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ORIGINAL RESEARCH ARTICLE

The neonicotinoid imidacloprid alone alters the cognitive behavior in *Apis mellifera L.* and the combined exposure of imidacloprid and *Varroa destructor* mites synergistically contributes to trial attrition

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Honey bee (*Apis mellifera L.*) populations have undergone a dramatic decline as an outcome of an increasing incidence of colony collapse disorder (CCD). The honey bee is a unique keystone species because its pollination activities are not only necessary for the viability of many flowering plant species in the wild, but are also necessary for many human agricultural practices. The increasing prevalence of CCD is a threat to the economic and nutritional security of all societies that rely on efficient pollination for large monoculture crops. Although there is ample evidence that Varroa mites, pesticides, disease, and climate change contribute to CCD, only a few studies have investigated the contribution of synergism across multiple factors to the development of CCD and CCD-like symptoms. However, there is increasing acknowledgement in the scientific community that synergism across different factors affects honey bee colony health and behavior. Here, we provide evidence that sub-lethal levels of imidacloprid neonicotinoid pesticide reduce learning and memory capabilities. Synergistic effects become apparent when Varroa mites together with imidacloprid exposure to individual bees more than doubled average mortality and attrition per trial.

Keywords: *Apis mellifera* L; honey bees; colony collapse disorder; Varroa; imidacloprid; neonicotinoids; pesticides

Introduction

The impact of honey bee (*Apis mellifera L.)* pollination has an estimated $215 billion value to the worldwide agricultural industry (Smith et al., 2013). As pollinator populations like honey bees decline, worldwide food production becomes vulnerable. Honey bees are uniquely valuable pollinators not only because they can pollinate large, monoculture crops due to their large colony sizes as compared to solitary bees, but also because they directly create honey, a valuable product itself (Gallai et al., 2009). The biology of honey bees made it possible for agriculture to grow pollinator-dependent crops in huge mono-cultures, and it is thus economically necessary to invest in the continued survival of the species.

The structure of complex honey bee colonies is both intricate in design and delicate in composition. Hundreds of thousands of bees enter and exit each hive every day. It follows that neurological malfunction, disease, predation, and parasites may alter the balance of comings and goings. Colony Collapse Disorder (CCD) is a world-wide phenomenon, wherein beekeepers have experienced sudden and catastrophic losses of whole colonies via a cryptic colony death, wherein adult bees appear to abandon their queen and brood, resulting in colony failure and swift hive death. Although there is a huge net loss of adult bees from collapsed hives, there is no evidence of death in the surrounding area. CCD is increasing in incidence, becoming a lead cause of over-winter death in U.S. managed hives since 2006 (Vanengelsdorp et al., 2009). Studies have shown that neonicotinoid pesticides and the Varroa mite are detrimental to colony survival and may contribute to CCD (Abbo et al., 2017; Blanken et al., 2015; Ellis et al., 2010; Farooqui, 2013; Gregorc et al., 2012; Martin, 2001; Nazzi et al., 2012; Sanchez-Bayo et al., 2016; Simon-Delso et al., 2014; Smith et al., 2013; Vanengelsdorp et al., 2009).

The increasing usage of neonicotinoid pesticides is quite alarming because these pesticides have grave implications on honey bee learning and behavior (Mohamed et al., 2009). Neonicotinoids are among the most widely used classes of pesticides, accounting for more than 25% of the global market-share (van der Sluijs et al., 2013). Neonicotinoids are amine-based pesticides that target nicotinic acetylcholine receptors (nAChR) of insect nervous systems, resulting in dysregulation. Studies have shown sublethal doses of imidacloprid (IMD, Figure 1), one of the most popular neonicotinoid insecticides, to be especially detrimental to *A. mellifera* health, learning, memory, and behavior (Abbo et al., 2017; Blanken et al., 2015; Bortolotti et al., 2003; Cresswell, 2011; Dively et al., 2015; Farooqui, 2013; Matsumoto, 2013; Medrzycki et al., 2003; Nguyen

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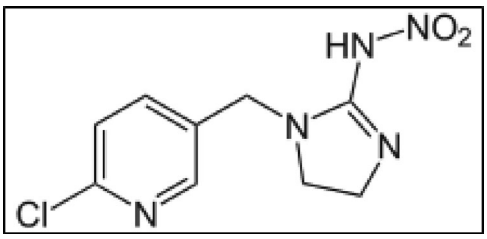

Figure 1. Structural Formula of Imidacloprid (By NEUROtiker - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=3384661).

et al., 2009; Sandrock et al., 2014; Suchail et al., 2001; Vanengelsdorp et al., 2009). Sublethal IMD impacts honey bee neurotransmitters and intracellular communication by acting as a nAChR agonist by mimicking acetylcholine (ACh). The cholinergic pathway is also key to the formation and retention of memories within the honey bee brain (Déglise et al., 2002). As an agonist, IMD overstimulates acetylcholine receptors, culminating in neural inflammation. This disrupts memory retention and olfactory function (Farooqui, 2013). Because Colony Collapse Disorder is marked by adult bees disappearing, it is not unreasonable to hypothesize that neonicotinoid pesticides such as IMD contribute to CCD by rendering foraging bees incapable of finding their way home.

Varroa mites are rampant, ubiquitous, and increasing in population size over the course of each season in honey bee colonies by infesting foraging bees (Anderson &Trueman, 2000). Varroa infestations impact honey bees from an early age, as female Varroa lay their eggs on unsealed larval honey bee cells. Studies have shown that Varroa mites contribute to honey bee immunosuppression through the destruction of the fat body. The honey bee fat body governs critical processes such as pesticide detoxification, metabolic and thermoregulation, synthesis and deposition of proteins and lipids integral to humoral immunity, and overwintering success. (Alaux et al., 2011; Degrandi-Hoffman & Chen, 2015; Gregory et al., 2005; Keeley, 1985; Koleoglu et al., 2017; Locke, 1980; Park et al., 2018; Ramsey et al., 2019; Seehuus et al., 2007; Yang & Cox-Foster, 2005); as vectors of several viral diseases, Varroa mites increase the frequency and intensity of pathogens that cause diseases such as Deformed Wing Virus (DWV) (Gregorc et al., 2012, Martin et al., 2012). Other studies have shown that Varroa mite infestation may correlate with colony losses (Dainat et al., 2012; Guzman-Novoa et al., 2010; Le Conte et al., 2010; Peck & Seeley, 2019; VAN DER Zee et al., 2015).

Scientists believe that CCD is likely a result of various factors acting synergistically, culminating in susceptibility to disease, colony weakness, and poor overwintering hive preparation (Bekic et al., 2014; Francis et al., 2013; Le Conte et al., 2010; Vanengelsdorp et al., 2009, 2010); decreased immunocompetence (Brandt et al., 2016); disrupted cognitive functions (Tison et al., 2017). However, there have been few studies that have researched the synergistic effects across these factors on honey bee health (Aufauvre et al., 2012; Blanken et al., 2015; Doublet et al., 2015; Gregorc et al., 2012; Johnson et al., 2009; Nazzi et al., 2012; Pettis et al., 2012; VAN DER Zee et al., 2015; Zheng et al., 2015). To date, there has been no study that has directly evaluated the synergistic effects of the Varroa mite and imidacloprid on honey bee cognition and vitality. This study aims to shed a novel light on CCD by contributing to the long overdue approach of looking at synergistic impacts on honey bee health.

Materials and methods

Proboscis extension response studies

Capture

An observation hive, inside a climate-controlled teaching laboratory, was monitored continuously for confounding variables. Forager bees were collected by placing a butterfly net in front of the hive entrance until 40-50 bees were caught each time. Bees with Varroa mite attachment and bees for all conditions were also collected from the GWU rooftop apiary. Due to the scarcity of bees with attached Varroa mites, non-foragers were also used and randomized across all conditions.

Proboscis extension response (per) evaluation

PER is innately elicited when honey bees come in contact with a food source. PER is also elicited when an unconditioned stimulus (2:1 sucrose: water solution) is introduced to the antennae (Bitterman et al., 1983; Felsenberg et al., 2011; Giurfa & Sandoz, 2012; Matsumoto et al., 2012; Menzel & Muller, 1996). 2:1 (sucrose: water) solution as the unconditioned stimulus, and lemongrass essential oil (100%, spritzed via cotton ball in transfer pipette) was used as the conditioned stimulus (Giurfa & Sandoz, 2012). Bees were evaluated and scored for cognitive function, which was assessed by their ability to perform PER upon exposure to the scent of lemongrass after having been successfully conditioned. Trials were run to investigate the incidence of successful PER elicitation on forager (1) control bees ($n = 587$), (2) bees fed 4 ng IMD/bee ($n = 416$), (3) bees with one Varroa mite attached ($n = 98$), and (4) bees with both Varroa mite attachment and fed 4 ng IMD/bee ($n = 71$).

Harnessing

Individual freeze-anesthetized bees were retrieved and placed into microfuge tube harnesses (Figure 2), so that the head protruded through an opening created by cutting the bottom off of the bottom of the tube. Cotton

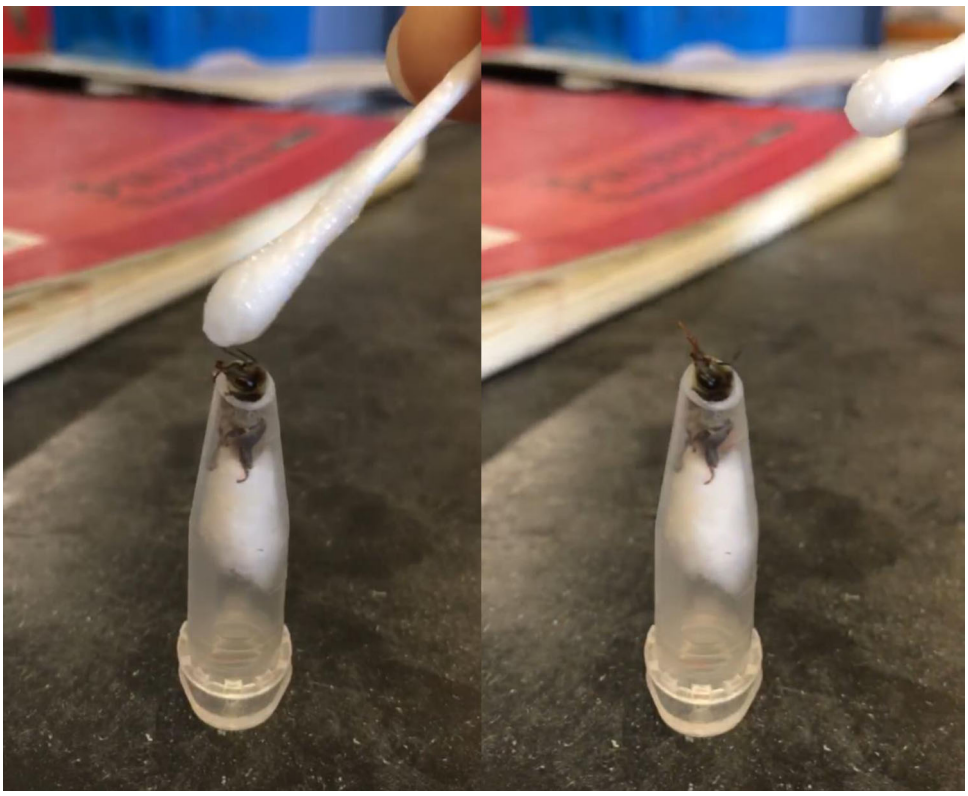

Figure 2. Bee in microfuge tube harness and demonstration of the Proboscis Extension Response.

was placed behind the abdomen in order to stabilize the body and to prevent escape.

Conditioning preparations

2:1 sucrose water solution was touched to the antennae of harnessed bees for approximately 3 s; each bee was observed for proboscis extension reflex (PER) elicitation. If PER was demonstrated, the bee was kept for classical conditioning. Bees that did not demonstrate PER were eliminated from further experimental use.

Classical conditioning

2:1 sucrose water solution (unconditioned stimulus) was applied to the antennae of harnessed bees to stimulate PER. If the bee failed to demonstrate PER, it was removed from the trial (Scheiner et al., 2013). If PER was elicited, lemongrass (neutral stimulus) was delivered for 5 sec. The lemongrass exposure procedure was performed on each harnessed bee that demonstrated PER for a total of four consecutive trials (5 minutes between each trial), eventually making lemongrass the conditioned stimulus. On the fourth trial, PER was again elicited, and the bee was then fed either 5 microliters of 0.8 ng/μL IMD 2:1 sucrose water solution (experimental) or 5 μL of 2:1 sucrose water solution (control). Once fed, bees were placed in a 30 °C humid incubator for 2 hours.

Retention

After the incubation period of two hours, the bees were exposed to lemongrass for 5 s and observed. Elicitation of PER with the scent of lemongrass alone was indicative of successful learning and memory retention. Failure ratios (number of total bees captured/number of bees with PER observed) were calculated.

Trial attrition

A number of bees eliminated per trial were recorded. Average attrition was calculated as the average number of bees that died during trials or were otherwise removed from trial due to cessation of PER response upon elicitation by the touch of antennae with sucrose solution.

Statistical analysis

PER failure ratios (number of total bees captured/number of bees with PER observed) were established from retention results. Non-parametric data were analyzed with chi-square analysis. Parametric (average attrition) data were analyzed by one-way ANOVA and Tukey HSD.

Results

The Proboscis Extension Response: Learning and Memory Reports of imidacloprid oral LD-50 in honey

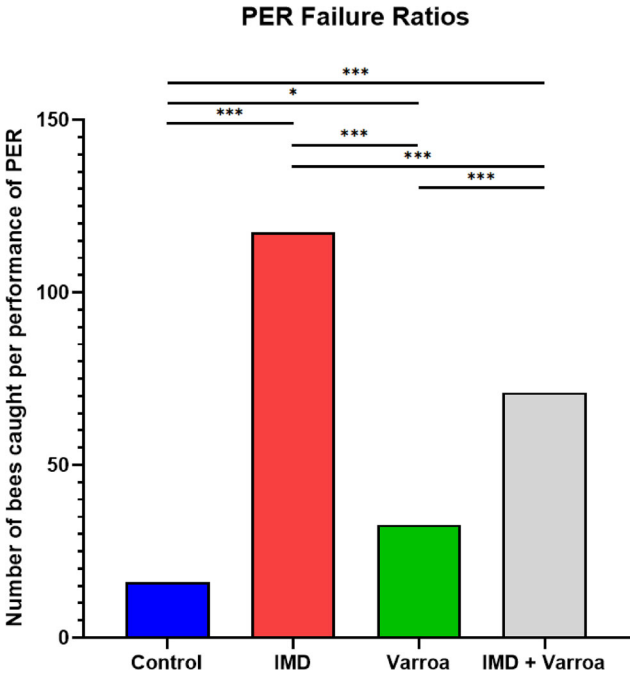

Figure 3. Failure ratios (number of total bees captured/number of bees with PER observed) for Control (sucrose water solution), IMD (4 ng/bee imidacloprid), Varroa (bees with latched Varroa mite), and IMD + Varroa (4 ng/bee imidacloprid with latched Varroa mite). (Chi-square analysis, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

bees have ranged from 3.7 ng/bee (Hertfordshire Pesticide Properties Database) to greater than 81 ng/bee (Nauen et al., 2001) at 48 hours, with many others falling in between these extremes (Schott et al., 2017). Because previous studies from our group have shown that 40 ng/bee is sufficient for 50% mortality, we considered this value as our oral LD-50. We selected 10% LD-50 IMD (4 ng/bee) to be used in conjunction with PER because preliminary data show no significant difference in the rate of death between bees fed sucrose solution and bees fed with 4 ng/bee IMD.

Learning and memory capabilities of control bees were much stronger than IMD bees, as they had a much lower failure ratio: One bee with PER for every 16 caught bees. For IMD, the failure ratio was much higher (117.4: 1). For Varroa alone, we observed one bee with PER for every 32.7 caught bees. When Varroa were paired with IMD in the IMD + Varroa condition, the results were intermediate (71: 1) between the Varroa condition and the IMD condition (figure 3). However, for the IMD + Varroa group, only one bee performed PER, and further data analysis was limited by sample size ($n = 71$).

There were significant differences across all groups. Control, Varroa, IMD, and IMD + Varroa ($\chi^2(3) = 103.02$; $p < .001$); Control and IMD ($\chi^2(1) = 77.08$; $p < .001$); Control and Varroa ($\chi^2(1) = 5.71$; $p = 0.02$); Control and IMD + Varroa ($\chi^2(1) = 34.78$; $p < .001$); IMD and IMD + Varroa ($\chi^2(1) = 11.42$; $p < .001$); and Varroa and IMD + Varroa ($\chi^2(1) = 14.71$; $p < .001$) (Table 1).

Table 1. Chi Square analysis of failure ratios of Groups (Control sucrose solution, 10% LD-50 imidacloprid, latched Varroa mite, and 10% LD-50 imidacloprid plus latched Varroa mite). There were statistically significant differences across all conditions and parameters ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

	n	df	χ^2	P
All Conditions	1172	3	103.02	$< 0.001^{***}$
IMD - Control	1003	1	77.08	$< 0.001^{***}$
IMD+Varroa - Control	487	1	34.78	$< 0.001^{***}$
Varroa - Control	514	1	5.71	0.02^{*}
IMD+Varroa - IMD	658	1	11.42	$< 0.001^{***}$
Varroa - IMD	685	1	47.84	$< 0.001^{***}$
Varroa - IMD+Varroa	169	1	14.71	$< 0.001^{***}$

Trial attrition

Bees exposed to both, IMD and Varroa mites showed a disproportionately high degree of trial attrition (the average number of bees removed from trial due to death or cessation of PER) during the four consecutive conditioning trials. There were statistically significant differences between IMD + Varroa and Control ($p < 0.01$) and IMD + Varroa and IMD ($p < 0.001$). IMD + Varroa more than doubled the likelihood of attrition from trials (8.82 bees per trial) as compared to the Control (3.10 bees per trial) and IMD (2.96 bees per trial) alone. Average attrition of the Varroa condition alone was slightly elevated (4.69 bees per trial) but not significantly different from any group. Trial attrition was comparable across the single variable groups of Varroa and IMD as well as the Control groups (Figure 4, Tables 2 and 3).

Discussion

These results suggest that imidacloprid contributes to neurological effects in honey bees, and the joint action of imidacloprid and Varroa mites synergistically contribute to significantly increased mortality (trial attrition). This synergism may be a contributing factor, possibly leading to CCD.

Proboscis extension response studies

Bees exposed to sublethal IMD dose/concentration (10% LD-50), Varroa mite pressure, or both had less retention of PER conditioning than those control bees that were given only sugar water and were not parasitized by Varroa (IMD:117.4 failed per 1 PER; Control: 16 failed per 1 PER; PER+Varroa: 71 failed per 1 PER; Varroa: 32.67 failed per 1 PER). Both sublethal IMD, as well as, Varroa mites negatively affected learning and memory in honey bees, but sublethal IMD appears to have had a more negative effect, as the failure ratios of control and Varroa bees were closer in value. In contrast, when IMD and Varroa were paired, failure rates

declined somewhat as compared to IMD alone. Overall, IMD alone corresponded with the most detrimental effects to honey bee cognition. Our results suggest that Varroa have a somewhat protective effect on honey bee retention ability, but the loss of most IMD + Varroa bees (attrition) did not allow for a proper analysis of post conditioning trials in this treatment group.

Behaviorally, IMD treated bees and Varroa-latched bees tended to act differently from control bees after the retention period. While control bees were typically still energized post-retention (kicking and twitching legs and antennae, general appearance), IMD-treated and Varroa-latched bees often appeared lethargic, with little movement or engagement (personal observation). These observations were consistent in the IMD + Varroa condition. As shown by average attrition, bees in the IMD + Varroa condition died or were otherwise removed from conditioning trials at more than twice the rate of those in the control or IMD condition. We believe that attrition affected failure ratios in two ways. First, it shrank our already small sample size of Varroa-latched bees for both the Varroa condition and in the Varroa plus IMD condition. Second, we believe that the pressures of IMD and Varroa together may have favored the strongest and most vigorous of bees, resulting in one single bee in this treatment group to demonstrate PER after conditioning. In consequence, this unexpected result gave us the opportunity to more deeply analyze the obtained attrition data during the conditioning runs.

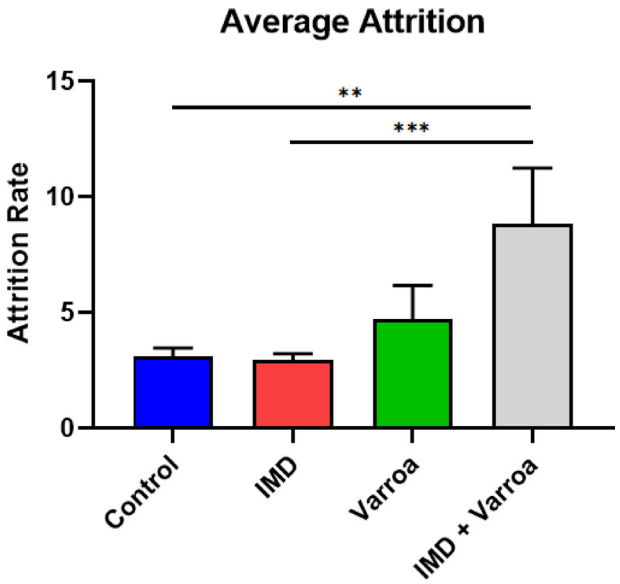

Figure 4. Average attrition across all groups. Average attrition refers to the average number of bees eliminated per trial. Bees were eliminated when they either died during trials or stopped responding with PER. The average attrition of control and IMD bees were comparable, however, when mites were introduced, average attrition was elevated. Bars represent standard error of mean (One-way ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Table 2. One-way ANOVA analysis across groups (Control sucrose solution, 10% LD-50 imidacloprid, latched Varroa mite, and 10% LD-50 imidacloprid plus latched Varroa mite). ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

	Sum of Squares	df	Mean Square	F Value	P
Group	112.8	3	37.60	7.742	$< 0.001^{***}$
Residuals	111.7	23	4.87		

Trial attrition

The training period of honey bees in each condition encompassed 4 five-minute training intervals, or twenty minutes of training. There were minimal, statistically non- significant losses of bees (attrition) in the Control and IMD groups (around 3 bees lost per trial for each group). For treatments of Varroa mites alone attrition was slightly elevated, but again, statistically not significant (4.69 bees lost per trial). However, attrition more than doubled for bees in the IMD + Varroa treatment group (significant with 8.82 lost bees per trial). Within twenty minutes, IMD and Varroa synergistically contributed to either neurological dysfunction or death in individual honey bees at more than twice the rate of those exposed to either sucrose solution or sublethal IMD alone. The magnitude of these synergistic effects was quite unexpected, but strongly suggest that the dual pressure of both sublethal IMD and Varroa mites swiftly weakens both neurological function and overall vitality.

Colony collapse disorder

Sublethal IMD hinders the ability of honey bees to learn and sublethal IMD, together with Varroa, more than doubled the chance of death and or loss of the innate

Table 3. Tukey HSD analysis of Average Attrition across groups (Control sucrose solution, 10% LD-50 imidacloprid, latched Varroa mite, and 10% LD-50 imidacloprid plus latched Varroa mite). There were statistically significant differences across all conditions and parameters. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Group	Mean difference	Lower Bound	Upper Bound	p adj.
IMD - Control	−0.143	−2.977	2.691	0.999
IMD+Varroa - Control	5.718	1.983	9.452	$< 0.01^{**}$
Varroa - Control	1.594	−2.141	5.328	0.645
IMD+Varroa - IMD	5.861	2.300	9.421	$< 0.001^{***}$
Varroa - IMD	1.736	−1.824	5.297	0.542
Varroa - IMD+Varroa	−4.124	−8.436	0.188	0.064

PER upon illiciation by an unconditioned stimulus. Learning and memory are extremely important for the ability of foraging honey bees to find their way back to their hives, and bees disappearing from their hives is a hallmark of CCD (Farooqui, 2013).In the long term, this may lead to a further weakening of the hive as the colonies not only lack those impaired members of the population, but also lack adequate food supplies as they prepare for the winter months. Moreover, the synergistic action of IMD paired with the Varroa mite is effective within the very short time period of twenty minutes, further suggesting that exposed foragers may not return to their hives Studies have shown that field-realistic levels of the neonicotinoid pesticides alter honey bee bumblebee foraging patterns (Henry et al., 2012, Muth & Leonard, 2019; Stanley et al., 2016). In a realistic scenario in which a forager bee either has or attains a latched Varroa mite while foraging and is exposed to a pesticide such as imidacloprid at the nutritional resource, it may only take minutes for this bee to either perish or find itself unable to make its way back to the hive (Abbo et al., 2017; Blanken et al., 2015; Ellis et al., 2010; Farooqui, 2013; Gregorc et al., 2012; Martin, 2001; Nazzi et al., 2012; Sanchez-Bayo et al., 2016; Simon-Delso et al., 2014; Smith et al., 2013; Vanengelsdorp et al., 2009).

Our data further show the detrimental effects of neonicotinoids on honey bee cognition and are suggestive of a devastating synergistic relationship between sublethal neonicotinoid pesticides and Varroa mites in honey bees. These results call for further studies of the joint action of imidacloprid and Varroa in honey bee colonies. Preliminary trials with a limited number of hives confirmed our suspicion that these synergistic factors of IMD + Varroa are also acting at the hive level (personal observation). Further avenues for research include studies of the joint action of other pesticides and stressors such as disease on honey bee cognition and vitality.

Colony Collapse Disorder is complex, and it appears to be a consequence of multiple factors acting synergistically (Aufauvre et al., 2012; Blanken et al., 2015; Doublet et al., 2015; Gregorc et al., 2012; Johnson et al., 2009; Nazzi et al., 2012; Pettis et al., 2012; VAN DER Zee et al., 2015; Zheng et al., 2015). Scientists must continue to study synergism across other factors that are thought to be contributing to CCD, such as climate change, miticides, and disease in order to curb its deleterious effects (Vanengelsdorp et al., 2009, 2010).

Disclosure statement

No potential conflict of interest was reported by the authors.

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