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Environmental Impacts of Proposed Management Options

A Combined LD_{50} for Agrochemicals and Pathogens in Bumblebees (*Bombus terrestris* [Hymenoptera: Apidae])

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Abstract

Neonicotinoid insecticides are the most commonly used insecticide in the world and can have significant sub-lethal impacts on beneficial insects, including bumblebees, which are important pollinators of agricultural crops and wildflowers. This has led to bans on neonicotinoid use in the EU and has resulted in repeated calls for the agrochemical regulatory process to be modified. For example, there is increasing concern about 1) the underrepresentation of wild bees, such as bumblebees, in the regulatory process, and 2) the failure to determine how agrochemicals, such as neonicotinoids, interact with other commonly occurring environmental stressors, such as parasites. Here, we modify an OECD approved lethal dose (LD_{50}) experimental design and coexpose bumblebees (*Bombus terrestris*) to the neonicotinoid thiamethoxam and the highly prevalent trypanosome parasite *Crithidia bombi*, in a fully crossed design. We found no difference in the LD_{50} of thiamethoxam on bumblebees that had or had not been inoculated with the parasite (*Crithidia bombi*). Furthermore, thiamethoxam dosage did not appear to influence the parasite intensity of surviving bumblebees, and there was no effect of either parasite or insecticide on sucrose consumption. The methodology used demonstrates how existing ring-tested experimental designs can be effectively modified to include other environmental stressors such as parasites. Moving forward, the regulatory process should implement methodologies that assess the interactions between agrochemicals and parasites on non-*Apis* bees and, in cases when this is not practical, should implement post-regulatory monitoring to better understand the real-world consequences of agrochemical use.

Key words: neonicotinoid, thiamethoxam, *Crithidia bombi*, *Bombus*, toxicity test

Neonicotinoids are systemic insecticides that are effective at controlling a broad range of pest species such as aphids, whiteflies, and pollen beetles (Simon-Delso et al. 2015). As neurotoxins they target the insect nervous system, acting as agonists of nicotinic acetylcholine receptors (nAChRs) (Moffat et al. 2016). Neonicotinoids can be used as a seed treatment or foliar spray, but are highly persistent in the environment, and may persist in soil for over a year (Goulson 2013, Bass et al. 2015, Bonmatin et al. 2015). Neonicotinoids can therefore contaminate the nectar and pollen of treated crops as well as neighbouring wildflowers, leading to exposure for bees and other flower visiting insects (Stewart et al. 2014, Botías et al. 2016). An analysis of global honey samples revealed that 75% of honey contained at least one neonicotinoid insecticide, with 45% containing two, confirming that bees are routinely exposed to neonicotinoids on a global scale (Mitchell et al. 2017). Such exposure can have significant negative effects on bee colony health, behaviour, and physiology (reviewed by Godfray et al. 2014, Goulson et al. 2015, Pisa et al. 2017, Main et al. 2018, Siviter et al. 2018b) which has led to bans and restrictions on their use globally, most notably in the European Union, where 3 commonly used neonicotinoids (imidacloprid, thiamethoxam, and clothianidin) are now banned. However, neonicotinoid use remains common globally, particularly in the United States and China (Simon-Delso et al. 2015).

Bumblebees are important pollinators of agricultural crops and wildflowers (Willmer et al. 1994, Garibaldi et al. 2013). Bumblebee nests routinely contain a plethora of different parasites and pesticides, suggesting that simultaneous exposure to both parasites and agrochemicals is the norm, not the exception (Goulson et al. 2018, Nicholls et al. 2018). When bees are exposed to multiple stressors, the stressors can interact and become more detrimental than when exposed to a stressor in isolation (Doublet et al. 2015, Tosi and Nieh 2019, Linguadoca et al. 2021). For example, Di Prisco et al. (2013) found that honeybees (*A. mellifera*) exposed to the neonicotinoid clothianidin had a reduced immune defence, which promoted the replication of DWV. Furthermore, coexposure to neonicotinoids and parasites can also increase the likelihood of adult, or larval mortality (Fauser-Misslin et al. 2014, Doublet et al. 2015). Therefore, understanding how, and to what degree, insecticides and parasites interact when bees are simultaneously exposed to both is of utmost importance.

Thiamethoxam is one of the most commonly used neonicotinoids in the world, and is routinely found in the nectar and pollen collected by bumblebees (Botías et al. 2017, Nicholls et al. 2018). *Crithidia bombi* is a trypanosome parasite that is highly prevalent in bumblebee populations, with infection levels ranging from 0 to 80%, depending upon the population and time of year (Shykoff and Schmid-Hempel 1991, Gillespie 2010, Kissinger et al. 2011, Jones and Brown 2014). *C. bombi* exposure when combined with stressors like nutrient limitation or hibernation can significantly reduce bumblebee survival (Brown et al. 2000), colony founding, growth and reproductive output (Brown et al. 2003, Yourth et al. 2008), and can also impair foraging behaviour and learning (Gegear et al. 2005, 2006; Otterstatter et al. 2005) but see (Martin et al. 2018). Previous studies investigating the interactions between thiamethoxam and *C. bombi* have shown various interaction effects (Fauser-Misslin et al. 2014, Fauser et al. 2017, Baron et al. 2017) and simultaneous exposure to both stressors can lower bumblebee queen survival (Fauser-Misslin et al. 2014). This suggests that toxicity assessment of thiamethoxam conducted in the regulatory process could underestimate the potential real-world consequences of thiamethoxam exposure on bumblebees infected with common bumblebee parasites.

Agrochemical regulatory processes differ between nations and governing bodies. The European Union, which is considered to have the most rigorous regulatory process, has a tiered system that is heavily reliant on toxicity tests in the lower tiers to determine whether agrochemicals (pesticides, insecticides, fungicides, herbicides) are hazardous to animals (EFSA 2013, OECD 2017, Sanchez-Bayo and Tennekes 2017). When determining whether an agrochemical is 'bee safe' or not, toxicity tests, such as LD_{50} and LC_{50} tests will be conducted on honeybees (Tier 1) to determine the amount of active ingredient that is required to kill 50% of the population when bees are orally (LD_{50}) or topically (LC_{50}) exposed. Based on this information, further higher tier assessments will, or will not, be conducted (EFSA 2013, Sanchez-Bayo and Tennekes 2017). In its current form bumblebee LD_{50} experiments can be conducted in Tier 1 of the regulatory process, but this is not mandated, and the potential interactions between insecticides and other environmental stressors are not considered (EFSA 2013, Sanchez-Bayo and Tennekes 2017). Regulators and policy makers therefore require methodologies that can be used within the current regulatory framework that 1) assess the impact of agrochemicals on non-*Apis*-bees and 2) test how agrochemicals interact with other environmental factors (EFSA 2013, Vanbergen & Insect Pollinators Initiative 2013, Franklin and Raine 2019; Siviter et al. 2021a, c).

Here we ask if simultaneous exposure to both thiamethoxam and *C. bombi* changes the LD_{50} values of thiamethoxam in bumblebees (*Bombus terrestris*). The acute, oral LD_{50} for bumblebees (*B. terrestris*) and thiamethoxam is known to be 5 ng of active ingredient per bee (EFSA 2015) and so if thiamethoxam and *C. bombi* significantly interact we would predict that this value would either increase or decrease. Our methodology was based on OECD guidelines (OECD 2017) but was modified to incorporate *C. bombi* inoculation. We hypothesised that when used in combination, thiamethoxam and *C. bombi* would lower the LD_{50} value of bumblebees (*B. terrestris*).

Methods

Six bumblebee colonies (*Bombus terrestris audax*) were ordered from Agralan (United Kingdom) and transferred into plastic colony boxes (28 × 22 × 12 cm) and maintained in a laboratory (25°C & 42% humidity), with ad libitum access to sucrose solution (50°Brix) and pollen (Agralan). The faeces of 15 workers from each colony were examined using a phase contrast microscope for common bumblebee parasites (*Apicystis bombi*, *Crithidia spp.* & *Nosema spp.* 400× magnification) (Rutrecht and Brown 2009). All colonies were unparasitized.

Parasite Inoculation

The aim of this experiment was to determine if inoculation with the parasite *C. bombi* changed the LD_{50} of thiamethoxam on bumblebees. To achieve this, we had a total of 21 treatment groups (2 control groups, 1 *C. bombi* group, 9 thiamethoxam groups and 9 groups exposed to both thiamethoxam & *C. bombi*; see Supp Table S1 [online ony]). We had 40 bumblebees in each treatment group and all bees were individually housed in Nicot cages (see below for details).

To create a *C. bombi* inoculum the faeces of 30 workers were taken from a commercial colony infected with multiple strains of *C. bombi*. These strains were originally isolated from bumblebee queens caught at Windsor Great Park (United Kingdom) and then propagated through commercial colonies in the laboratory. Faeces of infected workers from these colonies were placed in an Eppendorf tube containing 0.9% Ringer solution and centrifuged at 0.8 g for 2 min. The supernatant was removed, and clean Ringer solution added, a process that was repeated 7 times (8 times in total) to purify and concentrate the preinoculum (following a modified triangulation protocol based on [Cole 1970]). Cell counts were carried out using a Neubauer improved haemocytometer to determine the concentration of *C. bombi* cells. *The C. bombi* preinoculum was then combined with sucrose (50°Brix) to create an inoculum of 1,000 cells/ul.

Individual bumblebees from all treatment groups (see Supp Table S1 [online only] for list of treatment groups) were taken from queenright colonies, and individually housed in Nicot cages (148 × 130 × 11 mm) with ad libitum access to 50°Brix sucrose through a 1 ml syringe.

Prior to inoculation, workers from all the treatment groups underwent a starvation period of 3 h (Logan et al. 2005) after which all bees were removed from their Nicot cages and placed in an individual vial (9 × 2.5 cm). The inoculum was presented to each individual to drink with a 10 µl droplet of 50°Brix sucrose solution containing approximately 10,000 *C. bombi*. A dose of 10,000 cells has been determined to produce a reliable and high rate of infection (Ruiz-González and Brown 2006). A period of 15 min was allowed for the individual to consume the inoculum. Workers from control and thiamethoxam only treatment groups underwent the