppb)	Increased Nosema infections significantly.
Doublet et al.	Thiacloprid (0.1 mg/kg)	Additive interaction with black queen cell virus (BQCV) leading to increased larval mortality.
Lu et al.	Imidacloprid (20–400 μg/kg of HFCS)	Leading to colony collapse disorder (CCD).
Lu et al.	Imidacloprid or clothianidin (0.74 ng/bee/day)	Leading to colony collapse disorder (CCD).
Rondeau et al.	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.	Imidacloprid (10 ppb)	Reduction in feeding rate.
Cresswell et al.	Imidacloprid (125 μg/L)	Reduced mean daily locomotory activity.
Scholer and Krischik	Imidacloprid (16 ppb) Clothianidin (17 ppb)	Reduction in queen survival, worker movement, colony consumption, and colony weight.
Mayes et al. Morandin et al.	Spinosad (0.8 mg/kg)	Adverse health effects included adult mortality, brood development, weights of emerging bees, and foraging efficiency.
Whitehorn et al.	Imidacloprid (0.7–6 μg/kg)	Gained significantly less weights and produced less numbers of queens.
Bryden et al.	Imidacloprid (10 ppb)	Colonies fail with decreased birth rates and increased death rates.
Larson et al.	Clothianidin (0.45 kg a.c./ha)	Reduced foraging activity, increased worker mortality, delayed weight gain, and produced no new queens.
Smagghe et al.	Chlorantraniliprole (0.4 ppm)	Effect was on reproduction in colonies.
Gill et al.	Imidacloprid at (10 ppb)	Impairment to pollen foraging efficiency.
Gill and Raine	Imidacloprid at (10 ppb)	Chronic behavioral impairment, decrease in pollen foraging efficiency.
Feltham et al.	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.	Imidacloprid (30 ppb and 300 ppb)	Sub-lethal effects on larval development and longer developmental time for *Osmia lignaria*.
Tomé et al.	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. Catae et al.	Thiamethoxam (0.0428 ng a.i./L)	Malpighian tubules showed pronounced alterations for Africanized *Apis mellifera*.
de Almeida Rossi et al.	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.	Thiamethoxam (2.87 μg/kg) Clothianidin (0.45 μg/kg)	Fewer total brood cells, higher offspring mortality, and male-biased offspring sex ratio for solitary bee *O. bicornis* (red mason bee).

seeds. When maize plants reached anthesis, maize pollen was found to contain clothianidin and other pesticides that are readily available for bees to collect. They showed that among those 20 pollen samples collected directly from bees using pollen trap, 3 and 10 samples contained thiamethoxam and clothianidin, respectively. Fungicides were also frequently detected in which azoxystrobin and propiconazole were found in all pollen samples while trifloxystrobin was found in 12 of the 20 pollen samples. Concentrations (μg/g) of thiamethoxam, clothianidin, trifloxystrobin, azoxystrobin, and propiconazole in pollen collected from bees foraging in adjacent maize fields planted with treated seeds ranged from non-detect to 7.4, non-detect to 88, non-detect to 9.8, 4.3 to 66, and 3.2 to 23.8, respectively.

The concern of sources of pesticide exposure 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. [35] 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 corn plants 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 the elevated acute toxicity to bees. According to authors, 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. [35] concluded that this is a threatening scenario for bees and other pollinators that does not comply with an ecologically acceptable situation.

Since it is conceivably difficult to compare pesticide levels in samples collected from bees or 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. Were the field-realistic levels for a certain pesticide that bees would encounter in the environment existed, it is likely to encompass a very wide range of concentrations. The data presented in the above studies would support this statement. Regardless, the objective of this review is to focus on sub-lethal exposure to neonicotinoids that are ubiquitous in bees' foraging environment and relevant to declining bee population and the causation of CCD.

Table 4 Reference dose (RfD) and acceptable daily intake (ADI) for each neonicotinoid pesticide and their basic chemical and physical information

Name	CAS number	Molecular formula	Water solubility (mg/L)	$\log K_{ow}$	RfD (mg/kg/day)	ADI (mg/kg/body wt.)
Acetamiprid	135410-20-7	$C_{10}H_{11}ClN_4$	222.6	2.55	0.07	0.07
Thiamethoxam	105843-36-5	$C_8H_{10}ClN_5O_3S$	2862	0.80	0.006	0.08
Imidacloprid	138261-41-3	$C_9H_{10}ClN_5O_2$	4973	− 0.41	0.06	0.06
Clothianidin	210880-92-5	$C_6H_8ClN_5O_2S$	6685	0.64	0.01	0.1
Flonicamid	158062-67-0	$C_9H_6F_3N_3O$	6222	0.50	N.A.	0.025
Thiacloprid	138261-41-3	$C_{10}H_3ClN_4S$	231.9	2.33	0.004	0.01
Dinotefuran	165252-70-0	$C_7H_{14}N_4O_3$	6140	− 0.19	0.02	0.2
Nitenpyram	150824-47-8	$C_{11}H_{15}ClN_4O_2$	3453	0.40	N.A.	0.53

The Association of Pesticide Exposure 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, and no studies linking sub-lethal pesticide exposure to adverse chronic health effects in honeybees were published in peer-review scientific journals until 2009. This might signal the omission by the research and regulatory communities on the roles of pesticides at the sub-lethal levels on the deteriorating honeybee's health.

Brood Development, Adult Bee Longevity, and Metabolic Responses

Wu et al. [80] showed that worker bees reared in brood comb containing high levels of many pesticides have experienced multiple health effects, including reduced adult longevity, increased brood mortality, delayed larval development, or higher fecundity of *Varroa* mites. Delayed development was observed in the early stages (days 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 Wu et al., pesticides could migrate from comb containing high 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. [47] 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 by 2 °C from the optimal 35 °C, it significantly 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 at 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. [21] analyzed molecular profiles of worker-bee 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 a diminished buffering and stability of the developmental program in which would likely to cause an increased rate of developmental failure.

Foraging Difficulty

Decourtye et al. [19] demonstrated that feeding honeybee workers with 24 μg/kg of imidacloprid in sugar water can decrease their foraging activity and cause negative effects of olfactory learnt discrimination task. Yang et al. [82] also investigated the foraging behavioral changes by measuring the time intervals between two visits at the same feeding station in honeybee workers with sub-lethal dosages of imidacloprid. The normal foraging interval of honeybee workers was within 300 s after training to fly to an artificial feeder 35 m away from the hives. However, they found those honeybee workers delayed their return visit for more than 300 s when they were treated orally with sugar water containing imidacloprid. The delayed percentage, as reported by Yang et al. [82], is imidacloprid concentration dependent. The lowest effective concentration of imidacloprid was 50 μg/L, and when bees were treated with imidacloprid higher than 1200 μg/L, they all showed abnormalities in revisiting the feeding site. 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, and subsequently reduce colony fitness over the long term. Eiri and Nieh [25] 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 h 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 h post treatment.

In order to clarify the effects of sub-lethal exposure to pesticides on honeybee behaviors, Teeters et al. [65] 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 with each other 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 also spent significantly less time near the food source than control bees. The average "interaction" times for bees were also significantly affected by imidacloprid 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 imidacloprid, suggesting an obvious behavioral effect.

Henry et al. [37] tested the hypothesis of which sub-lethal exposure to thiamethoxam would indirectly increase 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 0.07 ppb of thiamethoxam (or 1.34 ng in a 20-ml sucrose solution). Bees were then released away from their hive with a microchip glued onto their thorax so they can be monitored by 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 failed to return to their colonies when foraging in treated crops on a daily basis. Schneider et al. [59] 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 (or 0.02 ppb assuming each bee weight 30 mg) for clothianidin and > 1.5 ng/bee (0.06 ppb) for imidacloprid during the first three hours after treatment. In the trials conducted with imidacloprid at 3 ng and clothianidin at 2 ng, only 25% and 21% of bees returned to the hives during the 3-h 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. [64] showed that sub-lethal exposure to 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 that dangerous feeder as compared with control bees, and subsequently 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. [57, 58] investigated the effects of sub-lethal dietary neonicotinoids exposure by feeding bees with pollen contaminated with thiamethoxam (5.0 ppb) and clothianidin (2.0 ppb). They reported 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. [79] illustrated that after 24 h 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. [31] 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 (75 ppb), and 11.25 ng/bee (112.5 ppb) of imidacloprid, and 1.25 mg/bee (12.5 ppm) of thiacloprid, would interfere 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 clothianidin and imidacloprid would either block the retrieval of a remote memory or alter this form of navigation memory.

Results from above studies 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 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 death of the colonies.

Cognition/Neurological Impairment

In addition to affecting honeybees' foraging activities, sub-lethal exposure to neonicotinoids has also been shown to disrupt honeybees' behavior and learning abilities, which would subsequently impair their foraging and homing abilities. Decourtye et al. [20] demonstrated that imidacloprid at the sub-lethal dose (12 ng per bee) decreased the acquisition and the retention performances tested in the conditioned proboscis extension reflex (PER) paradigm. El Hassani et al. [26] observed a significant reduction of sucrose sensitivity in honeybee workers at the dose of 1 ng/bee of fipronil treated 1 h 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. [27] postulated that acetamiprid at sub-lethal levels can affect gustatory, motor, and mnemonic functions in honeybees. They showed that after oral ingestion of sucrose solution with acetamiprid at doses between 0.1 and 1 μg/bee, acetamiprid could increase bees' sensitivity to antennal stimulation and impaired long-term retention of olfactory learning. 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. [50] showed that using recordings from mushroom body Kenyon cells in acutely isolated honeybee brain, imidacloprid (50 nM–10 μM), clothianidin (200 nM), and the oxon metabolite of miticide coumaphos (50 nM–1 μM), could cause a depolarization-block of neuronal firing, and subsequently inhibit nicotinic responses. These effects were observed at the concentrations (50 nM–10 μM) that are encountered by honeybees in the foraging environment and within their hive and are additive with repeated pesticide applications. Those findings provided a neuronal mechanism that may account for the cognitive impairments caused by neonicotinoids and OP-based miticides commonly used in honeybee 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 100 nM) of imidacloprid or/and coumaphos impaired olfactory learning and memory formation in honeybees have been made by Williamson and Wright [77]. In the experiment, they combined imidacloprid with coumaphos to simulate the situation where honeybees are exposed to pesticides in food and to miticides applied to their hives. 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. However, when bees exposed to these two pesticides simultaneously, the synergistic responses were observed. Williamson and Wright [77] concluded that simultaneous exposure to sub-lethal doses of cholinergic imidacloprid and coumaphos would significantly impair 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. [78] 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 intention of assessing acute effects of sub-lethal doses is unknown, the data presented in Williamson et al. [78] were inconsistent to the majority of papers published. Boily et al. [7] first reported an increased AChE activity for both in-field and laboratory data. Sub-lethal doses of neonicotinoids (0.08, 0.16, 0.24, and 0.30 ng/bee of imidacloprid, and 0.12 and 0.24 ng/bee of clothianidin) could increase AChE activity in caged bees, and after 2 weeks exposure in field experiments as well. The results suggest that the no observable effects level (NOEL) for imidacloprid alone should be at no 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. [7] 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), they 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. [2] demonstrated the interaction between imidacloprid (at the doses ranging from 0.7 to 70 ppb) 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 the control, Nosema, or imidacloprid-treated groups. Vidau et al. [74] 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 1 ppb, thiacloprid at 5.1 ppm, or untreated. They found exposures to fipronil and thiacloprid had no effect on the mortality of uninfected honeybees compared with 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. [81] also demonstrated higher proportion of bees reared from the high pesticide residue brood comb became infected with *N. ceranae*, and died at younger ages, compared with those reared in low residue brood combs. Although both Vidau et al. [74] and Wu et al. [81] studies have suggested

that developmental exposure to pesticides in brood comb could increase the susceptibility 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 in honeybees was further demonstrated by Pettis et al. [52]. They exposed honeybee colonies during three brood generations to imidacloprid at 5 and 20 ppb 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 with bees from control hives, suggesting an indirect effect of neonicotinoids on pathogen growth in honeybees. The results reported by Pettis et al. [52] also suggested that other than the known nAChR inhibition, sub-lethal neonicotinoids exposure could promote Nosema infection in bees, a new finding of adverse health outcomes to bees caused by neonicotinoids. In addition, Pettis et al. [53] 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. [53] used pollen traps to collect pollen pellets from foraging bees' 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 332 ppb, respectively. Esfenvalerate (216 ppb) and phosmet (14,700 ppb), 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 worrisome, but useful as the supplement to those reported by Mullin et al. [49] and Krupke et al. [39].

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 encoding NF-κB, Di Prisco et al. [23] demonstrated that neonicotinoids at sub-lethal levels could reduce immune defenses and subsequently promote the replication of the deformed wing virus (DWV) in honeybees bearing covert infection. Doublet et al. [24] 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. A similar trend of increased mortality in adult honeybees was observed by Doublet et al. [24] due to the synergistic interactions between *N. ceranae*, BQCV, and thiacloprid.

Synergic Effects of Neonicotinoids with Fungicides

It was not until 2013 when reports showed other pesticides could also play a potential synergistic role with neonicotinoids to compromise honeybees' health when exposure occurred simultaneously. Biddinger et al. [5] demonstrated a synergism of neonicotinoids and fungicides to honeybee 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. [66] 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

Although numerous papers that are previous discussed in this review have claimed the link of sub-lethal neonicotinoids exposures to colony collapse disorder (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 observation of CCD that is the 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. [43] 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 study design consisting of 4 independent apiary sites, and each apiary consisted of 4 hives treated with different doses of imidacloprid 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 without any symptom of diseases during the 13-week dosing regime and stayed healthy 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 at each apiary site

unequivocally augments the conclusion of which sub-lethal imidacloprid exposure via HFCS intake led to CCD after several brood generations. Lu et al. [44] continued to demonstrate 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 and subsequently leads to CCD. They found both control and neonicotinoid-treated hives progressed almost identically in terms of brood development 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 decrease. 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. [55] 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. Those findings reported by Rondeau et al. [55] supported the conclusions made by Lu et al. [43, 44], and indirectly provided an answer of why CCD often occurred in winter.

Regardless, several reports have discredited the causal relationship of neonicotinoids to honeybee CCD. Pilling et al. [54] 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 treated 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 in seed treatment on oilseed rape and maize. However, the methodological aspects of the study, as well as the conclusions made by Pilling et al. [54], have been 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' concerns of methodologies and conclusions are legitimate, they also believed that the contributions from Pilling et al. [54] 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. [10] also discredited the role of neonicotinoids in honeybee CCD by screening dead colonies from three apiaries in Spain for the presence of neonicotinoids in store pollen. They reported absence of neonicotinoids in pollen but made no mention of other pesticides that have reported to be commonly present inside hives. Although Cepero et al. [10] 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 mostly likely Cepero et al.'s opinions. More importantly, those general factors have long been co-existed with beekeeping practices and posed no biological plausibility to honeybee CCD.

Cutler et al. [16] reported a significant number of honeybee incidents in Ontario, Canada, where exposure to neonicotinoids dust during corn planting 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 was 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). Cutler et al. [16] 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 that are far greater than those 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 Cutler et al. [16] 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

(https://www.ontario.ca/page/neonicotinoid-regulations-growers?_ga=2.196107244.1373047601.1571753131-1752665599.1571753131).

The Association of Pesticide Exposure and Adverse Health Outcomes in Bumblebees (*Bombus spp.*)

Acute Toxicity/Direct Contact

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*), alfalfa leaf cutting bees (*Megachile rotundata*), and *Osmia lignaria*. They found clothianidin and imidacloprid are highly toxic to all three species, followed by deltamethrin and spinosad, whereas novaluron was found non-toxic to those 3 bees. Although they found bumblebees were generally more tolerant to pesticide toxicity by direct contact, results were not consistent. To establish whether imidacloprid would harm individual bees when ingested at environmentally realistic levels, Cresswell et al. [14, 15] exposed adult worker bumblebees to imidacloprid in feeder syrup at dosages between 0.08 and 125 ppb. They found bumblebees progressively developed a dose-dependent reduction in feeding rate with 10–30% declines over time and reduced average daily locomotory activity on dosed syrup 125 μg/L. Data from Scholer and Krischik [60] indicated that feeding sugar syrup containing imidacloprid or clothianidin at 20 ppb (actual concentrations for imidacloprid 16 ppb and clothianidin 17 ppb) has a significant reduction in queen's survival (37% in imidacloprid and 56% in clothianidin), worker movement, colony consumption, and colony weight compared with no neonicotinoids treatments. Feeding on imidacloprid or clothianidin can cause changes in behavior (reduced worker movement, consumption, wax pot production, and nectar storage) as well that leads to detrimental effects on colonies (queen survival and colony weight).

Colony Vitality/Brood Development

Gels et al. [32] reported the effects of imidacloprid, chlorpyrifos, carbaryl, and cyfluthrin on native pollinators, specifically bumblebees after turf applications where bees forage on the weed flowering. This is the earliest study aiming to assess the toxicity of various types of pesticides to 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 application methods, they found that foraging workers did not avoid pesticide-treated field at all. Both Morandin et al. [48] and Mayes et al. [46] 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. Mayes et al. [46] have reported that spinosad is an insecticide derived from the bacterial species *Saccharopolyspora spinosa* that was reported by Mayes et al. [46] with very little or no effect on brood development in honeybees.

Whitehorn et al. [75] conducted a study to simulate the likely effects in wild bumblebee colony exposed to flowers of imidacloprid-treated rapeseed. Colonies received either control, low (0.7–6 μg/kg), or high (1.4–12 μg/kg) dosages 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 [41], however, provided somewhat conflicted 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. [42] 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 dosages, 39 and 98 μg/kg, whereas no detectable effects were found at dosages between 1 and 11 μg/kg. By comparison with previously published data, they concluded that brood production in bumblebee workers is more sensitive to imidacloprid than thiamethoxam. Bryden et al. [8] showed 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 pose a threat to the survival of pollinators. Larson et al. [40] 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 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 171 ppb 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. [62] 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 0.4 ppm 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. Lastly, Cutler and Scott-Dupree [12] 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 [12] suggested that exposure during pollen shed to corn grown from neonicotinoid-treated seeds poses low risk to bumblebee, it should be noted that those observations were collected at the cross-sectional manner within a relatively short period of time in summer. It is unlikely that bumblebees would have developed any adverse health endpoints, such as brood development and body weight, right after exposure.

Foraging Impairment

Gill et al. [34] 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 10 ppb level could cause impairment to pollen foraging efficiency, leading to increased colony demand for food as shown by increased worker recruitment to forage. Consequently, it would 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 [33] used the RFID technology to identify effects of imidacloprid on overall foraging activity. They found that bees exposed to 10 ppb 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. [30] reported a consistent finding as of Gill et al. [34] and Gill and Raine [33] 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. Those consistent findings 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 Exposure and Adverse Health Outcomes in Other Bees (*Hymenoptera*: *Apidae*: *Meliponinae, Osmia lignaria*)

Abbott et al. [1] examined the lethal and sub-lethal effects of imidacloprid and clothianidin on *Osmia lignaria* (Cresson) and *Megachile rotundata* (Hymenoptera: Megachilidae) by exposing their larvae to control, low (3 or 6 ppb), intermediate (30 ppb), and 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 dose (300 ppb) of imidacloprid. Tomé et al. [67] 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 imidacloprid negatively affects the development of mushroom bodies in the brain and impairs the walking behavior of newly emerged adult workers.

Rossi Cde et al. [56], Catae et al. [9] and de Almeida et al. [17] showed the effects of imidacloprid and thiamethoxam in the non-target organs of Africanized *Apis mellifera*. They 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 Cde et al. [56] 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 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 concluded that sub-lethal doses of imidacloprid have cytotoxic effects on exposed bee brain, including optic lobes region. Sandrock et al. [57, 58] 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 with 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.

Conclusion

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 Tables 1, 2, and 3, as well as the reference dose (RfD) and the acceptable daily intake (ADI) for each neonicotinoid in Table 4. The weight-of-evidence of this review clearly demonstrated bees' susceptibility to insecticides, in particular to neonicotinoids, and the synergistic effects resulting from multiple pesticide exposure 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 relatively 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 such 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 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 only reflect the fact that neonicotinoids are ubiquitous and persistence in the environment once applied. Instead, we should focus on understanding the adverse health effects resulting from sub-lethal exposure to neonicotinoids over a longer period of time than a simple cross-sectional assessment carried out under the so-called field realistic levels.

The rising awareness of protecting honeybees and other pollinators worldwide is directly related to the recent emergence of honeybee colony collapse disorder (CCD). It is very clear from the literature that the detrimental effects of neonicotinoids affect not only individual bees but also the survival of honeybee colonies. More importantly, unlike other diseases associated with bees, CCD could not be prevented, managed, or treated by beekeepers. The deliberate omission of the recognition of the existence and the causes of CCD will only put additional pressure on the recovery of honeybee colonies. 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. The effectiveness of those regulatory restrictions on neonicotinoids uses and its impact to agriculture will be thoroughly assessed in the near future.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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