

What's really out there? Investigations into the effects of pesticides and pathogens
on bee health

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Abstract

Bees provide crucial pollination services to both cultivated and wild plants. In recent decades there were large declines in the populations of several native bee species and in the health of managed honey bee colonies. Neonicotinoid pesticides were suspected to negatively impact the health of native and managed bees, although this topic was hotly contested. Unfortunately, we lacked the knowledge regarding the typical duration that bees were exposed to neonicotinoids. Therefore, I first quantified the dose, duration, and types of chemicals honey bee colonies were exposed to near agricultural corn fields (Chapter 2). I found that honey bee colonies were exposed to a cocktail of chemicals, out of which neonicotinoids were the most likely to pose a health risk. I also found that honey bees were exposed to neonicotinoids for up to four months – the majority of the honey bee’s active season. I then performed a controlled experiment, where I exposed honey bee colonies to neonicotinoids in a manner that mimicked the field exposure (Chapter 2 and 3). I found that this field realistic exposure to neonicotinoids reduced worker life span, increased queenlessness, and impacted both social and innate immunity. Then, I studied the genetic underpinnings of neonicotinoid sensitivity in honey bees (Chapter 4). I found that survival after neonicotinoid exposure was heritable and was associated with natural polymorphisms found in two detoxification genes. Although survival after exposure is a convenient trait to study under laboratory conditions, it offers little insight into the plethora of phenotypes sublethal neonicotinoid exposure can affect. Thus, I used transcriptomics to look into the effects of field and field realistic exposures to neonicotinoids on the brain gene expression of forager and nurse honey bees (Chapter 5). I found that neonicotinoids affected the brain states of foragers and nurses in a different manner, possibly reflecting a consequence of developmental alterations. I then applied transcriptomics tools to a declining bumble bee, *Bombus terricola* (Chapter 6). I discovered that bumble bees near agriculture had signatures of stress due to pesticides and pathogens. Overall, I found that neonicotinoids and agricultural landscapes put undue stress on the health of bees. My research also highlights the importance of conducting season-long studies and quantifying multiple stressors and phenotypes at a time in ecotoxicological research.

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Chapter 1 Overview

Bees play a significant role in pollination of agricultural crops (Rucker, Thurman, and Burgett 2012) and non-agricultural plants (Potts et al. 2016). These pollination services are provided by both managed and wild bees (Garibaldi et al. 2013, Greenleaf and Kremen 2006, Kremen, Williams, and Thorp 2002). In recent years, many bumble bee species have experienced steep population declines (Cameron et al. 2011, Williams and Osborne 2009, Colla and Packer 2008, Colla et al. 2012). At the same time, high honey bee colony losses have been reported in North America, Europe, and Middle East (Cox-Foster et al. 2007, Anderson and East 2008, Hayes Jr, Underwood, and Pettis 2008, Evans et al. 2009, Brodschneider, Moosbeckhofer, and Crailsheim 2010, Potts et al. 2010, Van der Zee, Pisa, Andonov, Brodschneider, Charriere, Chlebo, Coffey, Crailsheim, et al. 2012). These pollinator losses threatened both food security and critical ecosystem services (Steffan-Dewenter, Potts, and Packer 2005, Klein et al. 2007).

Determining the drivers behind pollinator declines is critical for both devising effective conservation plans for at-risk pollinators (Potts et al. 2016), and for applying appropriate interventions for managed pollinators (Grozinger and Zayed 2020). Decades of research has identified the most important drivers behind pollinator declines. They are habitat loss, pesticide use, decreased resource diversity, invasive species, pathogen spread, and climate change (Potts et al. 2010, Cameron and Sadd 2020). Unfortunately, much of the research linking these potential drivers of declines are correlational (Cameron and Sadd 2020). Additionally, although these drivers rarely act in isolation, they are often studied in isolation (Potts et al. 2010, Vanbergen 2013). Moreover, only a subset of these stressors may be impacting any given population at a time (Szabo et al. 2012). These limitations have a dramatic effect on our ability to manage the health of pollinators.

One of the suspected causes of bee decline is unintended exposure to pesticides (Mullin et al. 2010, Krupke et al. 2012). Neonicotinoids (NNIs) are the most commonly used insecticide in the world (Goulson 2013). They act as nicotinic acetylcholine receptor (nAChR) agonists and have a generally low toxicity to mammals (Tomizawa and Casida 2005). They are systemic in nature, which enables NNIs to be taken up via the roots for transport to other parts of the plant

(Elbert et al. 1991, Elbert et al. 2008). This property contributed to NNIs dominating the seed treatment market (Elbert et al. 2008). NNIs are effective against a wide variety of sucking, biting, and chewing insects without the need to spray (Kundoo et al. 2018). They also have an alternative mode of action to the traditionally used organophosphate, carbamate, and pyrethroid insecticides, thus NNI use prevents the buildup of resistances in the pests (Kundoo et al. 2018). These properties made NNIs attractive for farmers resulting in massive use of these products since their commercial availability in the early 1990's (Goulson 2013).

The number of papers in the last two decades on bee decline and conservation has increased dramatically, with many of them focusing on NNIs (Cameron and Sadd 2020). Even so, there has been a controversy regarding their actual role in bee mortality even in the most studied species, the honey bees (Blacquiere et al. 2012, Goulson 2013, Mullin et al. 2015, Carreck and Ratnieks 2014, Cresswell, Desneux, and VanEngelsdorp 2012).

Several studies that treated bees in laboratory setting with sublethal doses of NNIs (typically 1 to 10 parts per billion, ppb) documented negative effects on foraging (Gill, Ramos-Rodriguez, and Raine 2012, Ramirez-Romero, Chaufaux, and Pham-Delegue 2005, Schneider et al. 2012), learning (Decourtye et al. 2004, Ramirez-Romero, Chaufaux, and Pham-Delegue 2005), innate immunity (Retschnig, Neumann, and Williams 2014, Di Prisco et al. 2013) and colony fitness (Whitehorn, O'Connor, et al. 2012, Sandrock et al. 2014, Gill, Ramos-Rodriguez, and Raine 2012, Chensheng, Warchol, and Callahan 2014). However, these studies have been criticized for using unrealistic doses and duration of exposure (Carreck and Ratnieks 2014, Dicks 2013, Eisenstein 2015, Cresswell 2011). At the same time, several field studies have failed to show a negative effect of NNIs on honey bee colonies (Cutler and Scott-Dupree 2007, Cutler et al. 2014, Rundlof et al. 2015, Pohorecka et al. 2012), although these studies also themselves been criticised for lacking the statistical power to detect sublethal effects (Cresswell 2011). Other lines of inquiry concluded that NNIs are unlikely to be the cause of honey bee colony losses (Cresswell, Desneux, and VanEngelsdorp 2012, Staveley et al. 2014), which further casted doubt over the role of NNIs on pollinator declines.

There is a large gap in knowledge that is critical for resolving the NNI debate. We lack data on the amount of NNIs present in nectar and pollen that is collected by bees under field conditions throughout the field season (Carreck and Ratnieks 2014, Dicks et al. 2013). Without such data, laboratory experiments can be dismissed as pointing out the obvious – high doses of insecticides may kill bees in lab experiments, but these high doses would – supposedly – be rarely encountered in the field. Thus, first we must understand how and for how long bees are exposed to pesticides in the field. Then, we need to mimic this exposure in a controlled manner to determine what kind of effects this exposure has on bees. At the same time, we must examine other variables that might contribute to the variation of responses we find in the literature, such as bee genetics. Finally, we need to examine the bias we inadvertently introduce when we pick certain traits to study for ecotoxicology, as it may be that our choice of traits to measure can influence our conclusions regarding the safety of pesticides to pollinators (Grozinger and Zayed 2020, Trapp, McAfee, and Foster 2017).

Do neonicotinoids impact honey bee health (Chapters 2 and 3)

First, I set out to determine how and for how long honey bees are exposed to NNIs in an agricultural system where NNIs have been suggested to have a substantive influence on honey bee health. In 2012-2013 beekeepers in Ontario and Quebec reported unusually high mortality rates, which coincided with the planting of corn (PMRA 2013). Practically all of the corn in Canada is grown from NNI-treated seeds (Stewart and Baute 2013). Moreover, corn production represents the largest use of arable land in Canada (Hamel and Dorff 2014). Therefore, I chose to focus on honey bee colonies located near corn fields.

In Chapters 2 and 3, I documented the levels, duration, and route of exposure to NNIs and other agrochemicals, as well as their associated health impacts on honey bee colonies. I found that honey bee colonies near NNI-treated corn fields were exposed to NNIs for up to four months (Tsvetkov et al. 2017) and that this exposure was associated with impacts on the bees' social and innate immunity. Realistic experiments showed that NNI exposure increased worker mortality and increased queenlessness over time, as well as reduced social and innate immunity. I also discovered that the acute toxicity of NNIs doubles in the presence of a commonly found

fungicide. This work demonstrated that field exposure to NNIs can reduce honey bee health near corn.

Heritability of neonicotinoid susceptibility (Chapter 4)

A large scale study on the effects of NNIs on honey bees across Europe found country-specific effects (Woodcock et al. 2017). The authors note that these inconsistent results suggest that there are interacting factors. Indeed, several researchers found an interaction effect between NNIs and pathogens (Sánchez-Bayo et al. 2016), nutrition (Archer et al. 2014), and other agrochemicals (Tsvetkov et al. 2017). These environmental interactions could explain the inconsistencies found in the literature (Woodcock et al. 2017).

However, one important factor is missing in the current discussion: bee genetics. Woodcock et al. (2017) used different honey bee stocks in each country they studied, yet they did not mention how genetics may have influenced their results. There is some tangential evidence that bee genetics can modulate NNI toxicity (Sandrock et al. 2014, Rinkevich et al. 2015) although heritability (i.e. a genetic predisposition) of NNI susceptibility has yet to be proven.

In Chapter 4, I examined whether a worker's genetics had an effect on her survival following exposure to an NNI and whether natural variation in specific detoxification genes can explain the variation in survival. I found that genetics had a significant effect on survival and estimated the broad sense heritability as 37.8%. I then sequenced the primary NNI detoxification genes (Manjon et al. 2018) and found associations between survival and haplotypes of two of the three genes. This study showed that there is a substantial genetic component to NNI susceptibility following lethal exposure, and that the natural variation found in two detoxification genes may be partially responsible.

Transcriptomic insights into bee health (Chapter 5 and 6)

Certain traits, such as survival after exposure, are convenient for laboratory studies, but they are limited in terms of capturing the full effects toxins can have on an organism.

Additionally, placing bees in laboratory cages removes them from their natural environment and alters their physiology (Alaux et al. 2009, Grozinger et al. 2003). Although studying bees under laboratory conditions is easier than studying bees in the field, this leads to a ‘streetlight effect’, which limits our understanding (Battaglia and Atkinson 2015).

One way to reduce the ‘streetlight effect’ is to quantify a very large number of phenotypes. RNA sequencing (RNAseq) provides a way to simultaneously quantify the expression of thousands of genes in response to a stressor (Grozinger and Zayed 2020, Lozier and Zayed 2017). Moreover, given the large body of knowledge linking genes with biological processes and molecular functions in insects (Kanehisa and Goto 2000, Ashburner et al. 2000, Consortium 2019), it is possible to use transcriptomics to provide insight about the typical traits that are impacted by a particular stressor.

In Chapter 4, I looked for differentially expressed genes in the brains of honey bee workers following natural NNI exposure from the field and following experimental exposure. I found that field and field realistic exposure affected gene expression differently in nurses and foragers. Foragers, who carry out food collection outside the hive, had shifts in gene expression associated with cognition and development, while nurses, who take care of the brood inside the hive, had shifts in gene expression associated with metabolism. This study was able to provide insight into the effects of field exposure to NNIs on the brain states of both foragers and nurses.

In Chapter 5, I investigated the gene expression in the abdomens of the declining *Bombus terricola* (Cameron et al. 2011, Colla and Packer 2008). I found that workers collected near agricultural areas had upregulated genes associated with pesticide and pathogen stress. In addition, I was able to detect five pathogens in the abdomens, three of which are common in managed honey bee and bumble bee colonies. This study landed support for the pathogen-spill-over hypothesis, which stipulates that managed bees act as a pathogen reservoir that spills into the wild bee population (Colla et al. 2006), and implicated pesticides in the decline of *B. terricola*.

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Neonicotinoid insecticides (NNIs) are highly toxic to insects (Tomizawa and Casida 2005) and have been implicated in the decline of pollinators (Goulson 2013, Rundlof et al. 2015) and other wildlife (Hallmann et al. 2014). Many studies that experimentally treated bees with sublethal doses of NNIs documented negative effects on bee health (Gill, Ramos-Rodriguez, and Raine 2012, Ramirez-Romero, Chaufaux, and Pham-Delegue 2005, Di Prisco et al. 2013, Sandrock et al. 2014). However, these studies have been criticized for using unrealistic doses and duration of exposure (Carreck and Ratnieks 2014). Although recent surveys have quantified agrochemical residues in several environments (Mullin et al. 2010, Krupke et al. 2012, Long and Krupke 2016), they have done so during one or two time periods in the season. We thus lack knowledge of the typical duration that pollinators are exposed to NNIs—a fundamental parameter in ecotoxicology and one that is central to the current debate regarding the safety of NNIs. Addressing this knowledge gap is essential for developing evidenced-based policy on the use of NNIs.

Honey bees (*Apis mellifera*) experienced high colony mortality in Indiana, Ontario, and Québec’s corn-growing regions early this decade (Krupke et al. 2012, PMRA 2013). Corn production represents the largest use of arable land in North America (Hamel and Dorff 2014), and almost all corn is grown from NNI-treated seeds (Stewart and Baute 2013). The timing of honey bee deaths in Ontario, Québec, and Indiana, along with the presence of NNI residues in dead bees and hives in the spring (Krupke et al. 2012, PMRA 2013), suggested that NNI-contaminated dust generated during seeding was the main route of acute exposure (PMRA 2013). However, in the absence of season-long data, it is impossible to rule out that honey bees are also chronically exposed to sublethal levels of NNIs after planting. Here, we present the findings of a 2-year study that quantified the duration and magnitude of NNI exposure in Canada’s corn-

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growing regions and experimentally evaluated the influence of field realistic NNI exposure on honey bee health.

We quantified agrochemicals in 55 bee colonies that were randomly allocated to five apiaries close to corn (exposed sites, <500 m) or six apiaries away from agriculture (unexposed sites, >3 km) in 2014. We conducted our study after Canada mandated the use of seed fluency agents (PMRA 2014) to reduce NNI-contaminated dust generated during corn planting. We detected 26 different agro-chemicals that included miticides, fungicides, herbicides, NNIs, and other insecticides (Table S 2.1 and Table S 2.5). NNIs included clothianidin, thiamethoxam, imidacloprid, and acetamiprid. We detected agrochemicals in significantly more samples in exposed, relative to unexposed, sites (Welch's t test: $t_{7,92} = -3.48, P = 0.008$). NNIs were detected in significantly more time periods in exposed, relative to unexposed, sites ($t_{8,02} = 5.88, P < 0.001$); and the period of contiguous exposure to NNIs was longer in exposed (83.4 ± 13.47 SEM days), relative to unexposed, sites ($22.7 \pm 10.7; t_{8,07} = 3.53, P = 0.007$) (Figure 2.1 and Figure S 2.1). Honey bee colonies near corn are thus chronically exposed to NNIs for a substantial proportion of the active season in temperate North America.

Agrochemicals and NNIs were most prevalent in pollen (Figure S 2.2). However, pollen from seed-treated crops was rarely found in NNI-positive samples (1 in 21 for corn and 5 in 21 for soy bean), and, when present, it constituted a minute proportion of the pollen grains (0.2% for corn and a mean of $0.6\% \pm 0.22$ SEM for soybeans). Most pollen from NNI-positive samples originated from non-target plants common in Ontario and Québec (Table S 2.2). Our findings are consistent with recent studies that documented NNIs in pollen from bee-attractive wildflowers in the United Kingdom and USA (Long and Krupke 2016, Botías et al. 2015).

Although we detected many agrochemicals in 2014, the concentration of NNIs found in bee samples combined with their high toxicity (Table S 2.3) rendered them the most likely compounds to influence honey bee health (Figure S 2.3). We carried out an experiment to investigate the effects of clothianidin exposure—the most common NNI found in our study—on honey bees by chronically treating colonies with an artificial pollen supplement containing clothianidin over a 12-week period in 2015. We approximated field realistic exposure by treating

colonies with progressively smaller concentrations of clothianidin, mirroring typical levels found in pollen collected from naturally exposed colonies in 2014 (Figure S 2.4).

We first investigated the effect of clothianidin exposure during larval development on adult traits by removing sealed brood from treated and control colonies after the first 3 weeks of exposure and tagging the emerging workers with radio frequency identification chips before introducing them into a common untreated observation hive. We observed age by treatment differences in the number and duration of flights taken by experimental workers (Figure S 2.5), consistent with previously documented effects of NNIs on navigation in honey bees (Henry et al. 2012). The treated workers, which were exposed to contaminated brood food during the first 9 days of their lives as larvae, had a 23% reduced life span relative to controls (Figure 2.2A) [$F_{(1,7)}=5.78, P=0.047, n=93$]. The presence of sublethal levels of NNIs in colony pollen for 3 to 4 months is thus expected to shorten the life span of many cohorts of workers produced in the spring and summer. The high forager mortality brought upon by chronic sublethal NNI exposure can, in theory, lead to cycles of precocious foraging that reduce colony fitness and cause colony failure (Perry et al. 2015).

We quantified hygienic behavior and the presence of a laying queen in treated colonies and control colonies over the course of our 12-week experiment. We hypothesized that phenotypic effects of exposure—if they exist—should manifest as a function of exposure time (Cleophas and Zwinderman 2012) (i.e., significant treatment by time interactions). We detected a significant treatment by time interaction on hygienic behavior [$F_{(1,23)}=14.86, P=0.001, N=34$]; the average hygienic behavior of clothianidin-treated colonies decreased over time but that of control colonies did not (Figure 2.2B). We observed a similar pattern in the field in 2014, where exposed colonies near corn had significantly lower hygienic behavior relative to unexposed colonies at the end of the season (Figure 2.2C) [$F_{(1,48)}=6.42, P=0.015, N=50$]. Our study is similar to a recent study that found an association between chronic exposure to imidacloprid and reduced hygienic behavior (Wu-Smart and Spivak 2016). Our findings indicate that NNIs impair the honey bee's social immune system.

We also observed a significant treatment by time interaction on queenlessness [generalized linear mixed model (GLMM), $z=2.242, P=0.025, N=54$] whereby the presence of a laying queen declined over time in the clothianidin-treated group (Figure 2.2D). Strong colonies, like many of our controls, typically become queenless in midsummer during swarming season, but then rapidly rear and sustain a replacement queen. However, that pattern of queen loss in treated colonies peaked well after Ontario's swarming period, and most treated colonies were not able to rear replacement queens by the end of our experiment. Our finding is consistent with a recent study (Williams et al. 2015) that documented NNI effects on queen mortality and reproductive physiology. The association between chronic clothianidin exposure and queenlessness is expected to have major consequences on colony fitness, because colonies that are unable to rear replacement queens eventually perish (Harman, Shimanuki, and Flottum 2005).

Finally, we studied possible interactions between NNIs and co-occurring agrochemicals on bee health. Clothianidin was most commonly found with herbicides (50%), of which linuron was the most common (31%). Thiamethoxam was commonly found with fungicides (79%), of which boscalid was the most common (45%). We investigated how field realistic doses of boscalid (mean 497 ppb in pollen) and linuron (mean 7.3 ppb in pollen) influenced the 24-hour oral toxicity of NNIs to honey bee workers. Boscalid and linuron did not, on their own, cause mortality to honey bees at field realistic doses (0% 24-hour mortality in triplicate trials). Linuron did not influence the median lethal dose (LD_{50}) of clothianidin [generalized linear model (GLM), $z=-0.700, P=0.487, N=45$] or thiamethoxam (GLM, $z=0.611, P=0.544, N=45$) (Figure 2.3). However, boscalid significantly reduced the LD_{50} of clothianidin (GLM, $z=2.317, P=0.026, N=45$) and thiamethoxam (GLM, $z=2.060, P=0.046, N=45$) (Figure 2.3). Both NNIs became nearly twice as toxic to honey bees in the presence of field realistic levels of boscalid.

Our study demonstrates that honey bees in corn-growing regions of Canada are exposed to toxicologically significant levels of NNIs for the majority of the active bee season despite the mandated use of dust reducing seed lubricants during planting. Pollen from nontarget plants represents the primary route of exposure to NNIs in our study. Like most bees, honey bees are diet generalists, and it is thus expected that native bees found in Canada's corn-growing regions

would be similarly chronically exposed to NNIs. We carried out experiments that approximated field realistic exposure and found biologically significant effects of clothianidin exposure on honey-bee worker mortality, hygienic behavior, and the abilities of colonies to sustain a laying queen over time. Finally, we uncovered that the acute toxicity of NNIs to honey bees increases in the presence of field realistic levels of a common fungicide. Our findings indicate that chronic NNI exposure reduces the health of honey bee colonies near corn crops.

Supplementary Materials for Chronic exposure to neonicotinoids reduces honey-bee health near corn

Materials and Methods

2014 Field Study

Overview: In Canada, 90% of corn is produced in the provinces of Ontario and Québec (Hamel and Dorff 2014). Nearly 100% of the corn seed is treated with NNI, and corn is commonly grown near soybeans that are often (ca. 60%) treated with NNIs as seed (OMAFRA 2015). We quantified agrochemicals in bees and hive food stores from 55 colonies that were randomly allocated to 5 apiaries close to corn (<500 m, hereafter called exposed sites) or 6 apiaries away from agriculture (>3 km, hereafter called unexposed sites) in 2014. The field study was not designed to determine the effects of exposure on bees, but rather to quantify the magnitude of agrochemical exposure using honey bee colonies as environmental sentinels.

Study sites: We studied a total of 5 exposed sites (i.e., <500 m from corn; 4 in Ontario, 1 in Quebec) and 6 unexposed sites (>3 km away from corn; 4 in Ontario, 2 in Quebec). The sites were chosen based on the following criteria: (i) presence of an apiary that was either adjacent (<500 m) or far away (>3 km) from corn crops; (ii) sites were far apart from each other (>3 km); (iii) sites were accessible by car; and (iv) one-way travel between sites and York University or Université Laval was 4 hours or less to facilitate sampling multiple sites within a reasonable time period. We had initially picked two exposed sites in Quebec, but the farms adjacent to one of these sites were not planted in the spring of 2014 and we removed this site from our experiment. Although we placed exposed colonies adjacent to corn, corn and soy are planted in very close proximity in Ontario and Québec, and thus the agrochemical inputs in exposed sites are derived from both corn and soy crops. We used Canada's public crop inventory map for 2014 to study land use patterns within a 3-km radius of our exposed and unexposed sites (Figure S 2.6). As expected, the average proportion of land covered by corn or crops was significantly higher in the exposed sites relative to the unexposed sites ($p < 0.01$ for both tests). The unexposed sites in Ontario were mostly surrounded by urban areas, while the unexposed sites in Quebec were mostly surrounded by forest. All exposed sites had a high proportion of land used for corn and

other crop production. We do not provide exact GPS coordinates for apiaries to protect the identity of the beekeepers and farmers involved.

Honey bee colonies: We used 55 standard Langstroth hives (single 9-5/8" D x 19-7/8" L x 16-1/4" W chamber with 10 frames) from three commercial beekeepers (two in Ontario, each providing 20 colonies, and one in Québec, providing 15 colonies). The colonies were inspected early in the spring of 2014 to ensure that they were healthy and had a laying queen. We randomly allocated the 40 colonies from Ontario (5 colonies per site) to either exposed sites in Middlesex county, Wellington county (2 sites), and Lambton county or to unexposed sites in York region, the city of London, and the city of Toronto (2 sites). We randomly allocated the 15 Québec colonies (5 colonies per site) to either the exposed site in the Montérégie region or to unexposed sites in the Estrie region (2 sites). Colonies were moved into exposed and unexposed sites prior to planting. We actively managed the colonies during the season, including adding empty honey 'supers' (i.e., a shallow 5-11/16" D x 19-7/8" L x 16-1/4" W chamber) and removal of swarm cells, but we did not chemically treat the colonies to control hive pests or diseases. Queenless colonies were re-queened or given frames of young larvae to raise new queens.

Collections: Colonies were sampled during six time points: in early May (pre-planting in Ontario and Québec), late May (post-planting in Ontario but pre-planting in Québec), June (post-planting in Québec), July, August, and September. In each time point, we quantified agrochemicals in dead bees, foragers who collect pollen and nectar for the colony, nurses who feed the brood, old larvae, pollen and nectar (i.e., 6 sample types at 6 time points for a total of 36 samples/site). During each sampling period, we collected approx. 1 gram of pollen, nectar, larvae, nurses, foragers and dead bees from each colony and pooled the samples by site for pesticide residue analysis. All samples were kept on dry ice in the field and were stored at -80 °C until analysis. Pollen: 5 g of freshly deposited pollen (lightly packed and dry) were collected from each site. The pollen was sampled from hive comb using a small spatula. On average, this involved sampling 150 to 200 cells per site (30 to 40 pollen cells per colony). This lightly packed pollen was likely collected by foragers within a 2-week period prior to the sampling date (Vásquez and Olofsson 2009). Nectar: 5 mL of nectar was collected per site (1 mL per colony) from comb, using a 1-cc syringe. The nectar was likely collected by foragers within a 5-day

period prior to the sampling date (Winston 1991). Larvae: 100 L4-stage larvae were collected per site (20 per colony) using a small spatula. Nurses: 50 nurse bees were collected per site (10 per colony). Nurses were identified as bees on uncapped brood frames that actively insert their head into cells containing larvae. Foragers: 50 forager bees were collected per site (10 per colony). We identified foragers as they returned into the hive with pollen on their legs. Dead bees: 65 dead bees were collected per site (13 per colony). We sampled recently deceased bees by placing a 1-m² white sheet in front of the hive entrance and collected the dead bees found on the sheet 24 hours later.

Hygienic behavior: During each sampling period, we picked a capped frame containing red-eyed brood from each colony to test for hygienic behavior using the freeze kill assay (Spivak and Downey 1998). A section of brood 7 cm in diameter was photographed, frozen using liquid nitrogen, allowed to thaw, and placed at the third frame position within the colony with the freeze-killed brood facing inward (i.e. towards colony center). After 24 hours, the frozen section of the frame was photographed to estimate the proportion of uncapped and removed dead brood. We could not carry out hygienic testing on brood-less colonies; these colonies were excluded from relevant analyses.

Residue Analysis

Samples were assigned generic serial numbers and shipped on dry ice to the Agriculture and Food Laboratory at the University of Guelph for multi-residue analysis. The analytical lab was blind to the exposure status of samples. A two gram sub-sample was taken from each sample to quantify a total of 231 agrochemicals that were extracted using the QuEChERS method (Schenck and Hobbs 2004). Briefly, samples were extracted into 1% acetic acid in acetonitrile in the presence of anhydrous sodium acetate and magnesium sulfate. The supernatant was then evaporated and diluted with methanol and 0.1 M ammonium acetate. Sample extracts were analyzed using liquid chromatography coupled with electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) following established methods (Wang and Leung 2008). Given our monthly sampling, we estimated the period of contiguous exposure in the spring and summer as: (15 days + last contiguous NNI detection) – (first contiguous NNI detection – 15 days), where

15 days represents the approximate midpoint between two sampling periods. We think this is more realistic than using the observed contiguous period of exposure, which assumes that NNIs instantaneously increase from zero to quantifiable values on the day they were detected, then decrease to zero immediately after they are last detected.

Palynology

Pollen subsamples were assigned generic serial numbers and sent to Laboratoire BSL (Quebec) for identification and quantification of pollen types using standard methods (Barth et al. 2010, LOUVEAUX, Maurizio, and VORWOHL 1970, Von Der Ohe et al. 2004). Briefly, 2 g subsamples of collected pollen were added to 50 ml tube containing 40 ml distilled water. The pollen was vortexed and left overnight to permit dissolution. After dissolution, samples were vortexed for 2 min, then a drop of the pollen preparation was transferred using a glass pipette to a slide on a warm histology plate. A small cube of glycerin jelly stained with basic fuchsin was added to the preparation and homogenized by stirring delicately with a mounted needle. A cover glass was applied to the preparation and the slide was sealed with melted paraffin. This procedure was repeated to obtain a second slide for each sample. The slides were left to dry at room temperature before microscopic analysis at 1000X magnification. Pollen grain identification began near the center then towards one side of the slide (Barth et al. 2010). The analysis continued following an S pattern until 500 pollen grains (or palynomorphs) were identified. The number and proportion of each pollen form identified (species, genus, family or pollen type) from each sample was used for analysis.

2015 Experimental Study

Overview: Our field study in 2014 established that honey bees in Canada's corn-growing regions encounter sublethal residues of NNIs for 3 to 4 months, mostly through contaminated pollen. Clothianidin was the most common NNI detected in our study, representing 53% of all NNI-contaminated samples. To investigate the impact of clothianidin exposure on honey bee health, we chronically treated honey bee colonies with an artificial pollen supplement containing clothianidin over a 12-week period in 2015, and compared them to colonies that were fed clothianidin-free supplements. We approximated field realistic exposure by treating bees with the

average amount of clothianidin found in pollen during the average period of contiguous exposure found in exposed sites in 2014.

Experimental setup: We used 10 standard honey bee colonies housed at the York University Research Apiary (Toronto, Ontario). The Research Apiary is far away ($>>3$ km) from agriculture. These colonies contained two deep chambers separated by a 1-inch spacer; the bottom chamber containing brood and food stores and the upper brood chamber containing empty foundation frames to allow colonies to grow. These colonies were randomly assigned to one of two groups (1 or 2) prior to the experiment. The weight of the colonies between the groups was not significantly different before the start of the experiment (t test: $t=-0.24$, $df=6.9$, $p=0.816$). The colonies were free from any visible signs of disease. Pollen traps were placed on all colonies and activated at the beginning of the experiment. Every 2-3 days (Mondays, Wednesdays and Fridays) each colony received a fresh 200 g artificial pollen patty, which was placed between the two chambers. We used artificial pollen patties in lieu of real pollen because real pollen often contains a number of agrochemicals, even when collected from natural areas, as shown herein and by others (Mullin et al. 2010, Krupke et al. 2012, PMRA 2014, Samson-Robert et al. 2014, David et al. 2016). The artificial pollen patties were mixed using 56% FeedBee pollen supplement (Bee Processing Enterprises LTD.), 33% sugar syrup and 11% water. The experimental treatment patties were spiked with pure analytical clothianidin (99.9%, Pestenal, Sigma-Aldrich) to make the corresponding concentrations: week one, 4.9 ppb; week two, 4.2 ppb; week three, 3.3 ppb; week four, 2.2 ppb; week five, 2.0 ppb; and up to week twelve, 2.0 ppb. After the spiked and control pollen patties were made, another researcher not involved with the project labelled the patties as group 1 or 2 and provided the labelled patties to the experimenters; this ensured that the experimenters in 2015 were blind to the identity of treated colonies and control colonies. The lead author (N.T.) carried out statistical analyses on the raw data while blinded; she was unblinded in November 2015 after the analysis was complete.

The treated and control colonies were inspected every 2 weeks. Shallow honey supers were added as needed. Colonies were not treated with any chemicals to control pests and disease. During each inspection, we determined the presence of a laying queen by either observing the queen directly or observing her eggs. We carried out hygienic tests using the freeze-kill assay as

described above. Unlike the 2014 field studies, the colonies were not actively managed to prevent swarming or to re-queen queenless colonies. In cases of queen death, the colonies were allowed to naturally rear replacement queens from emergency queen cells; we did not remove queen cells during colony inspections and thus did not interfere with colonies' abilities to naturally requeen. The queen of one colony was accidentally crushed during the first inspection. Because the cause of queen mortality was clearly not natural or associated with the experiment, we decided to exclude this colony from our study – we later discovered that this colony was one of the treated colonies.

Validation of experimental doses: We carried out multi-residue testing on 10 pollen patties (5 treatment, 5 control), as described above. We confirmed that clothianidin in the pollen supplements was within ± 0.3 ppb of the target dose (correlation between observed and target: $r^2=0.93$, $df=2$, $p=0.03$) and the control patties were free of clothianidin. All patties tested contained trace levels of two fungicides – likely used as preservatives: dimethomorph (mean \pm SEM in samples: 1.05 ± 0.03 ppb) and boscalid (2.55 ± 0.12 ppb). Both compounds are not toxic to honey bees at these trace levels (LD_{50} : 308,000 ppb and 1,550,000, respectively (Mullin et al. 2010). Dimethomorph is not known to interact with clothianidin, and boscalid is not known to interact with clothianidin at the observed levels, which are more than an order of magnitude lower than that used in our interaction experiments (2.55 ppb vs. 497 ppb).

Effects on individual workers: After 3 weeks of exposure, we removed late-stage pupae from control and experimental colonies and placed them in a 33°C incubator. Newly emerged bees were tagged with a RFID chip (mic3® -TAGs, MicroSenys RFID in Motion). Bees outfitted were first immobilized by chilling for less than one minute, before the chip was attached to the bee's thorax using non-toxic glue. After recovery, the unique ID of each RFID chip was recorded using the iID® PEN-USB mini (MicroSenys RFID in Motion) along with the bee's colony of origin. After this process, tagged bees were introduced to an eight-frame observation colony with an entrance tunnel that was rigged with two RFID readers (iID® MAJA reader modules 4.1, MicroSenys RFID in Motion) arranged successively 3 cm apart. The RFID readers were connected to an iID® HOST type reader (MAJA 4.1, MicroSenys RFID in Motion), which

captured the date using the iID ® DateCapture Software (MAJA 4.1, MicroSenys RFID in Motion).

LD₅₀ interaction experiments

We tested the oral LD₅₀ of several combinations of agrochemicals fed in solution to worker honey bees confined to laboratory cages. Solutions were made in 50% sucrose with pre-dilution in acetone as required (no solution contained greater than 1% acetone). We tested the effect of 5 different doses of clothianidin and thiamethoxam (99.6%, Pestenal, Sigma-Aldrich), assessed in triplicates, alone or in combination with boscalid (99.9%, Pestenal, Sigma-Aldrich) or linuron (99.7%, Pestenal, Sigma-Aldrich) and we calculated the LD 50 using a standard method (Randhawa 2009). Briefly, the probit mortality values are plotted against the log-dose and the dose corresponding to 50% mortality was estimated. The cage experiments were conducted as follows: Workers were collected from a non-experimental hive from the York University Research Apiary. Bees were shaken from honey frames into a plastic container and brought into the lab. The bees were placed in containers (10 per container), given 50% sugar in excess, and placed overnight in a 25°C incubator. In the morning, the sugar feeder was removed for 2 hours, after which, a pre-weighed 200 µL feeder containing the target agrochemical(s) was placed in each box for 4 hours. The feeder was then re-weighed and mortality was assessed before placing 50% sugar in excess. After 24 hours, mortality was assessed in each box and the bees were frozen. All of the bees were placed in a 25°C incubator for the day of the experiment. During each day the experiment was performed, at least 3 appropriate controls with less than 10% mortality after 24 hours were present. Our estimated LD₅₀ for clothianidin and thiamethoxam is within the range found in the literature (clothianidin 1.24-6.76 ng/bee and thiamethoxam 1.99-9.00 ng/bee) (Laurino et al. 2013).

Data processing and statistical analysis

Residue analysis: We estimated ‘Risk’ for each agrochemical found in a given sample as the amount of agrochemical detected in a sample divided by the median lethal dose (LD₅₀) (EPA 2014). Two compounds may be present at the same amount (e.g. 10 ppb) in a sample, but the compound with the smaller LD₅₀ is more toxic and more likely to pose a health risk. We carried

out this analysis to highlight the most likely agrochemicals to influence bee health in our study, and not to estimate risk in absolute terms. Unless otherwise indicated in Table S 2.3, LD₅₀ values were obtained from the Pesticide Ecotoxicity Database of the Office of Pesticide Programs, Ecological Fate and Effects Division of the U.S. Environmental Protection Agency. We converted the LD₅₀ values from µg/bee into ppb using the average bee weight of 128mg following standard methods (Johansen and Mayer 1990).

RFID: The RFID data set was first processed by removing duplicate observations, defined as observations recorded less than one second apart. The two RFID readers (iID ® MAJA reader modules 4.1, MicroSenys RFID in Motion) were tagged as 1 and 2, where a bee has to pass them successively in order to exit the observational colony. A flight was defined as two consecutive recordings at reader 2 at least 7 seconds apart. Using a longer time period (i.e., 30sec) to define flights did not change our findings. We deployed a total of 356 tags (159 treated, 197 control), of which 46 treated and 66 control workers were recorded flying. These differences are not statistically significant (Fisher's test, p=0.35) and are likely caused by the rejection of introduced bees into their unrelated host colony. The final data set (n=112 tagged workers) included members from all experimental and control colonies. Each bee was observed flying 49.6 times on average over the course of the experiment. We estimated RFID-tagged workers' age of death by observing the cessation of their flight activity (Decourtye et al. 2011). The average adult lifespan of control workers in our experiment (ca. 3 weeks as adults) is within the typical range (3 to 4 weeks) expected of summer workers in North America (Rueppell et al. 2007). The distribution of flight times (Figure S 2.7) contained a large number of short duration flights ranging between 30 to 360 sec. The duration of these short flights matched well to the duration of orientation flights taken by honey bee workers, which typically do not exceed 391 sec (Capaldi et al. 2000). We thus separately analyzed short-duration flights (<391 sec) that likely represent orientation behavior, and long flights that likely represent foraging behavior.

Hypothesis testing: We used a repeated measures analysis of variance, as implemented using Proc MIXED in SAS Version 9.4 for Windows, to study colony and individual parameters that were measured during multiple time points in our experiment. Colony parameters were analyzed without transformation, except for flight time (log₁₀) and number of flights (square

root), which were transformed to improved normality. We used the default containment degrees of freedom approximation, which is fairly robust (Schaalje, McBride, and Fellingham 2002) and has similar power to other methods for estimating denominator degrees of freedom assuming moderate (>30) sample sizes (Li and Redden 2015). The distribution of the residuals was normal for all analyses (Kolmogorov-Smirnov $p > 0.150$), except those involving flight time and number (Kolmogorov-Smirnov $p < 0.010$ for both). However, our analyses of flight time and number should be robust to deviations of this assumption because of the very large sample sizes involved ($N = 3081$ and 3082 for long and short duration flights, respectively) (Lumley et al. 2002). For analysis of colony phenotypes measured over time, we included treatment, time and their interaction as fixed effects. Colony was treated as a random factor nested within treatment. For analysis of individual traits measured over time, we included treatment, bee age, and their interaction as fixed effects, and bees were treated as a random factor that was nested within colony. Detailed information on the results of SAS mixed models are presented in Table S 2.4. For analysis of queen presence over time, we used the generalized linear mixed model function `glmer` in the R package `lme4` (Bates et al. 2014) using the logit method appropriate for presence/absence data. For this analysis, we included treatment, time and their interaction as fixed effects, and we treated colony as a random factor nested within treatment. We excluded time point zero from analysis of colony level traits in 2015 because it predated experimental NNI exposure (i.e., queen presence and hygienic behavior was quantified at time point zero prior to providing the treatment group with NNI contaminated pollen). We report two-sided p-values for all tests.

Figure Legends

Figure 2.1. Honey bees near corn are chronically exposed to neonicotinoids

A heat map showing total NNIs detected in bees and colony food stores in (A) exposed and (B) unexposed sites. Residues between sampling periods were extrapolated on the basis of adjacent measurements. White areas (ND) represent periods when NNIs were below the limit of detection (<0.4 to 1.1 ppb). Triangles represent corn planting. The NNI detected in Québec (QC) (acetamiprid, LD₅₀=63,180ppb) is considerably less toxic to bees than clothianidin and thiamethoxam, and the peak of exposure in Québec in July does not reflect acute exposure.

Figure 2.2. Chronic clothianidin exposure reduces honey bee health

(A) Adults exposed to clothianidin as larvae (n= 49) were significantly younger during their final recorded flight relative to controls (n=44). (B) We detected a significant treatment by time effect whereby the hygienic behavior of treated colonies (N=4) decreased over time but that of control colonies (N= 5) did not. (C) Colonies near corn (N=25) had significantly less hygienic behavior relative to colonies away from corn (N=25) at the end of the 2014 season. (D) We detected a significant treatment by time effect whereby the number of colonies with a laying queen substantially declined over time in the treated group relative to the control group. Means and SEM. *P< 0.05 (see text for details). Yellow and blue indicate treated or exposed and control or unexposed workers or colonies, respectively.

Figure 2.3. NNIs are twice as toxic to honey bees in the presence of a common fungicide

The median oral lethal dose (LD₅₀) of the neonicotinoid clothianidin and thiamethoxam are significantly lower in the presence of field realistic levels of boscalid. Field realistic levels of the herbicide linuron did not influence NNI toxicity to honey bees. Means and SEM. ns, Not significant; *P<0.05

Figure S 2.1. Presence of neonicotinoids in different sample matrices

Black squares indicate the presence of neonicotinoids in pollen (P), nectar (N), dead bees (D), foragers (F), nurses (Nu) and larva (L) in (A) exposed and (B) unexposed sites of the six sampling periods in 2014.

Figure S 2.2. Contamination of colony food stores

When present, (A) agrochemicals and (B) neonicotinoids were mostly found in colony food stores, especially pollen. Number of positive samples is depicted along the perimeter. Note that we analyzed an equal number of samples for each colony matrix. Colony food stores represented 33.33% of all samples tested for agrochemicals, but made up more than 60% of samples contaminated with agrochemicals and NNIs.

Figure S 2.3. Neonicotinoids pose the greatest toxicity risk to honey bees

The absolute concentration of agrochemicals (e.g. 1 or 10 ppb) found in colonies is not very informative on its own because agrochemicals differ in toxicity (i.e., LD₅₀) to honey bees. We quantified relative risk for each compound found in a sample as its concentration standardized by its LD₅₀. A relative risk of 1 indicates that the concentration of a compound in a sample is identical to the compound's LD₅₀. NNIs (n=49 samples) posed the greatest relative risk to honey bees relative to other insecticide's (n=11), miticides (n=91), fungicides (n=64) and herbicides (n=19). Boxes represent the first and third quartiles, the middle notch represents the median, while the whiskers represent maximum and minimum values.

Figure S 2.4. We exposed honey bee colonies to realistic but conservative levels of NNIs

We used NNI residue data from Ontario (2014) to estimate the total dietary NNI exposure under two scenarios. Conservative scenario (grey): We naively assigned 0 ppb to samples with no detectable levels of NNIs and averaged over these zero values when estimating mean dietary NNI exposure (i.e., pollen + nectar) over the course of the season. Extreme scenario (yellow): We assigned a value of 1 ppb to samples with no detectable levels of NNIs and averaged over

these assumed values. Our treatment experiment (blue) treated honey bees to lower levels and a shorter duration of NNI exposure relative to both best case and worst-case scenarios derived from the field toxicology data. Error bars (SEM) were estimated for time points containing more than 2 observations.

Figure S 2.5. Pre-pupal exposure to clothianidin influenced flight behavior as a function of age

(A) Treated bees (yellow) went on more short-duration flights per day while younger relative to control bees (blue; Age x Treatment: $F_{(34, 2895)} = 2.49$, $p < 0.0001$, $N = 3082$). While the difference in the number of short-duration flights between treated and control bees is biologically subtle, it occurred when both groups were very young (<10 days) – an age where flight activity most likely reflects orientation behavior (Perry et al. 2015, Capaldi et al. 2000). (B) We did not detect a difference in the duration of short flights between treated workers and controls (Age x Treatment: $F_{(34, 2895)} = 0.96$, $p = 0.531$, $N = 3082$). (C) Treated bees went on more foraging trips per day while younger relative to control bees ($n = 1428$; Age x Treatment: $F_{(34, 2903)} = 1.93$, $p = 0.001$, $N = 3081$), but the difference was biologically subtle. (D) Treated bees took longer foraging trips as they aged relative to controls (Age x Treatment: $F_{(34, 2903)} = 1.78$, $p = 0.004$, $N = 3081$). Flights made by treated workers between 30 to 38 days old were on average 39 minutes longer (mean $6528\text{sec} \pm 715\text{ SEM}$, $N = 25$) relative to same-aged controls (mean $4139\text{sec} \pm 408\text{ SEM}$, $N = 43$); we did not observe treated workers that were older than 38 days. Points were slightly ‘jittered’ around their true position to reduce over plotting. For plotting (A), (C), and (D), we restricted the range of the y axis to 15 trips/day (A and C) and 7000 sec (D) to better show the difference between treated workers and controls – these limits were only used for plotting (i.e., not for statistical analyses). Lines were generated using linear regressions and the shaded area represents the 95% confidence region.

Figure S 2.6. Land use within a 3-km radius of exposed and unexposed sites in 2014

The exposed sites, were located near (<0.5 km) corn crops, but were also in the vicinity of soybeans, which are often grown from seeds treated with NNIs. Unexposed sites were located far (>3 km) from corn crops and most other agriculture. The field study was not designed to

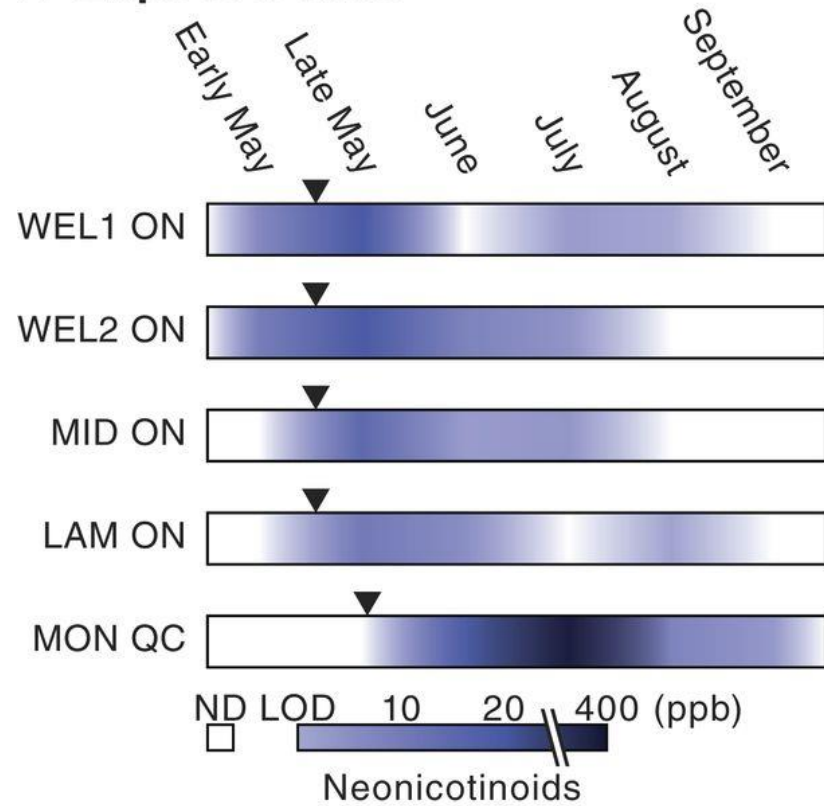
determine the effects of exposure on bees, but rather to quantify the magnitude of agrochemical exposure using honey bee colonies as environmental sentinels.

Figure S 2.7. The density of flight times taken by treated (yellow, n = 49) workers and controls (blue, n = 44)

Note the large number of short duration flights ranging between 30 to 360 sec, which matches the typical duration of orientation flights taken by young honey bee workers. We thus separately analyzed short-duration flights (<391 sec) that likely represent orientation behavior, and long flights that likely represent foraging behavior.

Figure 2.1

A Exposed sites



B Unexposed sites

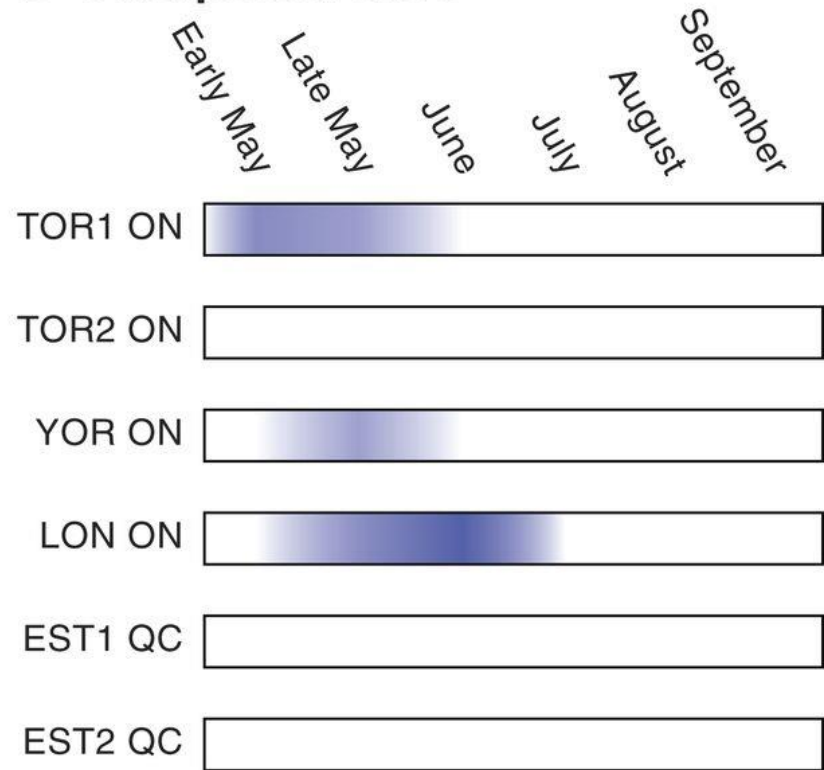


Figure 2.2

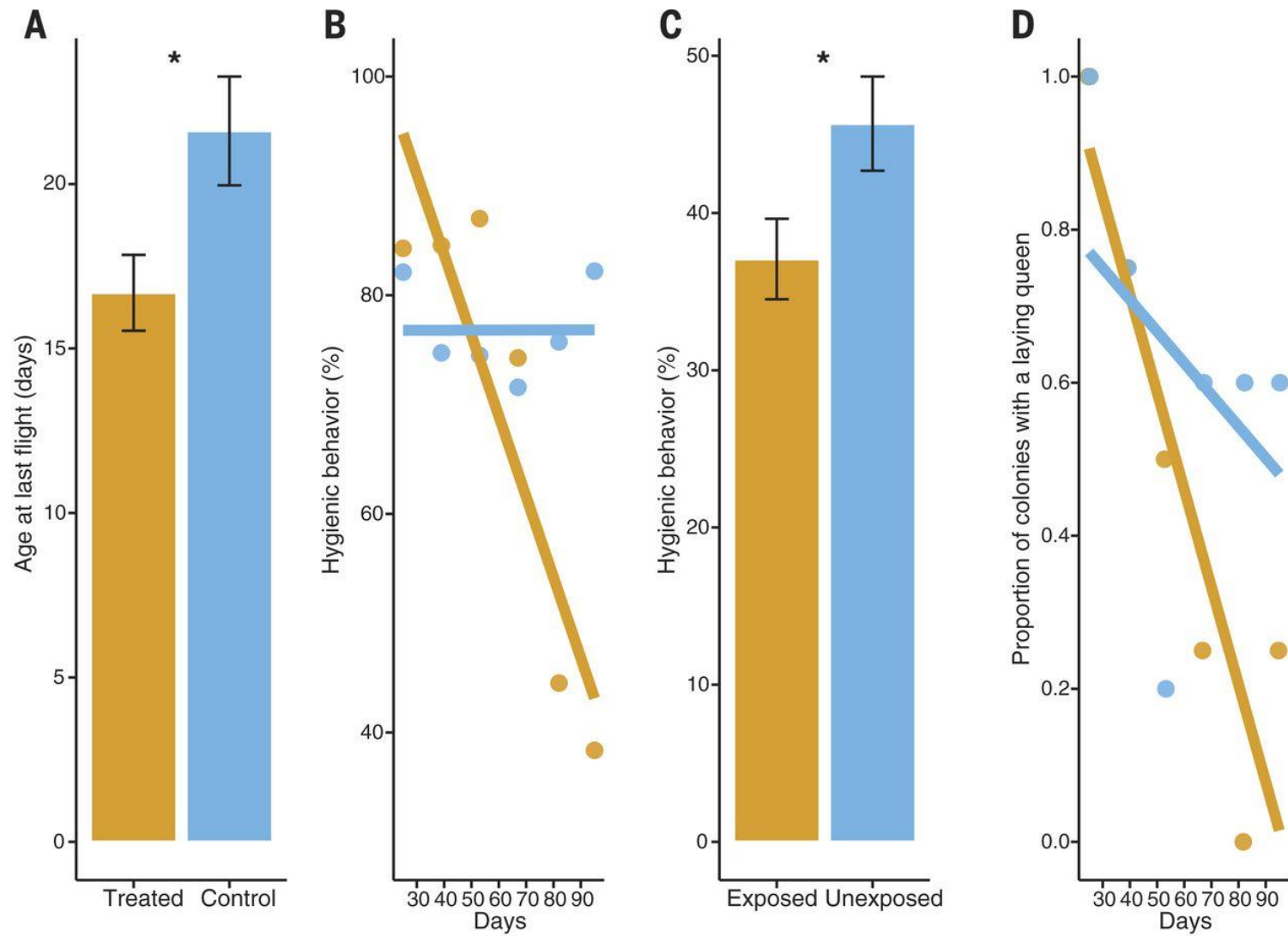


Figure 2.3

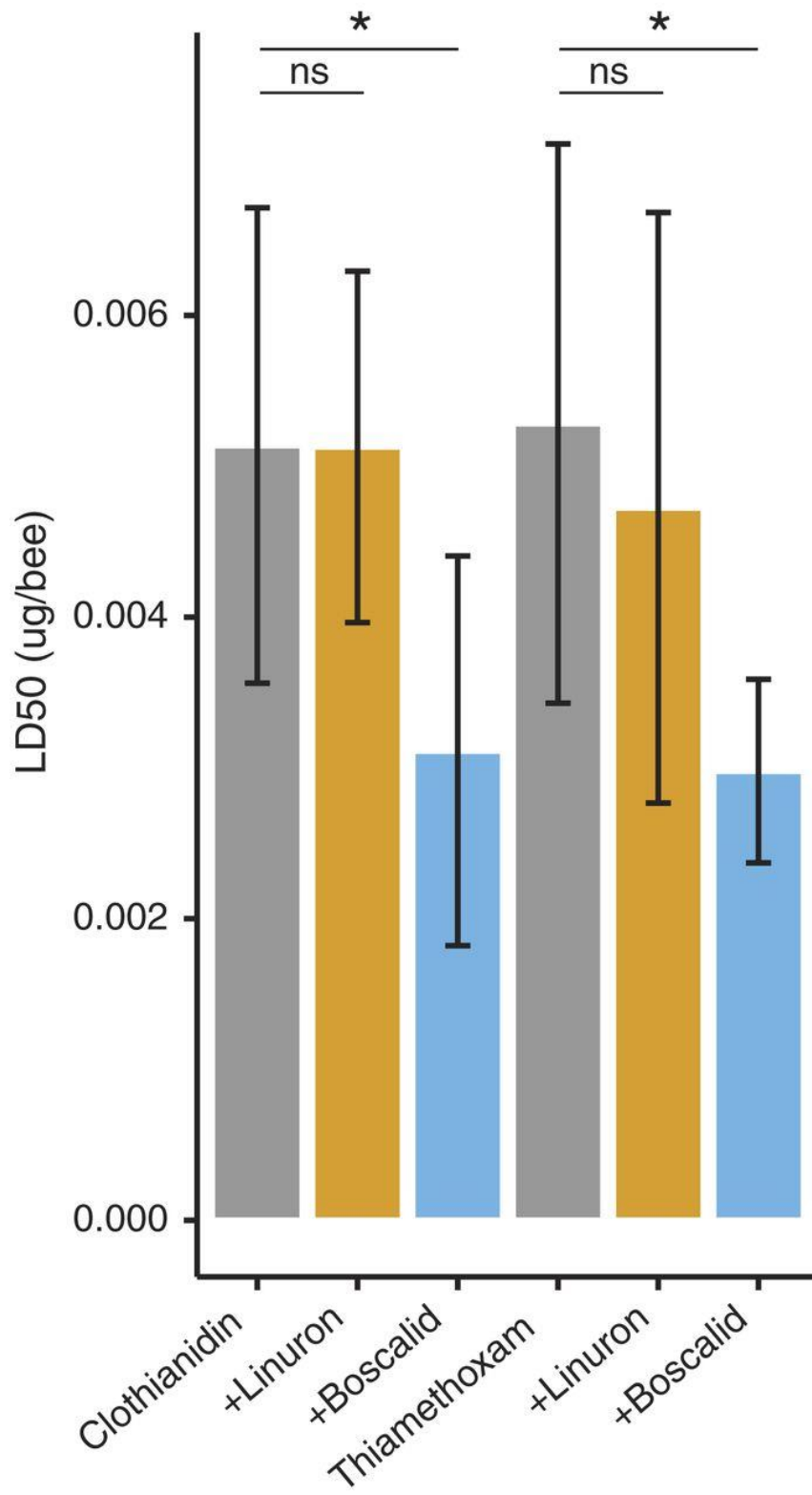


Figure S 2.1

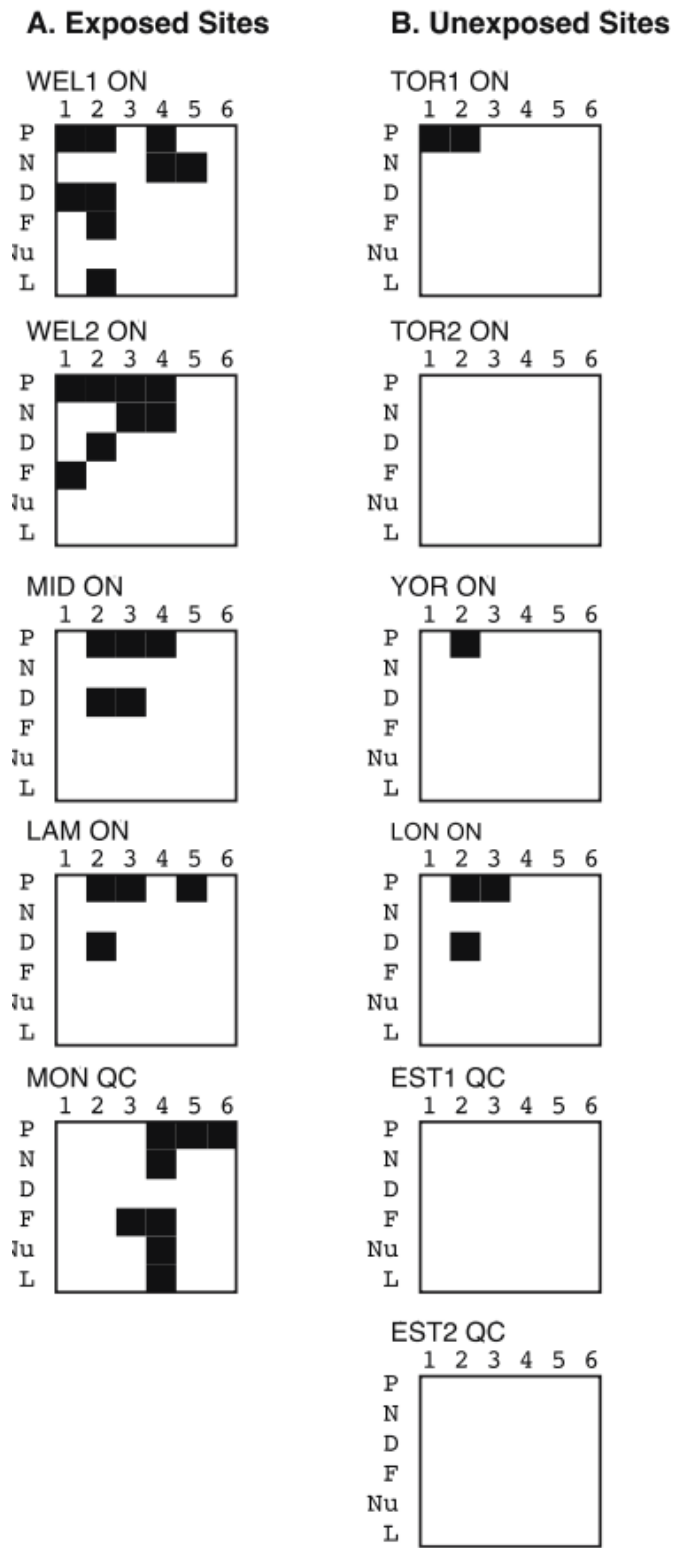
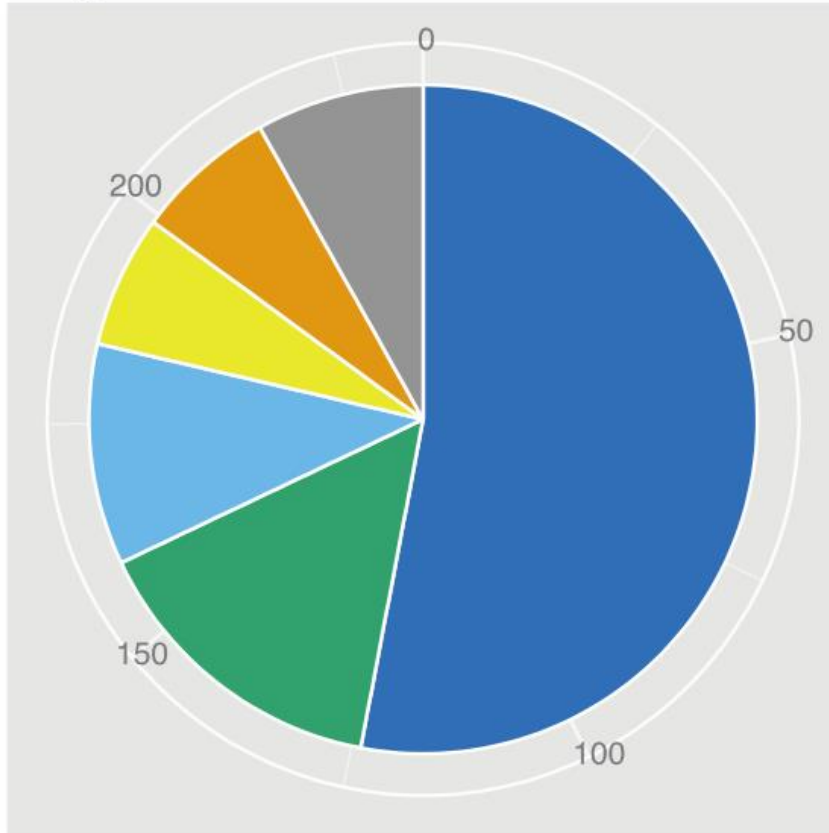
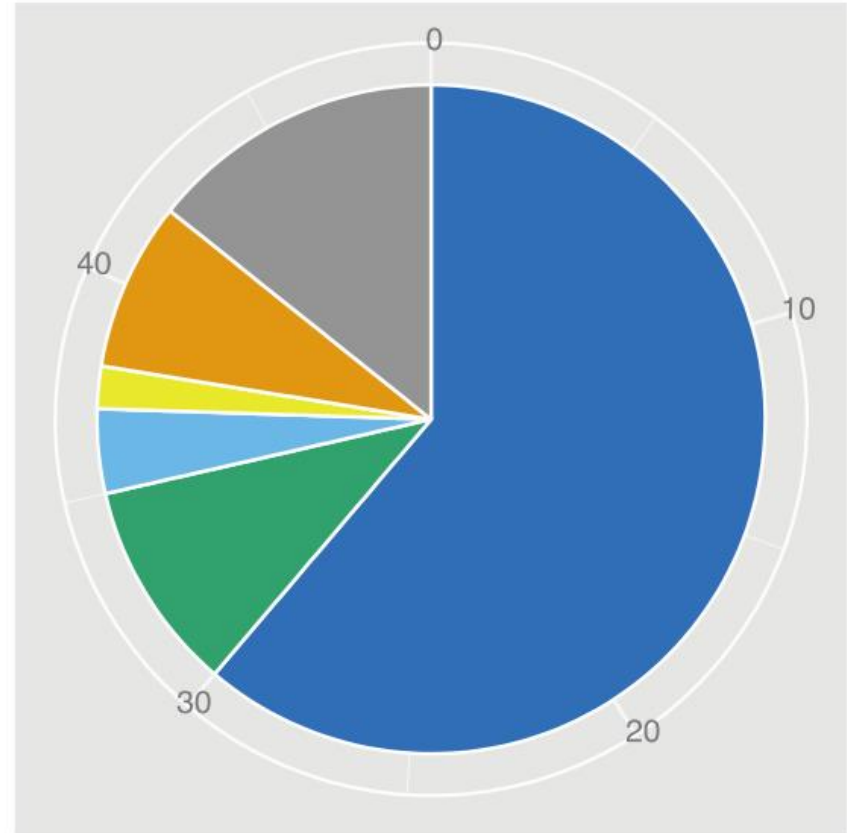


Figure S 2.2

A. Agrochemicals



B. NNIs



Sample DeadBees Foragers Larvae Nectar Nurses Pollen

Figure S 2.3

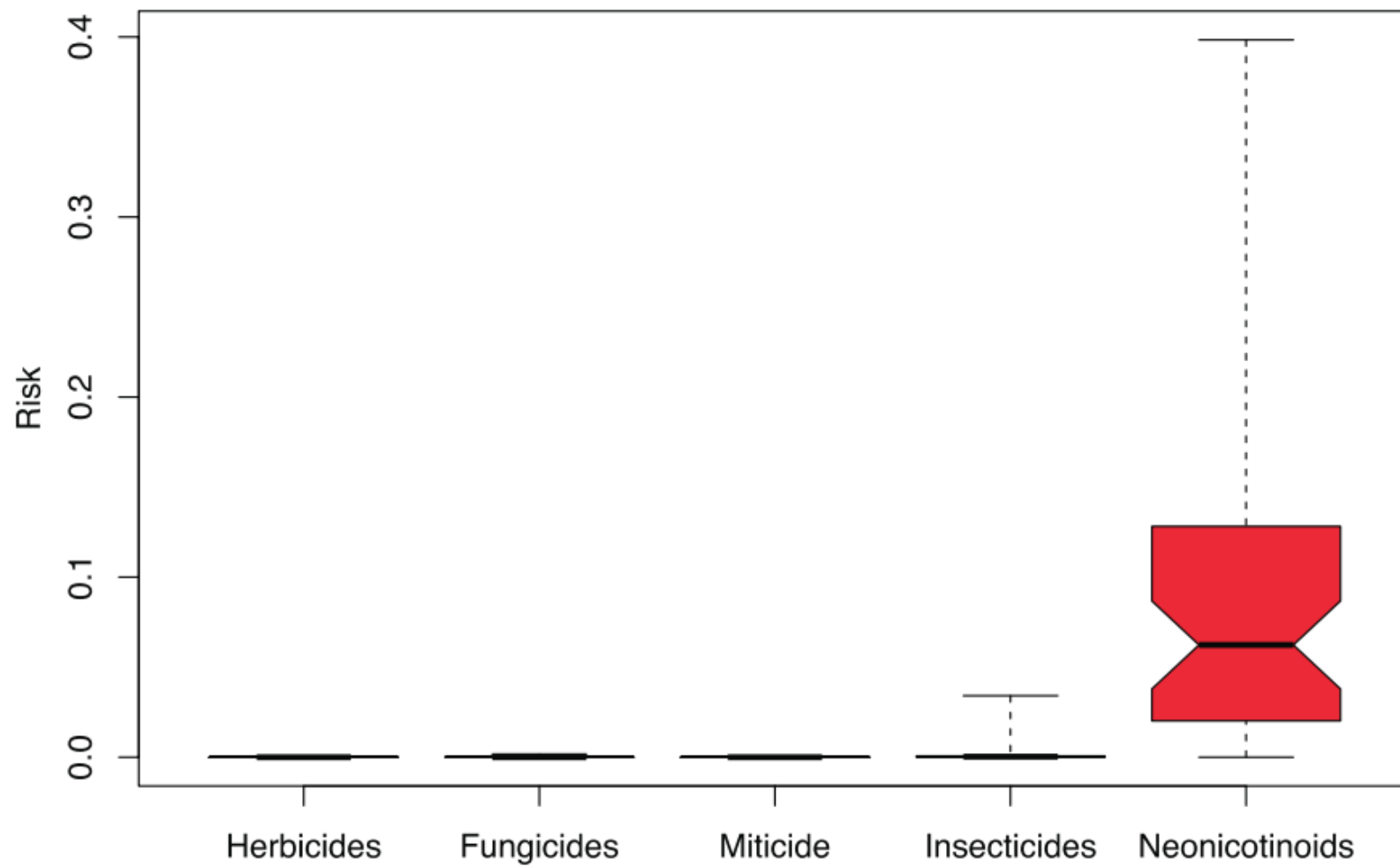


Figure S 2.4

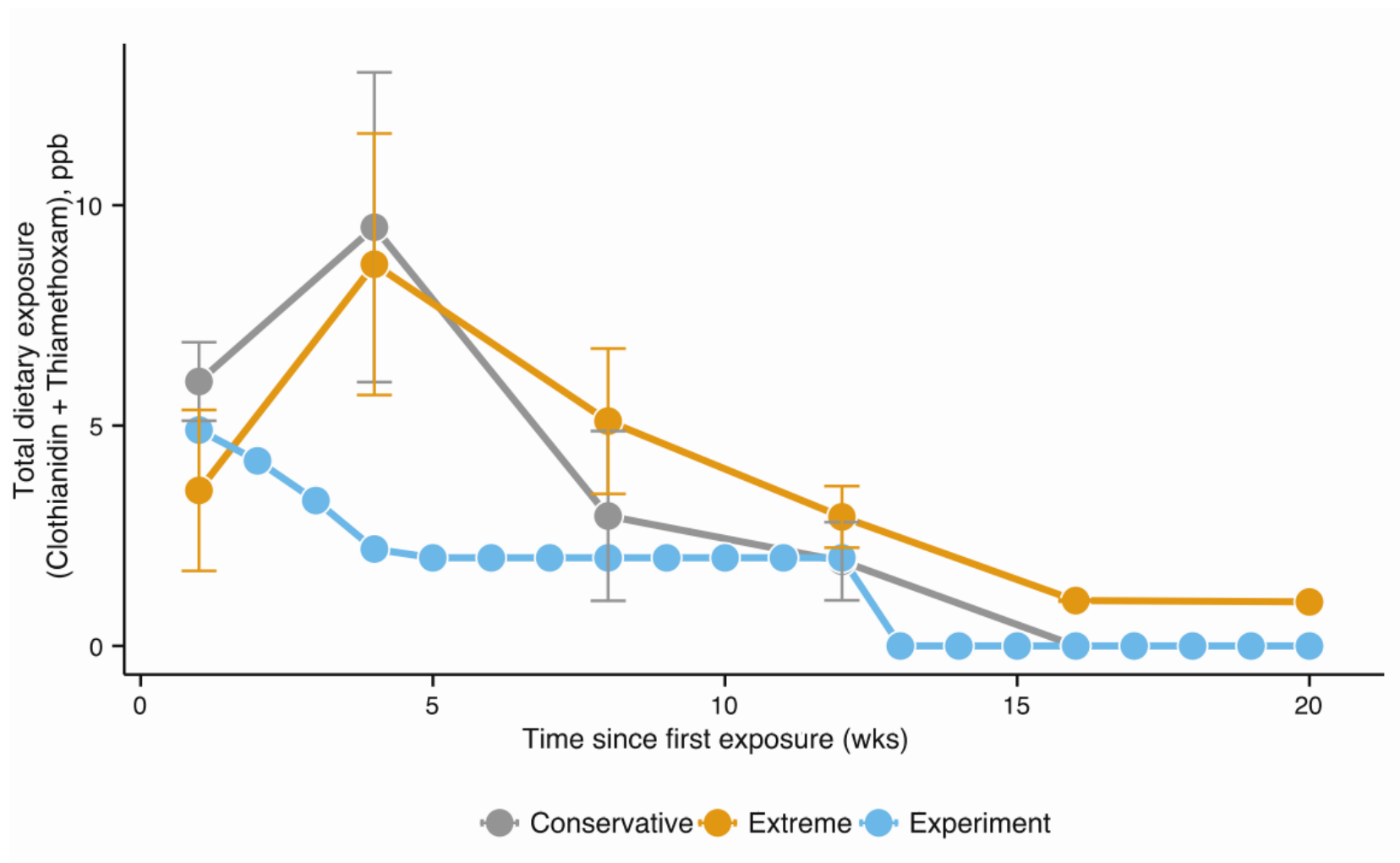


Figure S2.5

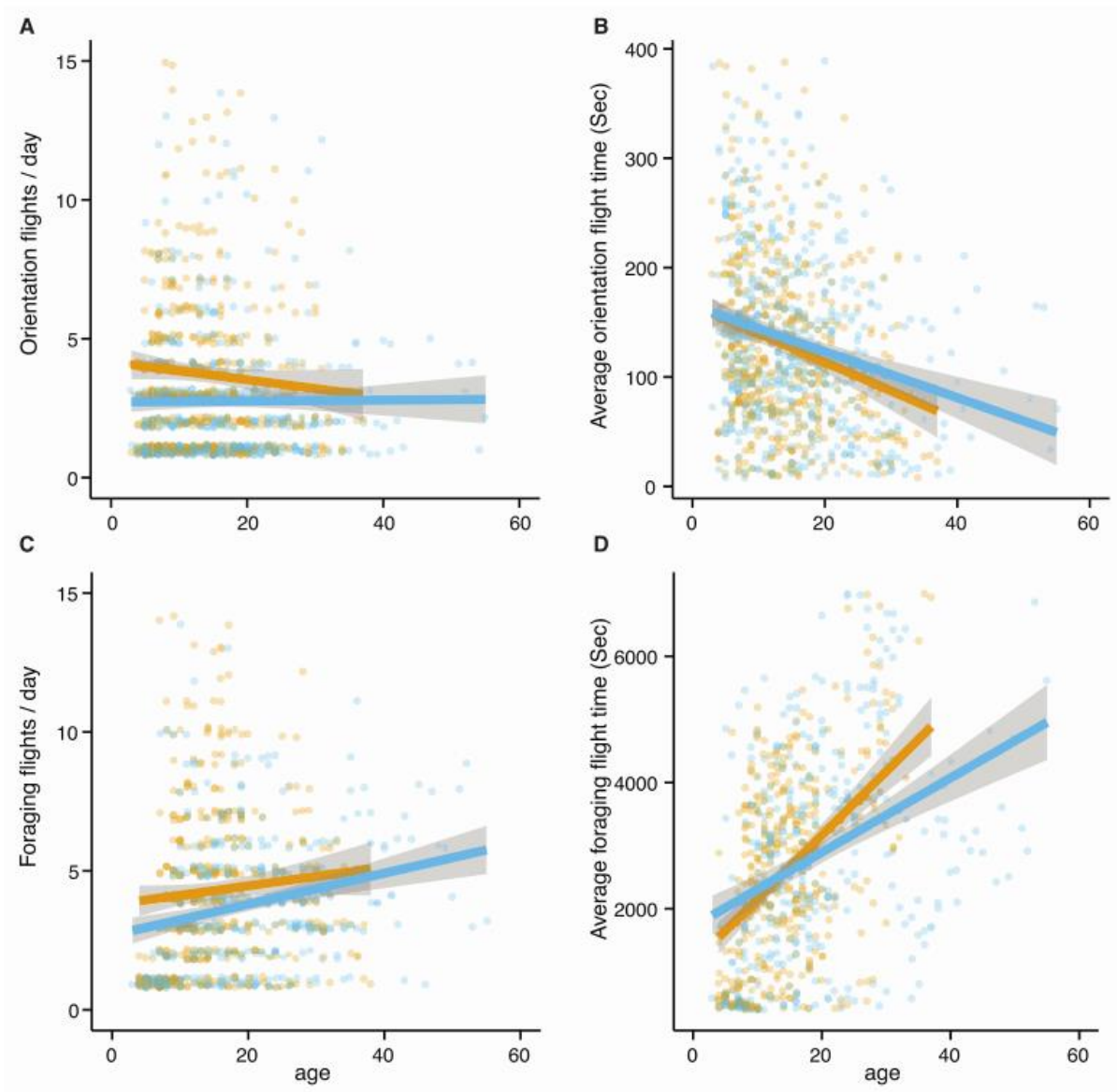


Figure S 2.6

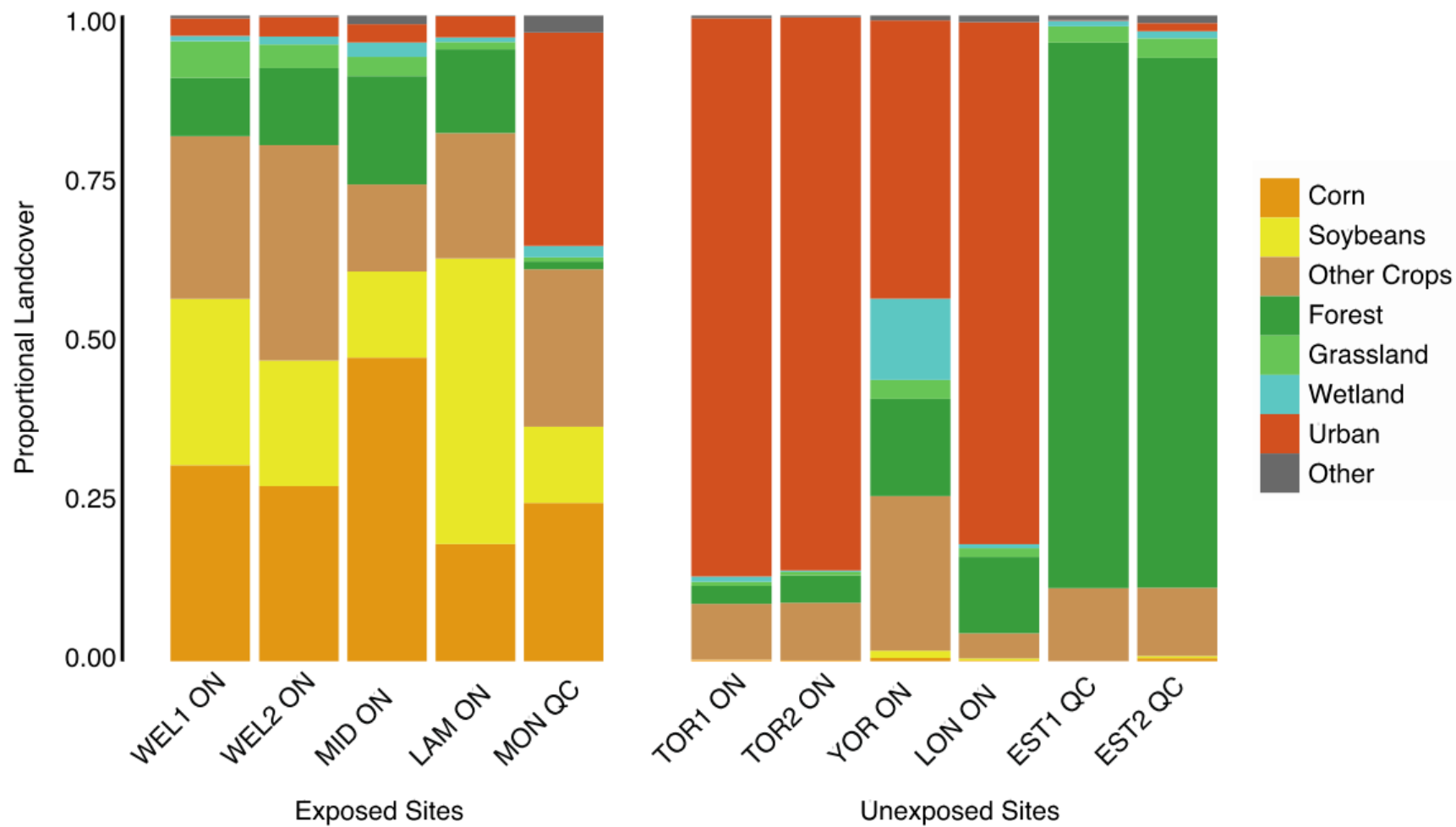


Figure S 2.7

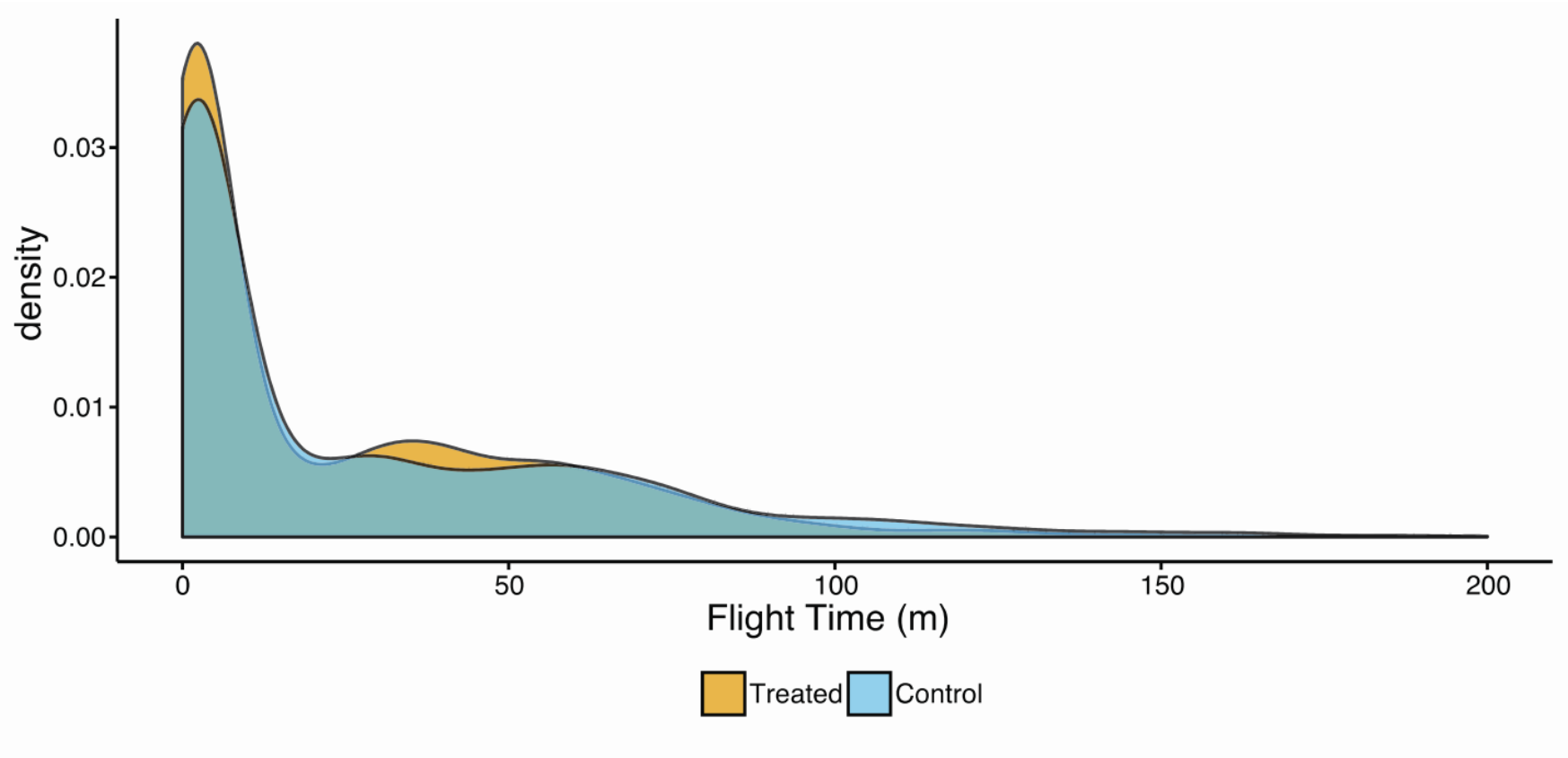


Table S 2.1. Mean levels of 26 agrochemicals detected in exposed and unexposed sites in 2014

We averaged over all samples with quantifiable measures of agrochemicals, even if the quantification was below LOD and LOQ. We did not include samples where no quantification was made for a given agrochemical when calculating the mean. SD represents standard deviation.

Compound	Type	Sample	Mean ± SD (LOD/LOQ) in ppb
Boscalid	Fungicide	Dead	<2.0 (0.7/2.0)
Boscalid	Fungicide	Foragers	2.7 (0.7/2)
Boscalid	Fungicide	Nectar	151.35 ± 208.7 (0.7/2.0)
Boscalid	Fungicide	Nurses	4.6 (0.7/2.0)
Boscalid	Fungicide	Pollen	497.34 ± 953.4 (0.7/2.0)
Carboxin	Fungicide	Pollen	<0.7 (0.7/2)
Flutolanil	Fungicide	Pollen	<1.4 (1.4/4.1)
Imazalil	Fungicide	Larvae	<2.7 ± 0.9 (2.7/10)
Imazalil	Fungicide	Pollen	<10.0 ± 2.5 (2.7/10.0)
Picoxystrobin	Fungicide	Dead	<0.9 ± 0.2 (0.9/2.7)
Picoxystrobin	Fungicide	Foragers	<0.9 ± 0.5 (0.9/2.7)
Picoxystrobin	Fungicide	Nurses	<2.7 ± 2.5 (0.9/2.7)
Picoxystrobin	Fungicide	Pollen	<3.1 ± 5.6 (1.0/3.1)
Propamocarb	Fungicide	Pollen	<1.0 (1.0/2.9)
Pyraclostrobin	Fungicide	Dead	<3.6 (1.2/3.6)
Pyraclostrobin	Fungicide	Foragers	<1.2 ± 0.4 (1.2/3.6)
Pyraclostrobin	Fungicide	Nurses	<3.6 (1.2/3.6)
Pyraclostrobin	Fungicide	Pollen	7.14 ± 6.6 (1.3/4)
Pyrimethanil	Fungicide	Foragers	<9.9 (3.3/9.9)
Pyrimethanil	Fungicide	Nurses	45.7 (3.3/9.9)
Pyrimethanil	Fungicide	Pollen	195.8 ± 328.1 (2.4/7.2)
Thiabendazole	Fungicide	Dead	25.2 (0.9/2.7)
Thiabendazole	Fungicide	Pollen	<1.1 (1.1/3.4)
Acetochlor	Herbicide	Pollen	9.5 ± 7.9 (2.8/8.5)
Chlorimuron-ethyl	Herbicide	Pollen	<5.0 ± 3.0 (1.7/5.0)
DimethenamidP	Herbicide	Foragers	<3.8 (1.3/3.8)
DimethenamidP	Herbicide	Pollen	14.25 ± 16.5 (2.3/6.8)
Diuron	Herbicide	Pollen	11.55 ± 9.2 (3.2/9.7)
Fluzifop-butyl	Herbicide	Nurses	<0.8 (0.8/2.3)
Linuron	Herbicide	Dead	<2.8 (2.8/8.3)
Linuron	Herbicide	Foragers	<8.3 ± 0.8 (2.8/8.3)
Linuron	Herbicide	Pollen	7.3 ± 6.2 (1.6/4.7)
Napropamide	Herbicide	Pollen	<2.7 ± 1.4 (0.9/2.7)
Carbaryl	Non-Neonic Insecticide	Foragers	<8.0 ± 0.6 (8.0/23.9)
Carbaryl	Non-Neonic Insecticide	Nurses	<8.0 ± 0.3 (8.0/23.9)
Carbaryl	Non-Neonic Insecticide	Pollen	51.3 ± 71.4 (2.1/6.3)

Chlorantraniliprole	Non-Neonic Insecticide	Pollen	9.6 (3.2/9.5)
Methomyl	Non-Neonic Insecticide	Foragers	<0.5 (0.5/1.4)
Omethoate	Non-Neonic Insecticide	Nectar	<0.7 (0.7/2)
Omethoate	Non-Neonic Insecticide	Pollen	<0.7 (0.7/2)
Tebufenozide	Non-Neonic Insecticide	Dead Bees	1.8 (0.6/1.8)
Coumaphos	Miticide	Dead Bees	2.57 ± 1.4 (0.7/2.0)
Coumaphos	Miticide	Larvae	12.69 ± 14.0 (0.7/2)
Coumaphos	Miticide	Nectar	<0.7 ± 0.3 (0.7/2)
Coumaphos	Miticide	Nurses	<0.7 ± 0.1 (0.7/2)
Coumaphos	Miticide	Pollen	3.50 ± 4.0 (0.7/2.0)
Acetamiprid	Neonic Insecticide	Foragers	13.55 ± 7.3 (0.5/1.6)
Acetamiprid	Neonic Insecticide	Larvae	<1.6 (0.5/1.6)
Acetamiprid	Neonic Insecticide	Nectar	19.3 (0.4/1.3)
Acetamiprid	Neonic Insecticide	Nurses	<1.6 (0.5/1.6)
Acetamiprid	Neonic Insecticide	Pollen	113.27 ± 186.8 (1/3.1)
Clothianidin	Neonic Insecticide	Dead Bees	<3.6 ± 3.2 (1.2/3.6)
Clothianidin	Neonic Insecticide	Foragers	<1.2 (1.2/3.6)
Clothianidin	Neonic Insecticide	Larvae	<1.2 (1.2/3.6)
Clothianidin	Neonic Insecticide	Nectar	<1.4 ± 0.5 (1.4/4.1)
Clothianidin	Neonic Insecticide	Pollen	4.27 ± 2.8 (1.4/4.1)
Imidacloprid	Neonic Insecticide	Foragers	<1.4 (0.5/1.4)
Thiamethoxam	Neonic Insecticide	Nectar	2.65 ± 2.2 (0.3/0.9)
Thiamethoxam	Neonic Insecticide	Pollen	3.58 ± 2.7 (0.7/2.0)

Table S 2.2. The relative contribution of different plant taxa to pollen samples where neonicotinoids were present or absent

Name	Common Name	NNI Present (%)	NNI Absent (%)
Salix	Willow	21.98	8.56
Type Aster/Solidago	Goldenrod	0.92	20.31
Type Trifolium hybridum	Alsike Clover	19.05	8.86
Rhamnus Type cathartica	Buckthorn	15.48	2.01
Lotus	Lotus	5.43	10.31
Type Melilotus	Sweet Clover	0.92	9.67
Acer Type negundo	Maple Ash	1.44	5.85
Type Brassica	Mustard Vegetables	5.73	1.77
Rosaceae fruit trees type	Apple	5.56	2.29
Acer Type rubrum	Red Maple	0.42	4.3
Liliaceae	Lily	2.82	0.36
Type Taraxacum	Dandelion	1.28	2.55
Type Trifolium pretense	Red Clover	1.53	2.03
Other	Other	<2.00	<2.00

Table S 2.3. Lethal median concentration for the 26 agrochemicals detected in 2014

Compound	LD ₅₀ (µg/bee)	LD ₅₀ (ppb)	LOD Range (ppb)
Acetamiprid	8.1	63180	0.4–1.0
Acetochlor	>200	1560000	1.7–2.8
Boscalid	>200	1560000	0.7
Carbaryl	0.23	1802	1.2–8.0
Carboxin	100*	780000	0.7
Chlorantraniliprole	119	928200	0.9–3.2
Chlorimuron-ethyl	12	93600	0.8–1.7
Clothianidin	0.0037	29	1.2–1.4
Coumaphos	20†	156000	0.7
Dimethenamid-P	94	733200	0.8–2.3
Diuron	145	1131234	1.5–11.5
Fluazifop-butyl	>200	>1560000	0.5–1.4
Flutolanil	>650	>5070000	0.3–1.1
Imazalil	35‡	273000	0.7–2.7
Imidacloprid	0.09	702	0.5–0.7
Linuron	121	943800	1.6–2.8
Methomyl	1	7800	0.4–1.3
Napropamide	113	885300	0.4–0.9
Omethoate	0.05§	390	0.7
Picoxystrobin	>200	>1560000	0.5–1.0
Propamocarb	100	780000	0.1–1.0
Pyraclostrobin	>100	>780000	0.4–1.3
Pyrimethanil	>100	>780000	2.4–3.3
Tebufenozide	234	1825200	0.6–1.4
Thiabendazole	4	31200	0.9–5.3
Thiamethoxam	0.024	187	0.3–0.7

When toxic limits are reported (i.e., > minimum value), we used the minimum value for analyses. LD₅₀ values were obtained from the U.S. Environmental Protection Agency's Pesticide Ecotoxicity Database, with the following exceptions: *(Authority 2010), †(Johnson, Pollock, and Berenbaum 2009), ‡(Group 2012), §(Fiedler 1987), and ||(Stoner and Eitzer 2016).

Table S 2.4. Summary results of SAS mixed models

	Num DF	Den DF	Statistic	p-value
Model: Worker lifespan				
Random factors: Colony				
Fixed factors:				
Treatment	1	7	5.78	0.0471
Model: Worker Short duration flight number				
Random factors: Colony, Bee				
Fixed factors:				
Treatment	1	105	0.03	0.8562
Age	46	2895	2.49	<0.0001
Age*Treatment	34	2895	2.49	<0.0001
Model: Worker Short duration flight time				
Random factors: Colony, Bee				
Fixed factors:				
Treatment	1	105	1.59	0.2097
Age	46	2895	2.2	<0.0001
Age*Treatment	34	2895	0.96	0.531
Model: Worker Long duration flight number				
Random factors: Colony, Bee				
Fixed factors:				
Treatment	1	91	1.93	0.001
Age	51	2903	2.48	<0.0001
Age*Treatment	34	2903	1.93	0.001
Model: Worker Long duration flight time				
Random factors: Colony, Bee				
Fixed factors:				
Treatment	1	91	1.99	0.1621
Age	51	2903	12.25	<0.0001
Age*Treatment	34	2903	1.78	0.0036
Model: Hygienic behavior, treatment experiment 2015				
Random factors: Colony				
Fixed factors:				
Treatment	1	7	10.89	0.0131
Time	1	23	17.27	0.0004
Time*Treatment	1	23	14.86	0.0008
Model: Hygienic behavior, field study 2014				
Random factors: Colony, Site				
Fixed factor:				
Treatment	1	48	6.42	0.0146

Table S 2.5. Raw toxicology data from bee matrices collected from colonies near or far from Corn in 2014

LOD and LOQ represent the limits of detection and quantification, respectively. File available Online at: <https://science.sciencemag.org/content/suppl/2017/06/28/356.6345.1395.DC1>

Chapter 3 Field realistic exposure to neonicotinoids has a subtle impact on the honey bees' immune system and pathosphere

N. Tsvetkov, H. S. Patel, O. Samson-Robert, V. Fournier, and A. Zayed²

Introduction

Elevated honey bee colony mortality has been observed across several continents over the past decades (Brodschneider, Moosbeckhofer, and Crailsheim 2010, Potts et al. 2010, Hayes Jr, Underwood, and Pettis 2008, Evans et al. 2009, Cox-Foster et al. 2007, Anderson and East 2008, Van der Zee, Pisa, Andonov, Brodschneider, Charriere, Chlebo, Coffey, Dahle, et al. 2012, Kulhanek et al. 2017). Several causes have been proposed, including pesticides and pathogens (Vanbergen 2013, Potts et al. 2010, Lever et al. 2014, McMenemy and Genersch 2015, Goulson 2013, Rundlof et al. 2015). These factors can act individually, but often act in combination resulting in synergistic interactions that have a greater influence on the health of pollinators (Vanbergen 2013, Goulson et al. 2015, Sánchez-Bayo et al. 2016). The effects of neonicotinoids (NNIs) on honey bee pathogens is of particular interest, since NNIs are now the most commonly used insecticides in the world (Goulson 2013), and pathogens have been directly implicated in honey bee colony losses (McMenemy and Genersch 2015).

The combined effects of NNIs and pathogens can have marked impact on honey bee health. Bees that are exposed to NNIs and are challenged with *Nosema ceranae*, a common fungal pathogen, have increased mortality (Alaux et al. 2010, Vidau et al. 2011, Doublet, Labarussias, Miranda, et al. 2015, Pettis, Johnson, and Dively 2012). The combination of thiacloprid, an NNI, and black queen cell virus (BQCV) elevate larvae mortality (Doublet, Labarussias, de Miranda, et al. 2015). Several studies have also shown that NNIs can suppress the honey bee's immune system (Brandt et al. 2016, Brandt et al. 2017) and promote deformed wing

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virus (DWV) replication (Di Prisco et al. 2013), further supporting the synergistic nature of NNIs and pathogens.

Although these studies demonstrate that NNIs impact the honey bees' immune system, evidence from whole colony exposure, rather than individual bees, have sometimes yielded contradictory results. Whole colony exposure to NNIs did not increase *Nosema* infection (Pettis, Johnson, and Dively 2012, Siede et al. 2017), acute bee paralysis virus load, or *Varroa destructor* infestations (Siede et al. 2017), a common honey bee parasite and a vector for viruses, particularly DWV (McMenamin and Genersch 2015). The combined exposure to two NNIs, thiamethoxam and clothianidin, did not increase DWV or *Varroa* levels (Straub et al. 2019). On the other hand, another study found that colonies located near NNI treated corn had higher levels of *Varroa*, but not DWV (Alburaki et al. 2015). In addition, they had higher levels of BQCV, but only after corn bloom (Alburaki et al. 2015). Another field study showed that colonies near NNI treated canola fields had the same levels of *Varroa* and several other viruses, including DWV and BQCV, as colonies near non-treated fields (Osterman et al. 2019). Even the expression levels of immune genes after NNI exposure significantly differs between honey bees exposed in cages and in whole colonies (De Smet et al. 2017). Overall, it remains unclear whether field realistic levels of NNI exposure on whole colonies have an effect on honey bee immunity and pathogen loads.

We conducted a season-long study on honey bees kept near and far from corn in Canada to determine typical routes and exposure to NNIs. Corn production represents the largest use of arable land in North America (Hamel and Dorff 2014) and almost all corn is grown from NNI-treated seeds (Stewart and Baute 2013). We then performed a controlled experiment mimicking the NNI exposure we found in the field (Tsvetkov et al. 2017). To examine whether NNI exposure leads to immune suppression in honey bees, we measured the expression of two immune genes, in addition to quantifying the loads of four common bee viruses and *N. ceranae* using real time quantitative polymerase chain reaction (RT-qPCR). We found no effect of NNI exposure on the expression levels of the immune genes. However, we did find that honey bees exposed to NNIs and infected with *N. ceranae* had higher levels of BQCV in both our field study and in our apiary experiment.

Materials and Methods

Honey bees

The honey bees were collected from colonies described in detail by Tsvetkov et al. (2017). Briefly, in the 2014 field study, 55 bee colonies were randomly allocated to five apiaries located near (<500m) NNI treated corn ('exposed sites') or to six apiaries located at least 3km from agriculture ('unexposed sites'). Four of the exposed and four of the unexposed sites were located in Ontario and the other sites were located in Quebec. Each site contained five colonies. At allocation, the weights of the colonies between the groups was the same (t test: $t = -0.24$, $df=6.9$, $p=0.816$) and all colonies were free from any visible sign of disease. Honey bees were collected from each colony's honey frames using a 50mL centrifuge tube. They were collected in early May, late May, June, July, August, and September. Bees were transported to the laboratory on dry ice and later stored at -80°C until analysis.

In 2015, we carried out an experiment mimicking the NNI field exposure found in 2014 (Tsvetkov et al. 2017). Ten colonies in a single apiary received artificial pollen for a 12-week period. Half of the colonies received a pollen patty spiked with clothianidin, the most commonly found NNI in the 2014 study. The exposure started with 4.9ppb of clothianidin in week 1 and was lowered each week until the dose reached 2.0ppb at week 5 and stayed the same until week 12 (Figure S 3.1). Honey bees were collected, transported, and stored in the same manner as described above.

RNA Extraction and cDNA synthesis

We followed well established protocols for RNA extraction and RT-qPCR analysis (Di Prisco et al. 2013, Osterman et al. 2019). Briefly, we used 50 bees per extraction and used the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). cDNA was synthesized using the iScript™ Select cDNA Synthesis Kit (BioRad). cDNA was stored at -20°C until RT-qPCR and excess RNA was stored at -80°C .

All samples were run in triplicate with a negative control for each pathogen/gene. Each replicate contained 1µL of diluted cDNA, 5µL of SsoAdvanced SYBR® green supermix, 3µL of DEPC H₂O, 0.5 µL Forward primer and 0.5 µL Reverse primer of the corresponding pathogen/gene (Table S 3.1). Relative quantification (RQ) was obtained using a variant of the $2^{-\Delta\Delta CT}$ method with quantification of the primer efficiencies (Livak and Schmittgen 2001, Pfaffl 2001). We used actin as a reference gene, which was amplified at the same time as the target genes. The Ct values of target genes that had no amplification after 40 cycles were imputed as 41. A colony far from corn in 2014 and an unexposed colony from 2015 were used as calibrators for their respective years' study.

Statistical Analysis

The expression data was logged and we removed outliers using cook's D (Cook 1977). We used a repeated measures mixed model, as implemented using Proc MIXED in SAS Version 9.4 for Windows. We used the default containment degrees of freedom approximation, which is fairly robust (Schaalje, McBride, and Fellingham 2002) and the autoregressive covariate structure. Tests for simple effects were performed using the Slice statement with adjustment for multiple testing using Tukey-Kramer method (Kramer 1956).

The absence or the presence of a pathogen were analyzed by fitting a generalized linear mixed-effects model (GLMM) using R (V.3.6.3)(Team 2005) package 'lme4' with colony as random effect, sampling time as a repeated measure, and a binomial family structure. Correlation analyses were performed in R and the p-values were corrected for multiple testing using the Holm-Bonferroni method (Holm 1979). Differences in correlations between treatments were tested using the 'cocor' package in R (Diedenhofen and Musch 2015) using the Fisher's z test (Fisher 1925).

Results

2014 Field Study

The results of the repeated measured mixed model for the 2014 field study are summarized in Table 3.1. We did not detect Kashmir bee virus (KBV) in a large proportion of our 2014 samples ($n=221/294$), thus we only analyzed the prevalence (i.e. presence/absence) of this virus. We detected seasonal variation in the abundance of all of the pathogens and in apidaecin (Table 3.1, $p<0.05$), but not in dorsal (Table 3.1, $p=0.139$).

Pathogens

Colonies located far from corn had higher levels of DWV expression than colonies located near corn fields (Figure 3.1A; Table 3.1, $p<0.001$). DWV levels increased through the summer (Table 3.1, $p<0.001$), but no interaction effects between location and time was found (Table 3.1, $p=0.907$).

Sacbrood virus (SBV) increased from early spring to mid-summer, when it seems to have tapered off (Figure 3.1B; Table 3.1, $p<0.001$). We found a statistically significant effect of location and of the interaction between time and location (Table 3.1, $p<0.05$). An ad-hoc test revealed that two months were statistically significantly higher in the colonies located near corn: July (Table S 3.2, $p=0.026$) and September (Table S 3.2, $p=0.001$). The intermediary month of August was marginally significant (Table S 3.2, $p=0.088$).

KBV was detected more often in the beginning of the summer than at the end (Figure S 3.2A, GLMM, $z\text{-value}=-3.931$, $p<0.001$). We found a statistically significant interaction effect of location by time (GLMM, $z\text{-value}=2.116$, $p=0.034$), where by the end of the experiment in September, we detected KBV in exclusively exposed colonies.

BQCV abundance increased to mid-summer and then decreased until late summer (Figure 3.1C, Table 3.1, $p<0.001$). We found no statistically significant effect of location nor did we find an interaction effect between location and time (Table 3.1, $p>0.2$).

Nosema ceranae abundance was relatively stable throughout the summer, but increased drastically in September (Figure 3.1D, Table 3.1, $p < 0.001$). We found no statistically significant effect of location nor did we find an interaction effect between location and time (Table 3.1, $p > 0.2$).

Prior research indicated that *Nosema* infections tend to co-occur with BQCV and increase BQCV replication (Chen and Siede 2007, Ribière, Ball, and Aubert 2008). Thus, we examined if *Nosema* infections affected BQCV load. We found that BQCV expression levels were higher in samples with detectable levels of *N. ceranae* in colonies located near NNI treated corn fields (Figure 3.2A; Kruskal-Wallis rank sum test, $\chi^2 = 6.692$, $df = 1$, $p = 0.029$), but not in colonies located far from corn fields (Kruskal-Wallis rank sum test, $\chi^2 = 3.152$, $df = 1$, $p = 0.152$).

Immune genes

Apidaecin levels increased over time (Figure 3.1F; Table 3.1, $p < 0.001$), but we found no effect of location nor did we find an interaction between time and location (Table 3.1, $p > 0.1$). We found no statistically significant effect of time, location, or their interaction on the abundance of dorsal (Figure 3.1E; Table 3.1, $p > 0.1$).

Apidaecin expression correlated with DWV expression (Figure S 3.3A; Pearson's Correlation, $t = 2.785$, $df = 271$, $p = 0.006$, $r = 0.167$). No differences in correlations were found between colonies located near corn or far from corn (Fisher's z , $z = -1.091$, $p = 0.275$). No other correlations between immune gene expression and viral loads were found to be statistically significant after correcting for multiple testing for either apidaecin or dorsal (Pearson's Correlation, $p > 0.1$).

2015 Apiary Experiment

The results of the repeated measures mixed model for the 2015 apiary experiment are summarized in Table 3.2. Similar to the 2014 field study, we only detected KBV in a small number of samples in 2015 ($n = 15/50$), thus we only analyzed KBV's prevalence. We detected seasonal variation in the abundance of DWV, SBV, BQCV, and apidaecin (Table 3.2, $p < 0.05$),

but found only marginally significant differences in the abundance of *N. ceranae* (Table 3.2, $p=0.60$) and dorsal (Table 3.2, $p=0.066$).

Pathogens

We found a statistically significant effect of time by treatment for DWV abundance (Figure 3.3A, Table 3.2, $p=0.045$). An ad-hoc analysis revealed that the exposed colonies had marginally higher levels of DWV at day 70 of the experiment (Table S 3.3, $p=0.072$). All other comparisons were not statistically significant (Table S 3.3, $p>0.1$).

SBV expression decreased over the progress of the experiment (Figure 3.3B; Table 3.2, $p=0.045$), but we found no treatment effect (Table 3.2, $p=0.477$) nor an interaction effect of time by treatment (Table 3.2, $p=0.926$).

We found no statistically significant effect of time (Figure S 3.2B; GLMM, z -value=1.641, $p=0.101$), treatment (GLMM, z -value=1.014, $p=0.311$), or their interaction (GLMM, z -value=-1.169, $p=0.242$) on KBV prevalence in our treatment experiment.

In 2015, BQCV exhibited a similar abundance profile over time as in 2014: an increase in abundance followed by a decrease (Figure 3.3C; Table 3.2, $p<0.001$). BQCV levels were higher in unexposed colonies relative to exposed colonies throughout the experiment (Table 3.2, $p=0.021$). No interactions between time and treatment were found (Table 3.2, $p=0.735$).

Nosema ceranae abundance decreased marginally over the course of the experiment (Figure 3.3D; Table 3.2, $p=0.060$). We found no treatment effect or an interaction effect on *N. ceranae* levels (Table 3.2, $p>0.1$).

Similar to 2014, colonies treated with NNIs and with detectable levels of *N. ceranae* had statistically significantly higher levels of BQCV expression than treated colonies without *N. ceranae* (Figure 3.2B; Kruskal-Wallis rank sum test, $\chi^2=8.877$, $df=1$, $p=0.012$). This relationship was not statistically significant for the control colonies (Kruskal-Wallis rank sum test, $\chi^2=1.984$, $df=1$, $p=0.159$).

Immune genes

Apidaecin abundance increased up to day 43 and then decreased until the end of the experiment (Figure 3.3F; Table 3.2, $p=0.001$). Apidaecin relative expression levels did not differ between exposed colonies and control colonies (Table 3.2, $p=0.982$), nor did we find an interaction effect (Table 3.2, $p=0.510$).

Dorsal abundance marginally varied over time (Figure 3.3E; Table 3, $p=0.0662$). No statistically significant effects of treatment (Table 3.2, $p=0.982$) or interaction between treatment and time were found (Table 3.2, $p=0.143$).

As in 2014, apidaecin expression correlated with DWV expression (Figure S 3.3B. Pearson's Correlation, $t=4.528$, $df=48$, $p=0.005$, $r=0.547$), but no differences were found between exposed and unexposed colonies (Fisher's z , $z = 0.836$, $p=0.403$). No other correlations were found to be statistically significant after correcting for multiple testing for either dorsal or apidaecin (Pearson's Correlation, $p>0.1$).

Discussion

The goal of our study was to examine the effects of field realistic NNI exposure on honey bee immunity and pathogen load. We did so by conducting a two-year study. First, we examined colonies near NNI seed-treated corn fields and colonies located far from such corn fields. Then, we conducted a treatment experiment where we exposed half of our colonies to clothianidin, mimicking the dose and duration of exposure we found in the field (Tsvetkov et al. 2017). The 2014 colonies located near NNI treated corn fields were exposed to multiple agrochemicals simultaneously, including several NNIs, while our 2015 exposed colonies were only treated with clothianidin. Overall, we found subtle effects on pathogen loads and no effect on dorsal and apidaecin expression. One pattern was consistent between the field study and apiary experiment: NNI exposed colonies with *N. ceranae* infections had higher levels of BQCV.

Immune genes

Dorsal is an NF- κ B transcription factor, a key component of the Toll pathway in the honey bees' innate immune system (Evans et al. 2006, Lourenço et al. 2018). Apidaecin, is a gene encoding an antimicrobial peptide under NF- κ B control (Evans et al. 2006). We found that the expression levels of dorsal and apidaecin did not differ between colonies located near NNI treated corn fields and colonies located far from corn fields. A field study conducted in Sweden in 2013 also failed to find any difference in apidaecin or dorsal gene expression between colonies placed in NNI treated canola fields or in fields without NNI treatments. They tested the expression levels of an additional six immune genes, all were not statistically different between the treatments (Osterman et al. 2019). In our 2015 apiary experiment, we also found no difference in apidaecin or in dorsal expression. This is in contrast to a laboratory study that found that clothianidin activated NF- κ B signaling (Di Prisco et al. 2013), but in agreement with whole colony experimental exposure that also failed to find NNI effects on dorsal and apidaecin (De Smet et al. 2017).

We found that apidaecin and DWV expression levels were positively correlated during both the 2014 and 2015 studies. Previously, apidaecin and DWV were found to be negatively correlated (Di Prisco et al. 2016). This further illustrates the differences found between field and laboratory studies.

Pathogens

In our 2014 field study we found that colonies located near NNI treated corn had higher levels of SBV than colonies located far from NNI treated fields. This effect only became evident in July, at which point colonies near corn had detectable levels of NNIs for up to three months (Tsvetkov et al. 2017). Additionally, we found that KBV prevalence was higher in colonies located near NNI treated corn, especially towards the end of the summer in August and September. These findings are in contrast to Osterman et al. (2019), who found no difference in viral loads or prevalence between colonies located near NNI treated canola fields and colonies located near untreated fields. However, their study investigated NNI exposure on a much shorter

time scale; their colonies were placed near the canola fields for 2-3 weeks, after which they were placed in a site far from agriculture.

In our 2015 apiary experiment, we found no effect of clothianidin treatment on the levels of SBV or on KBV prevalence. It appears that clothianidin exposure – on its own – has no effect on KBV prevalence or SBV infection levels, but the various stressors, including combinations of NNIs and other agrochemical, that honey bees face near corn fields (Tsvetkov et al. 2017, McMenamin and Genersch 2015) do have a negative impact on the colony's ability to fight off these infections.

We found that DWV levels were higher in colonies located far from corn fields throughout the study, but we note that these differences were present at the first sampling period and may simply have been the result of unequal viral loads between colonies allocated to the near sites caused by chance. Interestingly, Osterman et al. (2019) found that DWV prevalence was higher in colonies located near untreated canola fields, but they did not find a statistically significant treatment effect on DWV. Another study, which looked at DWV levels near NNI treated corn fields and untreated fields also found no treatment effect on viral levels (Alburaki et al. 2015).

In our 2015 apiary experiment, we found an interaction effect between time and NNI treatment on DWV abundance, with highest levels of DWV observed in exposed colonies at day 70 of the experiment. Previous work showed no NNI effects on DWV clinical symptoms (Straub et al. 2019). However, this difference could be explained by the increased sensitivity in our study both due to the testing method and the increased sampling rate.

We found no effect of NNI treatment on BQCV and *N. ceranae* levels in our 2014 field study. In the 2015 apiary experiment, we found no treatment effect on *N. ceranae*, but BQCV levels were consistently higher in unexposed colonies. We found no interaction effect between treatment and time, so it is possible that this result is due to the unexposed colonies having higher levels of BQCV from before the start of the experiment.

We did find that colonies exposed to NNIs and infected with *N. ceranae* had higher levels of BQCV in both our 2014 field study and 2015 apiary experiment. Interestingly this could explain the inconsistent findings from Osterman et al. (2019). They found that BQCV levels were affected by NNI treatment in one of the years, but not in the other. They did quantify *N. ceranae* levels, but they did not analyze the combined effects of *N. ceranae* and NNI treatment. This is particularly notable, since in the same year that they found NNI effects on BQCV levels, they also found different levels of *N. ceranae* between their exposed and unexposed colonies before they were placed in the field.

BQCV is known to co-occur with *Nosema* (Chen and Siede 2007) and *Nosema* infection affects the mid-gut, thus increasing susceptibility to BQCV (Ribière, Ball, and Aubert 2008). BQCV is one of the most prevalent honey bee viruses, often found in practically 100% of the samples (Osterman et al. 2019, Chen and Siede 2007, Ciglenc̆ki et al. 2014, Mondet et al. 2014). *Nosema ceranae* is also a relatively common pathogen (Chen et al. 2008). It thus seems likely that colonies would often be exposed to BQCV and *N. ceranae* infections simultaneously.

BQCV is a virus that primarily impacts developing queens, where infected dead larvae queens can be found sealed in inside their cells (Chen and Siede 2007), although it can affect workers, especially when co-infection with *N. ceranae* occurs (Doublet, Labarussias, de Miranda, et al. 2015). There is evidence that colony level exposure to clothianidin increases queen supersedure (Sandrock et al. 2014), impacts queen immune competence (Brandt et al. 2017), and results in lower levels of queen supersedure success (Williams et al. 2015, Tsvetkov et al. 2017). Our result that NNI exposure and the presence of *N. ceranae* increase the levels of BQCV adds to growing evidence of the negative impacts of clothianidin exposure on honey bee queens.

Laboratory studies have demonstrated that exposure to NNIs can suppress the honey bee's immune system (Brandt et al. 2016, Brandt et al. 2017) and increase pathogen load (Di Prisco et al. 2013). However, as critics have noted, honey bees experience vastly different conditions in the field than those in the lab (Carreck and Ratnieks 2014). Here, we demonstrate that the impact of NNIs on honey bee's immune system and pathogen load in the field is more subtle than those

found in the lab. Additionally, we demonstrate the importance of analyzing co-occurring stressors and performing season long studies, without which we would have failed to detect the impacts of NNIs on pathogen loads.

Acknowledgement

This project was funded through Growing Forward 2 (GF2) and a New Directions grant (ND2013-2084) from the Ontario Ministry of Agriculture, Food and Rural Affairs to A.Z. and V.F. We thank G. Thompson and the Toronto Beekeeping Cooperative for help in locating unexposed sites, B. Harpur for blinding the experimenters, F. McCune and J. Parent for assistance.

Figure Legends

Figure 3.1. Pathogen and Immune gene levels found in 2014

In 2014, (A) Colonies located far from corn had higher levels of DWV throughout the entire season (Table 3.1, $p < 0.001$). (B) Colonies located near corn had higher levels of SBV in late summer (Table 3.1, $p = 0.025$). We found no statistically significant differences between the levels of (C) BQCV, (D) *N. ceranae*, (E) Dorsal, or (F) Apidaecin in colonies located near or far from NNI treated corn (Table 3.1, $p > 0.05$).

Figure 3.2. *Nosema ceranae* infection increased BQCV levels in exposed colonies

(A) Colonies located near NNI treated corn with detectable levels of *N. ceranae* had significantly higher levels of BQCV than colonies without *N. ceranae* (Kruskal-Wallis rank sum test, $\chi^2 = 6.692$, $df = 1$, $p = 0.029$). This was not true for colonies located far from NNI treated corn fields (Kruskal-Wallis rank sum test, $\chi^2 = 3.152$, $df = 1$, $p = 0.152$). (B) Colonies exposed to NNIs in the 2015 apiary experiment with detectable levels of *N. ceranae* had significantly higher levels of BQCV than colonies without *N. ceranae* (Kruskal-Wallis rank sum test, $\chi^2 = 8.877$, $df = 1$, $p = 0.012$). This was not true for unexposed colonies (Kruskal-Wallis rank sum test, $\chi^2 = 1.984$, $df = 1$, $p = 0.159$). n.s.=not significant, $* = p < 0.05$. The violin plot shows the median at white dot, the interquartile ranges are marked with black thick lines, and the overall shape denotes the kernel density distribution.

Figure 3.3. Pathogen and Immune gene levels found in 2015

In 2015, (A) we found a time by treatment interaction for DWV abundance (Table 3.2, $p = 0.045$). (C) Unexposed colonies had higher levels of BQCV throughout the experiment (Table 3.2, $p = 0.021$). We found no statistically significant differences between the levels of (B) SBV, (D) *N. ceranae*, (E) Dorsal, or (F) Apidaecin in colonies located near or far from NNI treated corn (Table 3.2, $p > 0.05$).

Figure S 3.1. The NNI exposure colonies experienced in the field and in the apiary experiment

We exposed honey bee colonies to realistic but conservative levels of NNIs. We used NNI residue data from Ontario (2014) to estimate the total dietary NNI exposure under two scenarios. Conservative scenario (grey): We naively assigned 0 ppb to samples with no detectable levels of NNIs and averaged over these zero values when estimating mean dietary NNI exposure (i.e., pollen + nectar) over the course of the season. Extreme scenario (yellow): We assigned a value of 1 ppb to samples with no detectable levels of NNIs and averaged over these assumed values. Our treatment experiment (blue) treated honey bees to lower levels and a shorter duration of NNI exposure relative to both best case and worst-case scenarios derived from the field toxicology data. Error bars (SEM) were estimated for time points containing more than 2 observations. Originally published in (Tsvetkov et al. 2017).

Figure S 3.2. KBV prevalence

KBV prevalence had a statistically significant interaction effect between time and treatment in (A) 2014 (GLMM, z -value=2.116, p =0.0344), but not in (B) 2015 (GLMM, z -value=-0.273, p =0.785).

Figure S 3.3. Apidaecin and DWV expression levels correlated in both years

Apidaecin and DWV expression levels were positively correlated in (A) 2014 (Pearson's Correlation, t =2.785, df =271, p =0.006, r =0.167) and (B) 2015 (Pearson's Correlation, t =4.528, df =48, p =0.005, r =0.547). No difference between exposed and unexposed correlations were found in either years (2014: Fisher's z , z = -1.091, p -value = 0.275; 2015: Fisher's z , z = 0.836, p -value = 0.403).

Figure 3.1

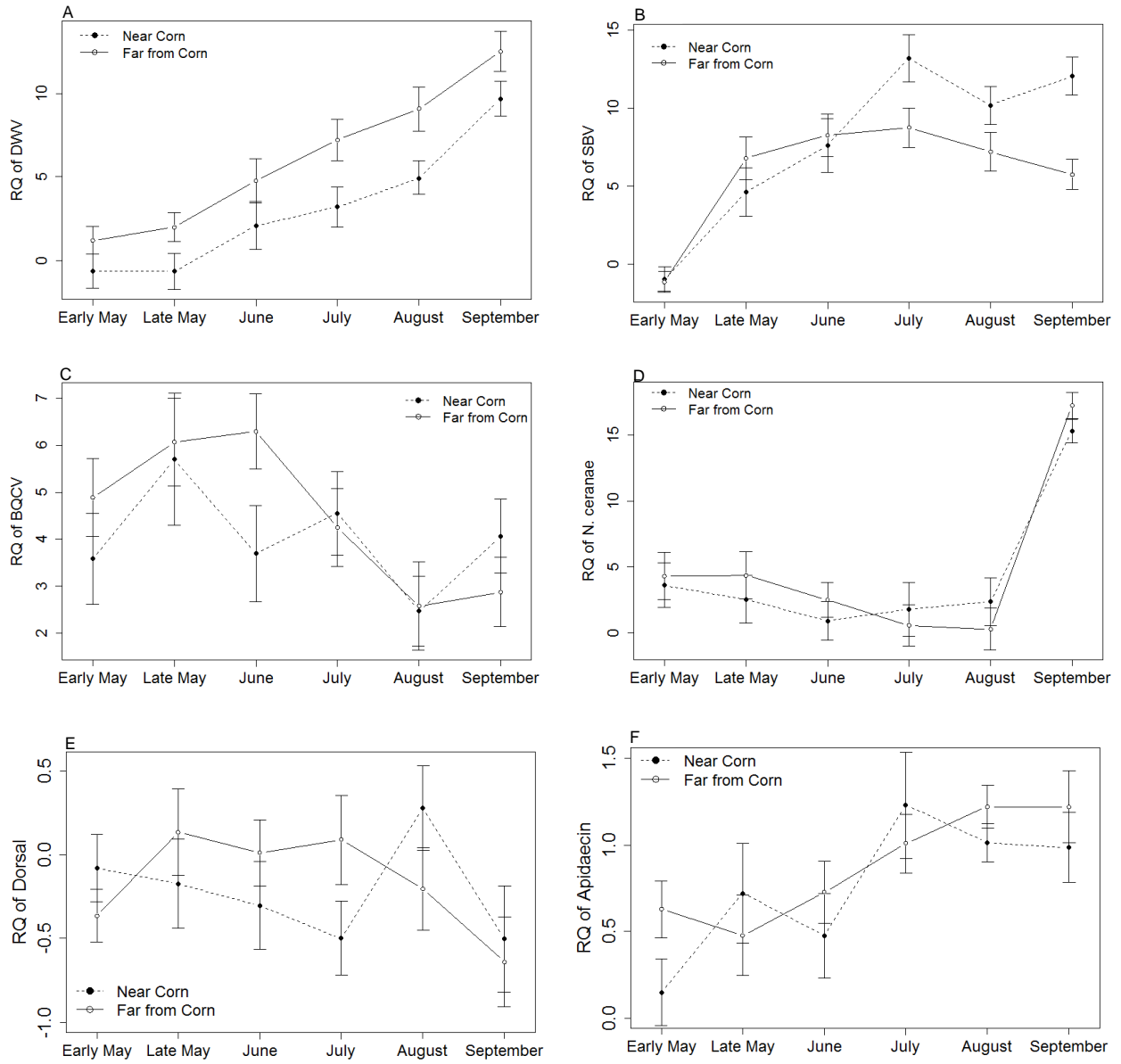


Figure 3.2

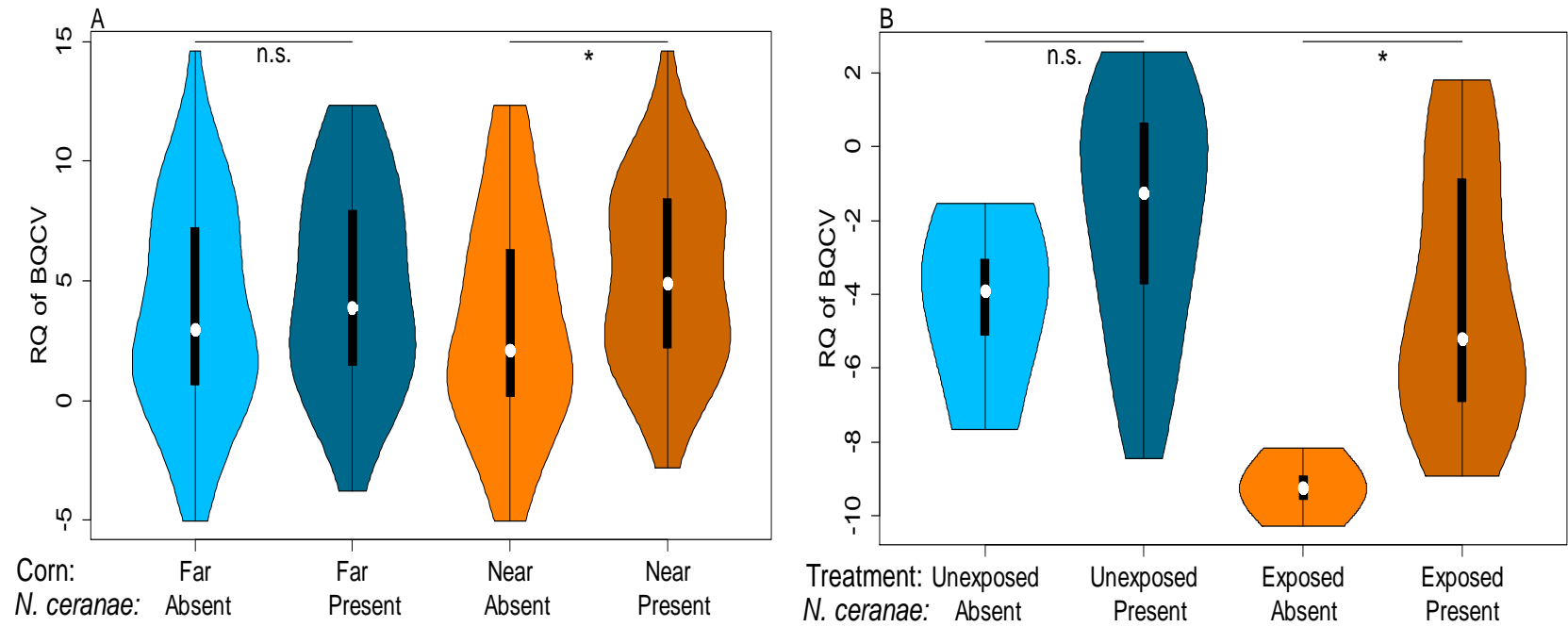


Figure 3.3

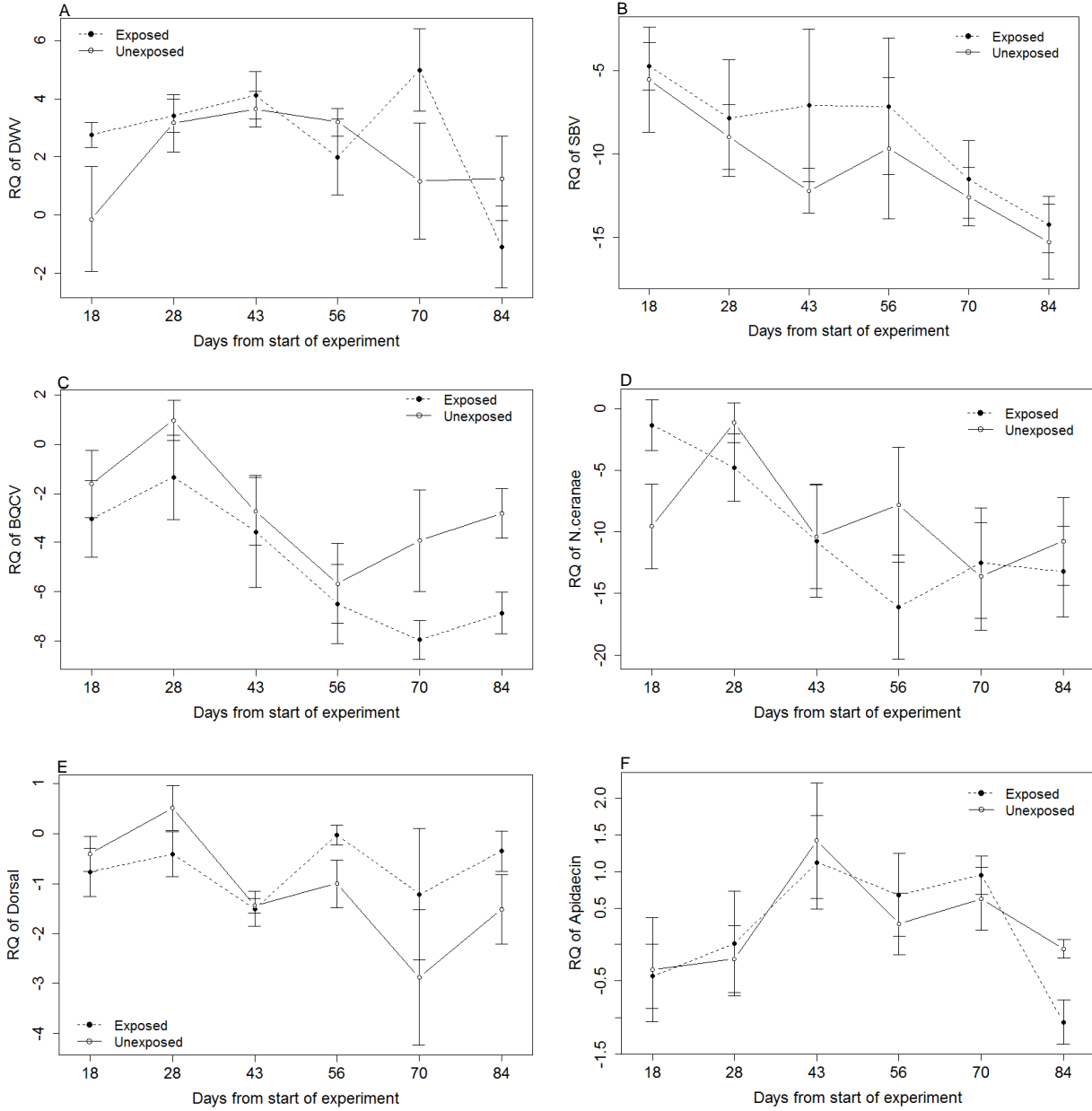


Figure S 3.1

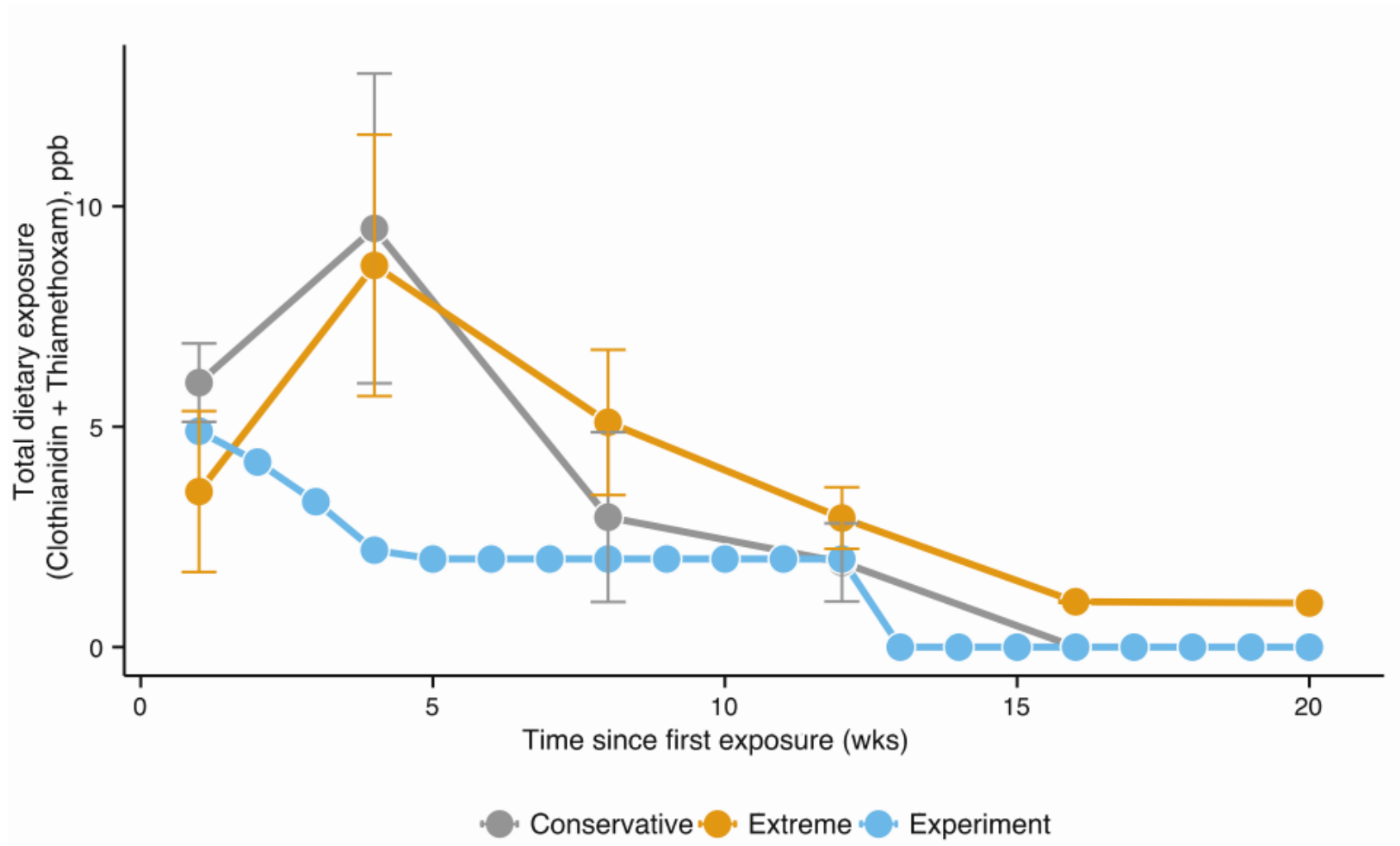


Figure S 3.2

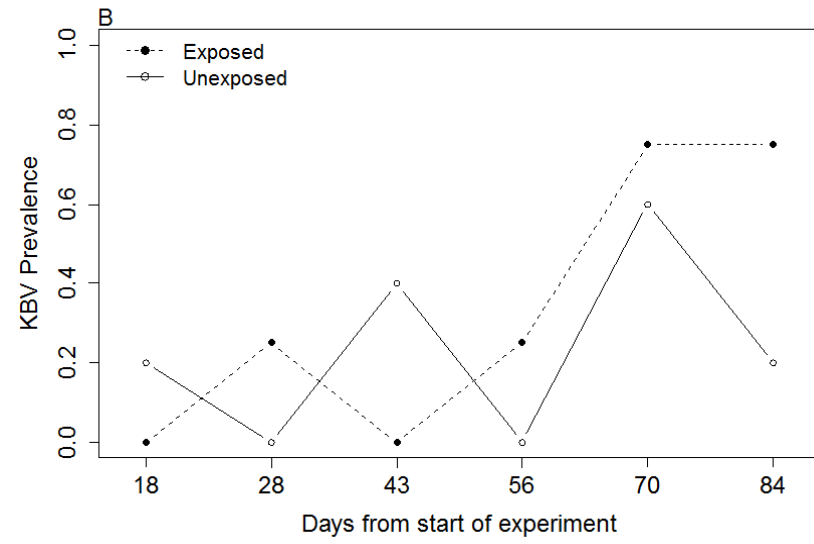
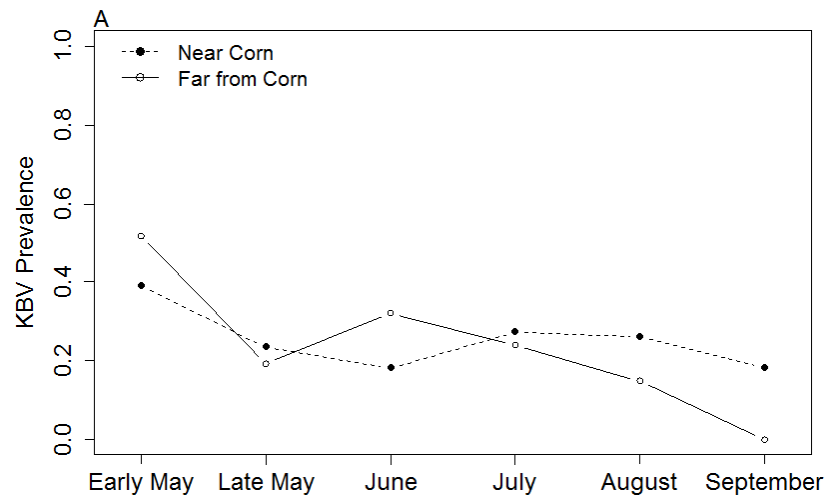


Figure S 3.3

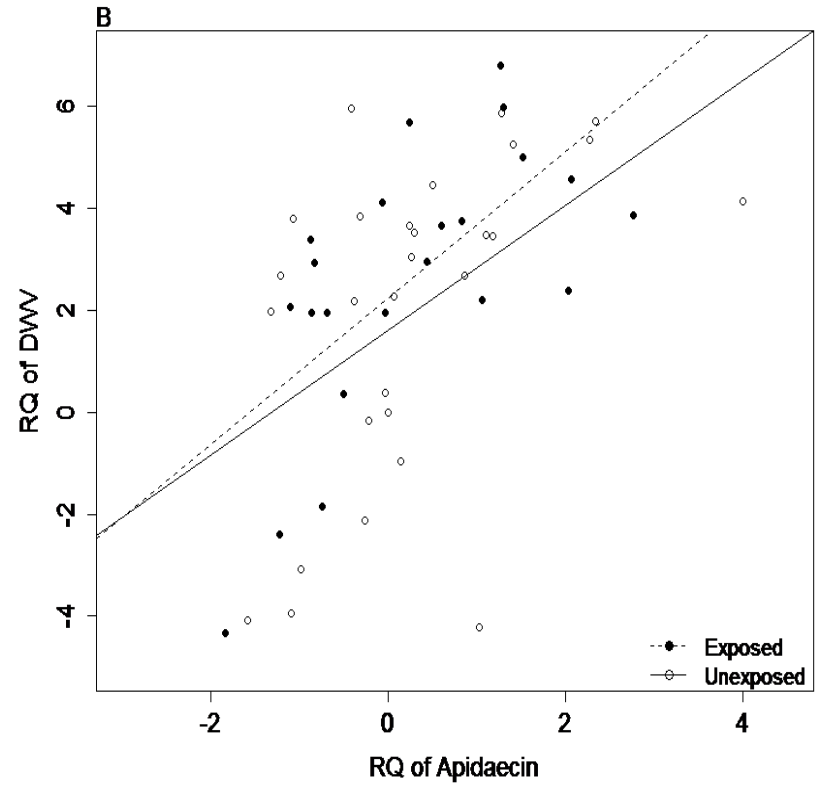
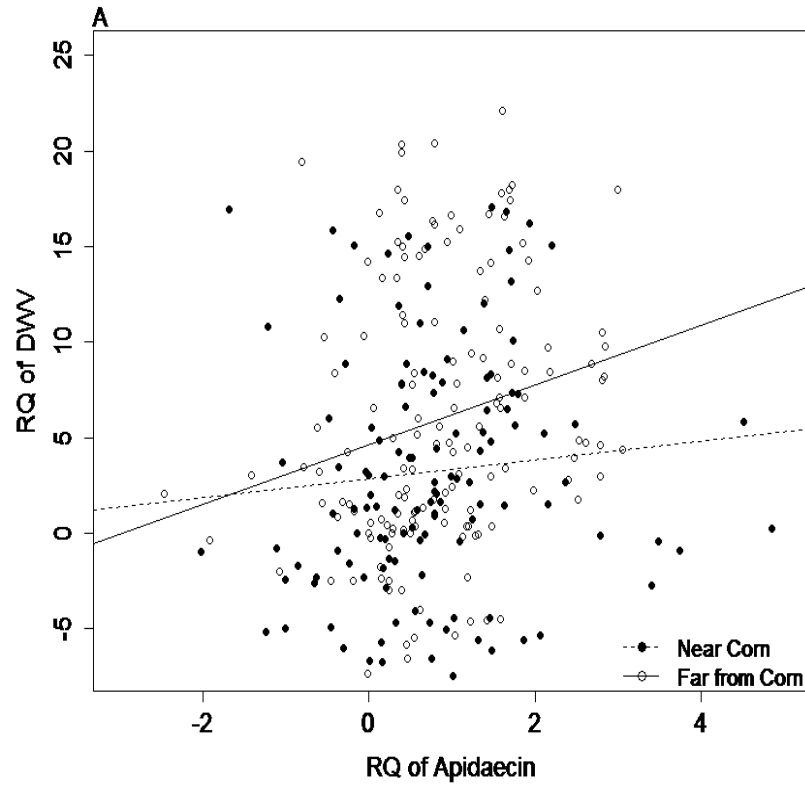


Table 3.1. Results of the repeated measures mixed model for the 2014 field study

	Effect	DF	F-value	P-value
DWV	Location	1, 53	15.74	<0.001
	Time	5, 218	21.22	<0.001
	Time*Location	5, 218	0.31	0.907
SBV	Location	1, 53	4.19	0.046
	Time	5, 222	19.90	<0.001
	Time*Location	5, 222	2.62	0.025
BQCV	Location	1, 53	0.36	0.554
	Time	5, 217	3.29	0.007
	Time*Location	5, 217	1.40	0.224
<i>N. ceranae</i>	Location	1, 53	0.21	0.645
	Time	5, 217	21.84	<0.001
	Time*Location	5, 217	0.54	0.746
Dorsal	Location	1, 53	0.12	0.734
	Time	5, 217	1.69	0.139
	Time*Location	5, 217	1.34	0.248
Apidaecin	Location	1, 53	0.66	0.420
	Time	5, 224	4.91	<0.001
	Time*Location	5, 224	1.18	0.321

Table 3.2. Results of the repeated measures mixed model for the 2015 apiary experiment

	Effect	DF	F-value	P-value
DWV	Treatment	1, 7	0.40	0.549
	Time	5, 31	3.00	0.025
	Time*Treatment	5, 31	2.59	0.045
SBV	Treatment	1, 7	0.56	0.477
	Time	5, 31	2.60	0.045
	Time*Treatment	5, 31	0.27	0.926
BQCV	Treatment	1, 7	8.72	0.021
	Time	5, 31	5.59	0.001
	Time*Treatment	5, 31	0.55	0.735
<i>N. ceranae</i>	Treatment	1, 7	0.17	0.693
	Time	5, 32	2.39	0.060
	Time*Treatment	5, 32	1.04	0.413
Dorsal	Treatment	1, 7	2.27	0.176
	Time	5, 31	2.33	0.066
	Time*Treatment	5, 31	1.79	0.143
Apidaecin	Treatment	1, 7	0.00	0.982
	Time	5, 32	5.64	0.001
	Time*Treatment	5, 32	0.87	0.510

Table S 3.1. Primers used for amplification of the pathogen, target and reference control genes as well as their slope and efficiencies

F= Forward primer; R= Reverse primer.

Pathogen/Gene	Primer Sequence (5'-3')	Slopes	Efficiency
Deformed Wing Virus	F: GAGATTGAAGCGCATGAACA R: TGAATTTCAGTGTGCCCCATA	-3.404	1.967009954
Sacbrood Virus	F: GGGTCGAGTGGTACTGGAAA R: ACACAACACTCGTGGGTGAC	-2.904	2.209798208
Kashmir Bee Virus	F: GAACGTCGACCTATTGAAAAA R: TCGATTTTCCATCAAATGAGC	-3.277	2.019183465
Black Queen Cell Virus	F: TTTAGAGCGAATTCGGAAACA R: GGCGTACCGATAAAGATGGA	-3.390	1.972237209
<i>Nosema ceranae</i>	F:CAATATTTTATTATTTTGAGAGA R:TATATTTATTGTATTGCGCGTGCA	-3.312	2.004159894
Dorsal	F: TCGGATGGTGCTACGAGCGA R:AGCATGCTTCTCAGCTTCTGCCT	-3.429	1.912926
Apidaecin	F: TTGCCTTAGCAATTCTTGTTG R: GTCGAGTAGGCGGATCT	-3.550	1.912926
Actin	F: TGTATGCCAACACTGTCCTTT R: TGGCGCGATGATCTTAATTT	-3.432	1.956029009

Table S 3.2. SBV level in each time point in 2014

2014 SBV Simple Differences of Time*Location Least Squares Means. Adjustment for Multiple Comparisons: Tukey-Kramer

Time	Estimate	Standard Error	DF	t-value	Adj P
Early May	-0.1877	1.8603	222	-0.10	0.9197
Late May	1.5945	1.8357	222	0.87	0.3860
June	0.8910	1.7438	222	0.51	0.6099
July	-4.0452	1.7970	222	-2.25	0.0254
August	-2.9819	1.7400	222	-1.71	0.0880
September	-6.3073	1.8880	222	-3.34	0.0010

Table S 3.3. DWV levels in each time point in 2015

DWV Simple Differences of Time*Location Least Squares Means. Adjustment for Multiple Comparisons: Tukey-Kramer

Time	Estimate	Standard Error	DF	t-value	Adj P
18	3.0840	1.9727	31	1.56	0.1281
28	0.2464	1.8421	31	0.13	0.8945
43	0.4795	1.8421	31	0.26	0.7964
56	-0.8404	2.00134	31	-0.42	0.6793
70	3.6394	1.9510	31	1.87	0.0716
84	-2.3617	1.8421	31	-1.88	0.2093

Chapter 4 Polymorphisms in CYP9Q genes explain large variation in honey bee sensitivity to neonicotinoid insecticides

N. Tsvetkov and A. Zayed

Introduction

The relationship between neonicotinoid insecticides (NNIs) and honey bee health is intensely debated (Havard, Laurent, and Chauzat 2020, Carreck and Ratnieks 2014, Cutler, Scott-Dupree, and Drexler 2014, Cutler and Scott-Dupree 2016). While many studies have found that sublethal exposure leads to a wide range of harmful effects in honey bees (Tsvetkov et al. 2017, Alkassab and Kirchner 2017), others have found no negative effects of NNIs exposure on honey bee health (Cutler et al. 2014, Osterman et al. 2019). While some of these discrepancies can be attributed to the differences in methodologies and study parameters, including the dose and duration of NNI exposure (Carreck and Ratnieks 2014), variation in NNI response within the same study is harder to explain (Woodcock et al. 2017). There is some evidence that suggests that background genetics plays a role in honey bee's susceptibility to NNIs (Rinkevich et al. 2015).

There is a large degree of variation in the LD₅₀ dose estimated for NNIs in honey bees (Iwasa et al. 2004, Thompson et al. 2014, Suchail, Guez, and Belzunces 2000, Rinkevich et al. 2015). The LD₅₀ dose is defined as the dose at which 50% of the test animals die (Randhawa 2009). Laurino et al. (2013) reported the LD₅₀ dose at 24 hour for clothianidin for five different colonies of *Apis mellifera ligustica*, which ranged from 9.69ppb to 41.96ppb. In another study, Rinkevich et al. (2015) tested multiple colonies from different honey bee stocks. The authors found that *Apis m. ligustica* were 34 time more sensitive to imidacloprid than *A. m. carnica*, although the authors did not control for the volume of NNI-spiked sucrose solution consumed by the different subspecies. While colony and subspecies differences in LD₅₀ may suggest genetic differences that modulate susceptibility of bees to NNIs, they can also reflect environmental differences between colonies (e.g. nutrition, exposure to other pesticides, and prevalence of disease). Controlled experiments are needed to determine if NNI susceptibility is indeed heritable, and to which degree genetics contribute to this important trait. Addressing this

knowledge gap is essential for understanding the impact NNIs have on bee health and for resolving the conflicting experimental evidence on the acute and sublethal toxicity of NNIs on honey bees currently found in the literature.

Resistance to NNIs has been studied in several pest insects. It is often modulated by increased metabolism of NNIs by cytochrome p450 monooxygenase (CYP) proteins, although resistance can also be caused by mutations in the nicotinic acetylcholine receptors, the target sites of NNIs (Matsuda, Ihara, and Sattelle 2020). Enhanced metabolism by CYP proteins can occur through mutations that increase protein expression or through amino-acid alterations in that alter protein structure in ways that can affect recognition, binding, and breakdown of the pesticides (Matsuda, Ihara, and Sattelle 2020, Zimmer et al. 2018). In honey bees, out of all the CYP proteins, it is the CYP3 family that is most associated with detoxification (Berenbaum and Johnson 2015). After testing all of the 27 genes in the CYP3 family, Manjon et al. (2018) showed that NNIs are metabolized by CYP9Q1, CYP9Q2, and CYP9Q3. We do not currently know if there is natural genetic variation in these genes and whether they are associated with NNI susceptibility.

In this study, we exploited patriline differences within honey bee colonies to estimate the heritability of NNI susceptibility. Honey bees are haplo-diploid and the queen is polyandrous (Rothenbuhler 1958). This means that, within a single colony, worker bees sired by the same father are, on average, 75% related, while workers sired by different fathers are, on average, 25% related. Since all of the workers within the colony experience the same maternal effects and environment, partitioning of the phenotypic variance to within and between partlines within a colony can be used to estimate broad sense heritability (H^2) - the proportion of phenotypic variance that is influenced by additive genetic variance (Fjerdingstad 2005, Laloï and Pham-Delegue 2010, Kovacs et al. 2010, Harpur et al. 2014).

We exposed individual worker bees from two colonies to the LD₅₀ dose of clothianidin (Rinkevich et al. 2015, Laurino et al. 2013) and recorded whether bees survived or died after 24 hours of exposure. We genotyped the workers at 11 microstallite loci to determine their patrilineages. This framework allowed us to estimate the broad sense heritability of NNI

susceptibility, here defined as the proportion of a patriline that survived the clothianidin treatment at 24h. We then sequenced the CYP9Q1, 2, and 3 genes from patrilines with high and low survival rates in order to determine whether particular mutations in these genes are associated with different survival rates. Our analyses shed new insights on the genetics and mechanisms underlying NNI susceptibility in honey bees.

Methods

Bees

The bees were obtained from two colonies with naturally mated queens located at the York University (Toronto, Ontario, Canada). Like most North American colonies, bees had a mixed genetic ancestry with major contributions from the East European population group (C group: *A. m. ligustica* and *A. m. carnica*) and minor contributions from the West European population group (M group: *A. m. mellifera*) (Harpur et al., 2012; 2013; Harpur and Zayed, 2013). Ready to emerge brood from two honey bee colonies were placed in a 33°C incubator. Every day, newly emerged worker bees were marked with a new color using non-toxic enamel paint (Testors®) and introduced into their respective colonies. When the bees were 7 days old they were collected, placed into individual petri dishes with air holes, given a large 50% sucrose feeder, and placed into a 33°C incubator overnight, separate from the brood.

Clothianidin Exposure

The next day, the bees were randomly allocated into 2 groups: lethal dose and control. The lethal dose was 29ppb of clothianidin representing the oral LD₅₀ value obtained from the U.S. Environmental Protection Agency Pesticide Ecotoxicity database. Clothianidin (99.9%, Pestal, Sigma-Aldrich) was first prepared using acetone and then diluted in 50% w/v sucrose (Sigma-Aldrich) solution, such that the final acetone concentration was 0.1%. The control group received 50% w/v sucrose solution containing 0.1% acetone. We followed standard protocol for acute oral toxicity test (Buschmann 2013), with the modification of testing one dose and each bee individually, as opposed to in groups. We also focused on 24 hour mortality, as opposed to

48 hour mortality, since we found no difference between 24 and 48 mortality in our pilot study (data not shown), which is in agreement with previous research (Laurino et al. 2013).

Briefly, the bees were starved for 2 hours, given a pre-weighed small feeder with 20 μ L of the appropriate sugar solution for 4 hours, and then the small feeder was removed, weighed, and a large feeder of 50% w/v sugar solution was introduced. These bees were then placed into the incubator and mortality was scored 24 hours after the removal of the small feeder. All of the bees were frozen at -80°C. The mortality in the control group never exceeded 10% per 24 hours. Bees that consumed less than 90% of the sugar solution were not included in the analysis (N=270).

DNA Extraction

DNA extraction was performed using the Mag-Bind® Blood & Tissue DNA HDQ 96 Kit (Omega Bio-Tek) and the KingFisher Flex extraction system (ThermoFisher Scientific). Briefly, half a bee's thorax was crushed using a pestle while being cooled in liquid nitrogen. Then, we added 350 μ L of TL buffer and 20 μ L of Proteinase K solution to the crushed thorax, vortexed for 10 seconds, and incubated at 50°C overnight. The next day, the sample was centrifuged for 10 minutes at 7000xg/rcf and 300 μ L of the clear supernatant was transferred into an intermediate tube. The intermediate tube was centrifuged for 10 minutes at 7000xg/rcf and 250 μ L of the clear supernatant was transferred into a KingFisher Microtiter deep-well plate. We then added 5 μ L of RNase A, mixed by pipetting, and incubated for 10min at room temperature. After that, we added 290 μ L of AL Buffer, 400 μ L of HDQ Binding bugger, and 20 μ L of Mag-Bind® particles HDQ. The plate was then placed into the KingFisher Flex instrument. The subsequent plates in the KingFisher Flex instrument contained: 600 μ L of VHB Wash Buffer, 600 μ L of VHB Wash Buffer, 600 μ L of SPM Buffer, 500 μ L of Nuclease-free Water, and 100 μ L of Elution Buffer. After the KingFisher Flex instrument protocol run was finished, we transferred the final elute into separate tubes. The DNA quality and quantity was tested using NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific)(Desjardins and Conklin 2010) and stored at -20°C until further analysis.

Microsatellite genotyping and Patriline determination

We amplified 11 microsatellites (Table S 4.1) using a poolplex reaction following a published protocol (Shaibi, Lattorff, and Moritz 2008). Briefly, a PCR reaction contained 10.0 μ L of Nuclease free water, 0.5 μ L of fluorescently labeled forward primer, 0.5 μ L of reverse primer, 12.5 μ L of TAQ 2X Master Mix (New England Biolabs), and 1.5 μ L of DNA sample. We used a x1000 touch™ thermocycler (BioRad) with the annealing temperature of 55.0°C. The samples were then sent to The Centre for Applied Genomics at The Hospital for Sick Children for automated fragment analysis. We used Geneious (version 11.0) with the Microsatellites plugin to assign alleles and call genotypes, which were checked manually for errors and miscalls. To distinguish patrilines, for each colony, we first deduced the genotype of the queen and assumed that workers with the same haplotype at the 11 microsatellites were sired by the same drone (Shaibi, Lattorff, and Moritz 2008, Harpur et al. 2014).

CYP9Q Genotyping

From each colony, the highest and lowest five patrilines in terms of survival with at least five tested bees were chosen for CYP9Q sequencing. We targeted the protein coding sequence (CDS) of three p450 genes (CYP9Q1, CYP9Q2, and CYP9Q3; Table S 4.2), where each gene contains one exon. The PCR reaction was performed as described above with the annealing temperatures of 59.0°C for CYP9Q1 and CYP9Q3, and 58.0°C for CYP9Q2. The samples were sent to The Centre for Applied Genomics at The Hospital for Sick Children for sanger sequencing and aligned to reference sequences for these genes from the published honey bee genome using the Geneious® (Ver. 11.0) software. CYP9Q1 sequences aligned from nucleotide 172 to 1580 of XM_006562301.3 (CDS: 74-1606), CYP9Q2 sequences aligned from 226 to 1739 of XM_392000.7 (CDS: 172-1770), and CYP9Q3 sequences aligned from 173 to 1567 of XM_006562300.3 (CDS: 85-1638). The coding sequences were translated into amino acid sequences in order to detect non-synonymous nucleotide changes.

Statistical Analysis

Statistical analysis was performed using R (version 3.6)(Team 2005). Effect of colony and patriline on survival was performed using the Pearson's Chi-squared test (Agresti 2018). Due to some patrilines having a low number of workers tested, the test was also carried out with the p-value computed by a Monte Carlo simulation (Hope 1968) with 5000 replicates. Both tests yielded similar p-values, therefore we only report the former.

The combined effects of colony and patriline were explored using a generalized linear model (GLM) with a binomial family structure, where patrilines were nested within colony. Broad sense heritability (H^2) of a phenotype can be estimated by contrasting the phenotypic variance that is explained by patriline relative to the total phenotypic variance. For haplo-diploid organisms, H^2 is twice the patriline variance divided by total phenotypic variance (Fjerdingstad 2005). Since we used a GLM with a binomial family structure, instead of variance explained, we used deviance explained or adjusted D^2 (Guisan and Zimmermann 2000) to estimate H^2 .

The effects of CYP9Q haplotypes on survival were explored using a binomial GLM weighed with number of bees ran per patriline, with haplotypes nested within colony and analysis of deviance analysis, type 2 for unbalanced design (Nelder and Wedderburn 1972, Langsrud 2003).

We examined how CYP9Q haplotypes were associated with survival rate by performing a classification tree analysis, which iteratively selects the attribute (gene) and value (haplotype) that can split the data into two groups (survived or perished after 24h exposure), while minimizing the variability within group and maximizing between group contrast (Breiman et al. 1984), as implemented in the R package 'rpart' (Therneau, Atkinson, and Ripley 2015). To prune the tree we chose the optimal complexity parameter (CP) value with the minimum cross-validated error. Visualization of the tree was performed using the 'rpart.plot' package (Milborrow 2016).

Results

The proportion of dead bees after 24 hours of exposure to 29ppb of clothianidin – the average oral LD₅₀ value published for honey bees by the US's Environmental Protection Agency (EPA 2017), was 69/247 (28%) bees for Colony 36 and 40/249 (16%) for Colony 37. This difference was statistically significant (Chi-squared test $\chi^2=9.510$, df=1, p=0.002). This lower than expected mortality (i.e. 50%) is consistent with the large degree of variability in NNI toxicity for different honey bee colonies and strains (Rinkevich et al. 2015, Laurino et al. 2013).

We found 26 patriline in Colony 36 and 21 patriline in Colony 37, within the range of published estimates of multiple mating for honey bee queens (Estoup, Solignac, and Cornuet 1994, Withrow and Tarpy 2018). In both colonies, patriline was found to have a statistically significant effect on survival (Figure 4.1; C36: Chi-squared test, $\chi^2=57.842$, df=25, p<0.001; C37: Chi-squared test, $\chi^2= 35.387$, df=20, p=0.029). When both colonies were analyzed together, patriline continued to have a statistically significant effect on survival (Analysis of deviance type II, LR $\chi^2= 98.769$, df=45, p<0.001).

The greatest amount of deviance was explained by the differences among workers within patriline (Table 4.1), with $D^2=0.791$. The patriline component explained 18.9% of the deviance, leading to a H^2 estimate of 37.8%. We repeated the analysis with patriline that had at least three workers tested and found similar levels of deviance explained by each factor (Colony: 2.2%; Patriline within colony: 17.2%; Bees within patriline within colonies: 80.6%; $H^2= 34.4\%$).

Variation within CYP9Q genes

We found non-synonymous mutations in all three CYP9Q genes (Figure S 4.1, Figure S 4.2, Figure S 4.3). We found a total of six, four, and seven haplotypes segregating within our sequenced bees for CYP9Q1, CYP9Q2 and CYP9Q3, respectively. Across all three genes, we identified 13 distinct CYP9Q haplotypes. These CYP9Q haplotypes had a statistically significant effect on patriline mean survival (Analysis of deviance type II, Likelihood Ratio (LR) $\chi^2=53.241$, df=14, p<0.001).

A classification tree analysis revealed that CYP9Q3 and CYP9Q1 haplotypes best predicted survival (Figure 4.2; split=2, CP=0.01). CYP9Q3 had the largest effect on survival. Three CYP9Q3 haplotypes, L, N, or P, were associated with lower survival relative to the other haplotypes (Figure 4.2). CYP9Q1 haplotypes modulated the effect of the survival reducing haplotypes of CYP9Q3 (Figure 4.2). The combination of CYP9Q3 haplotypes, L, N, or P and CYP9Q1 haplotype of B or E, had the lowest survival rates in our dataset. CYP9Q2 haplotypes did not improve survival prediction.

Discussion

In this study, we investigated the degree to which NNI susceptibility is heritable and whether genetic variations in the CYP9Q1-3 genes were associated with mortality after NNI exposure. We exposed workers from two naturally mated queens to an LD₅₀ dose of clothianidin. After 24 hours, we scored the workers as alive or dead and genotyped them in order to determine their patrilineage. We found that patriline had a statistically significant effect on survival and estimated the broad sense heritability as 37.8%. We identified non-synonymous mutations in three CYP9Q genes. CYP9Q3 haplotypes were the largest predictor of survival in our study. We also found that given particular CYP9Q3 haplotypes, CYP9Q1 haplotypes were also important for survival prediction, while CYP9Q2 haplotypes did not predict survival in our study.

CYP9Q1-3 proteins are part of the cytochromes p450 superfamily, which contain a heme cofactor and function as monooxygenases (Mao, Schuler, and Berenbaum 2011, Schuler 1996). Using molecular modeling, six putative substrate recognition sites (SRS) were identified (Mao, Schuler, and Berenbaum 2011). Amino acid changes near or within these sites are the ones most likely to have an effect on the protein's ability to metabolize substrates. Additionally, amino acid backbone size and hydrophobicity are the major factors affecting protein structure and function (Barnes and Gray 2003). Thus, here we highlight these amino acid changes in the CYP9Q haplotypes that were associated with a lower survival rate in our analysis.

In CYP9Q3, haplotype L had a notable amino acid change AA288M→T, which is located near the fourth putative substrate recognition site (SRS4) (Mao, Schuler, and Berenbaum 2011). Methionine (M) is non-polar, large amino acid, while threonine (T) is polar with a shorter

backbone (Barnes and Gray 2003). Haplotype N has a notable amino acid difference at position AA238G→R, which is located immediately before the third putative substrate recognition site (SRS3). Glycine (G), is non-polar and hydrophobic, while arginine (R) is positively charged, not hydrophobic, and much larger. Finally, haplotype P had an AA301T deletion inside SRS4. Threonine often forms hydrogen bonds with polar substrates (Barnes and Gray 2003), thus this deletion is particularly notable.

CYP9Q1 haplotypes B or E were also associated with lower survival in conjunction with CYP9Q3 haplotypes L, N, and P. Haplotype E had a 4 base pair deletion, which resulted in a frameshift and a stop codon shortly after. This would produce a protein that would be 280 amino acids long and probably non-functioning, given that the wildtype CYP9Q1 protein is 510 amino acids in length (Mao, Schuler, and Berenbaum 2011). Haplotype B had two notable mutations: AA320M→V and AA325T→A, which are located near SRS3. Although both methionine (M) and valine (V) are non-polar, M has a longer carbon chain that contains sulfur. Threonine (T) is polar, while alanine (A) is non-polar and smaller than T (Barnes and Gray 2003).

We found that CYP9Q2 haplotypes did not predict survival. This could be due to the similarity between the substituted amino acids we found in the CYP9Q2 haplotypes: AA248I → V and AA467H →Y. Isoleucine (I) and valine (V) are both aliphatic and hydrophobic, while histidine (H) and tyrosine (Y) are both aromatic and hydrophobic. These types of amino acid substitutions are unlikely to cause structural change in the protein (Barnes and Gray 2003).

Further inquiry is required in order to determine whether the above mentioned amino acid changes in CYP9Q1 and CYP9Q3 affect protein function. These amino acid changes were all found within or near the proteins' substrate recognition sites, which may lead to an altered cavity for substrate docking or changes in hydrogen bonds with the substrate (Wu, Podust, and Guengerich 2005). Therefore, testing the CYP9Q1 and CYP9Q3 haplotypes with clothianidin as a substrate will be critical in determining the mechanisms behind the large variation in NNI susceptibility.

Conclusion

Previous research has shown that NNI susceptibility is affected by genotype, but neither the degree nor the underlying genetic variance was explored (Rinkevich et al. 2015). Here, we demonstrate that NNI susceptibility is affected by patriline and has an estimated broad sense heritability of 37.8%. We also show that polymorphisms in CYP9Q3 and CYP9Q1 haplotypes predict survival after clothianidin exposure. We implicate certain mutations that are likely to cause structural change to these p450 proteins. Our study indicates that the natural variation within the primary NNI detoxification genes in honey bees helps explain the large variation in NNI susceptibility. We believe that this natural variation could explain some of the conflicting results found in the literature (Osterman et al. 2019, Sandrock et al. 2014), particularly where conflicting results are found within the same study (Woodcock et al. 2017). While survival after a lethal challenge does not imply that some honey bee genotypes are ‘resistant’ to NNIs, our study clearly shows that genetics do play a role in how honey bees respond to NNIs. Additional work is needed to establish how genetics influence the sublethal and chronic toxicity of NNIs to honey bees; conditions that are much more likely to be realistically encountered in the field (Tsvetkov et al. 2017).

Acknowledgements

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Figure Captions

Figure 4.1. Patriline affects survival after clothianidin exposure

(A) Colony 36 (Chi-squared test, $\chi^2=57.842$, $df=25$, $p<0.001$) and (B) Colony 37 (Chi-squared test, $\chi^2= 35.387$, $df=20$, $p=0.029$). The numbers within each bar indicate the total number of workers tested for each of the patrilines.

Figure 4.2. CYP9Q3 and CYP9Q1 haplotypes predicted bee survival

Each node shows (top to bottom): predicted class, predicted probability of survival, and the percentage of observations in the node.

Figure S 4.1. CYP9Q1 haplotypes

Putative substrate recognition sites (SRS) are underlined and based on Mao, Schuler, and Berenbaum (2011). Bolded amino acid residues represent the sites of non-synonymous mutations. Haplotypes with a grey background were found to be associated with a lower survival rate.

Figure S 4.2. CYP9Q2 haplotypes

Putative substrate recognition sites (SRS) are underlined and based on Mao, Schuler, and Berenbaum (2011). Bolded amino acid residues represent the sites of non-synonymous mutations.

Figure S 4.3. CYP9Q3 haplotypes

Putative substrate recognition sites (SRS) are underlined and based on Mao, Schuler, and Berenbaum (2011). Bolded amino acid residues represent the sites of non-synonymous mutations. Haplotypes with grey a background were found to be associated with a lower survival rate.

Figure 4.1

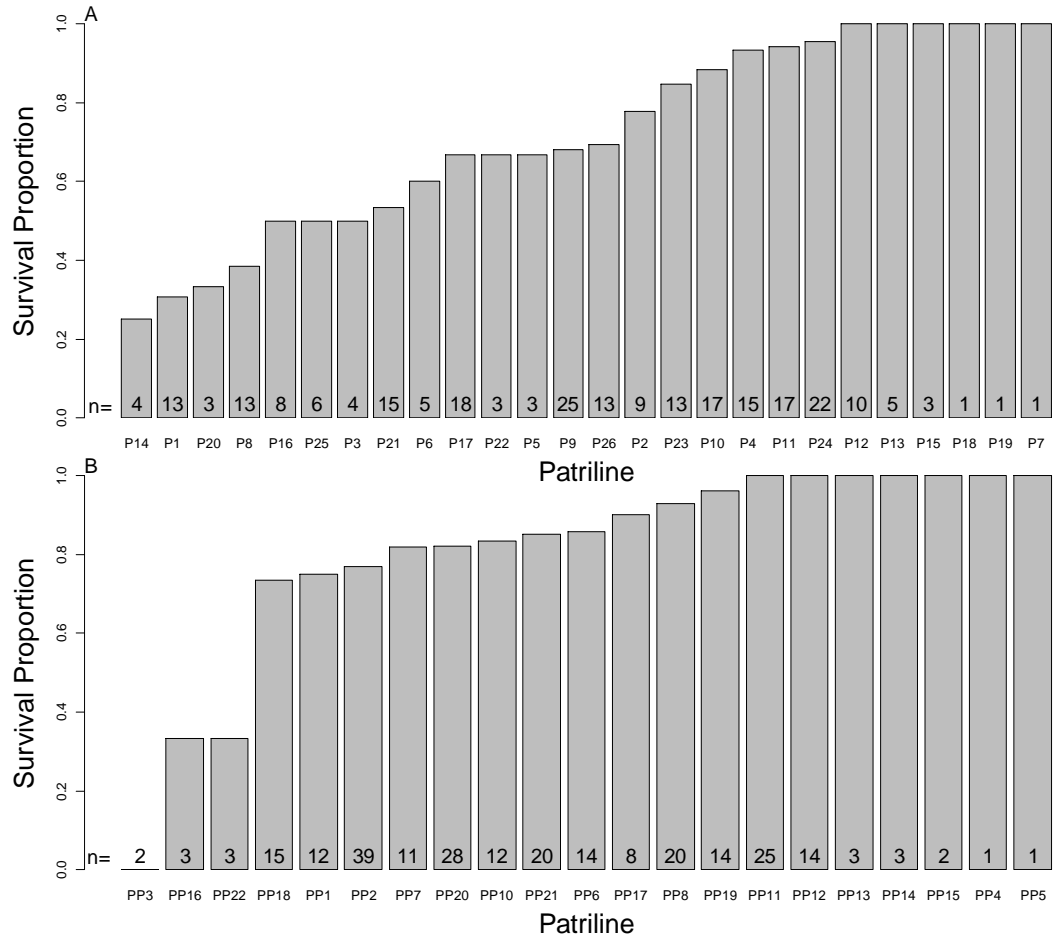


Figure 4.2

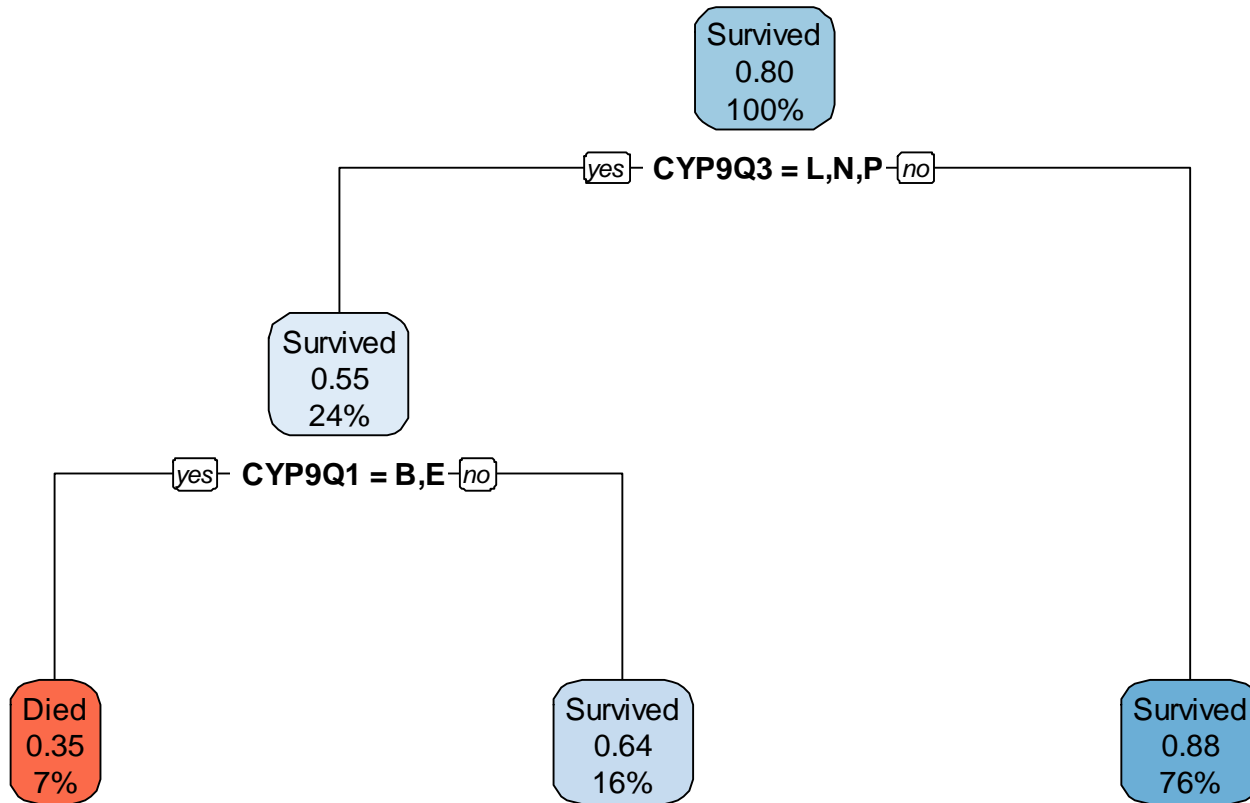


Figure S 4.1

NCBI_CYP9Q1	MDYLQGLGLTLLAILVAVYYLSTRNHKLLKRHGIVHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
Haplotype_C	-----VHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
Haplotype_A	-----VHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
Haplotype_F	-----VHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
Haplotype_D	-----VHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
Haplotype_B	-----VHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
Haplotype_E	-----VHIPPTPLFGNLGPLVRRKCHMEDVIO ⁶⁹
NCBI_CYP9Q1	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
Haplotype_C	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
Haplotype_A	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
Haplotype_F	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
Haplotype_D	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
Haplotype_B	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
Haplotype_E	RYVGMYEFTTPLIIIRDPELIKTI ⁷⁰ GVKEITNFTNHRPFVDVGVDPMLGEVLFAMQ ⁷¹ GDRWREHRTMLTTL ⁷²
NCBI_CYP9Q1	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
Haplotype_C	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
Haplotype_A	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
Haplotype_F	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
Haplotype_D	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
Haplotype_B	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
Haplotype_E	FTSSKIKSMFVLMSDCAKRFADYLSKVEREIELKSVLTRYTNDVIARC ⁷³ VYGVSVDSVNEPENIFRYR ⁷⁴ GQ ⁷⁵
NCBI_CYP9Q1	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
Haplotype_C	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
Haplotype_A	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
Haplotype_F	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
Haplotype_D	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
Haplotype_B	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
Haplotype_E	VASQLST ⁷⁶ FKQNLMI ⁷⁷ FVHRNS ⁷⁸ PRLAR ⁷⁹ L ⁸⁰ FN ⁸¹ LK ⁸² IL ⁸³ PV ⁸⁴ HI ⁸⁵ E ⁸⁶ KK ⁸⁷ FF ⁸⁸ H ⁸⁹ RL ⁹⁰ VD ⁹¹ T ⁹² IE ⁹³ TR ⁹⁴ RR ⁹⁵ EG ⁹⁶ VH ⁹⁷ GL ⁹⁸ D ⁹⁹ ML ¹⁰⁰ Q ¹⁰¹ L ¹⁰² MD ¹⁰³ M ¹⁰⁴ Q ¹⁰⁵
NCBI_CYP9Q1	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
Haplotype_C	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
Haplotype_A	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
Haplotype_F	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
Haplotype_D	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
Haplotype_B	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
Haplotype_E	SRRKESEEGKRGMTV ¹⁰⁶ TDIANHAF ¹⁰⁷ SFF ¹⁰⁸ FGSV ¹⁰⁹ DT ¹¹⁰ MT ¹¹¹ Q ¹¹² ISLISHMLAVNPDV ¹¹³ Q ¹¹⁴ RL ¹¹⁵ Q ¹¹⁶ EE ¹¹⁷ ID ¹¹⁸ E ¹¹⁹ VL ¹²⁰ S ¹²¹ ASE ¹²² DK ¹²³ Q ¹²⁴
NCBI_CYP9Q1	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
Haplotype_C	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
Haplotype_A	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
Haplotype_F	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
Haplotype_D	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
Haplotype_B	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
Haplotype_E	VG ¹²⁵ YD ¹²⁶ VI ¹²⁷ Q ¹²⁸ EM ¹²⁹ K ¹³⁰ YL ¹³¹ DA ¹³² VM ¹³³ SE ¹³⁴ AM ¹³⁵ RY ¹³⁶ HP ¹³⁷ IL ¹³⁸ LF ¹³⁹ VD ¹⁴⁰ RV ¹⁴¹ CG ¹⁴² ET ¹⁴³ FEL ¹⁴⁴ PPAL ¹⁴⁵ PG ¹⁴⁶ AR ¹⁴⁷ PF ¹⁴⁸ K ¹⁴⁹ LER ¹⁵⁰ GM ¹⁵¹ NI ¹⁵² WF ¹⁵³ PV ¹⁵⁴ KA ¹⁵⁵ I ¹⁵⁶ HH ¹⁵⁷ DPK ¹⁵⁸
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Haplotype_C	YFENPDRFDPDRFLRDGKGIASSGAYMPFGMGPRK ¹⁵⁹ IGSRFALTEMKILLFNILAKCSFKVGSKT ¹⁶⁰ MVPL ¹⁶¹
Haplotype_A	YFENPDRFDPDRFLRDGKGIASSGAYMPFGMGPRK ¹⁵⁹ IGSRFALTEMKILLFNILAKCSFKVGSKT ¹⁶⁰ MVPL ¹⁶¹
Haplotype_F	YFENPDRFDPDRFLRDGKGIASSGAYMPFGMGPRK ¹⁵⁹ IGSRFALTEMKILLFNILAKCSFKVGSKT ¹⁶⁰ MVPL ¹⁶¹
Haplotype_D	YFENPDRFDPDRFLRDGKGIASSGAYMPFGMGPRK ¹⁵⁹ IGSRFALTEMKILLFNILAKCSFKVGSKT ¹⁶⁰ MVPL ¹⁶¹
Haplotype_B	YFENPDRFDPDRFLRDGKGIASSGAYMPFGMGPRK ¹⁵⁹ IGSRFALTEMKILLFNILAKCSFKVGSKT ¹⁶⁰ MVPL ¹⁶¹
Haplotype_E	YFENPDRFDPDRFLRDGKGIASSGAYMPFGMGPRK ¹⁵⁹ IGSRFALTEMKILLFNILAKCSFKVGSKT ¹⁶⁰ MVPL ¹⁶¹
NCBI_CYP9Q1	KFKEGVFN ¹⁶² PAKNGFWL ¹⁶³ KIERRENSC ¹⁶⁴
Haplotype_C	KFKEGVFN ¹⁶² PAKNGFWL ¹⁶³ KI-----
Haplotype_A	KFKEGVFN ¹⁶² PAKNGFWL ¹⁶³ KI-----
Haplotype_F	KFKEGVFN ¹⁶² PAKNGFWL ¹⁶³ KI-----
Haplotype_D	KFKEGVFN ¹⁶² PAKNGFWL ¹⁶³ KI-----
Haplotype_B	KFKEGVFN ¹⁶² PAKNGFWL ¹⁶³ KI-----
Haplotype_E	-----

Figure S 4.2

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NCBI_CYP9Q2      MEFLSLALVLA AISIIAYYYCFVRKNFNLFQE HGILHVPPSPLVGNFGPLIRGKENVHDTIQRIYNIHP69
Haplotype_H      -----YYCFVRKNFNLFQE HGILHVPPSPLVGNFGPLIRGKENVHDTIQRIYNIHP
Haplotype_J      -----YYCFVRKNFNLFQE HGILHVPPSPLVGNFGPLIRGKENVHDTIQRIYNIHP
Haplotype_I      -----YYCFVRKNFNLFQE HGILHVPPSPLVGNFGPLIRGKENVHDTIQRIYNIHP
Haplotype_G      -----YYCFVRKNFNLFQE HGILHVPPSPLVGNFGPLIRGKENVHDTIQRIYNIHP

NCBI_CYP9Q2      DAKYVGIFEFLTPVIMIRDLDL IKSITMKNFDQFPDHRPMFCKSVDPMLGEMLFIMDGERWKEHRNMLS138
Haplotype_H      DAKYVGIFEFLTPVIMIRDLDL IKSITMKNFDQFPDHRPMFCKSVDPMLGEMLFIMDGERWKEHRNMLS
Haplotype_J      DAKYVGIFEFLTPVIMIRDLDL IKSITMKNFDQFPDHRPMFCKSVDPMLGEMLFIMDGERWKEHRNMLS
Haplotype_I      DAKYVGIFEFLTPVIMIRDLDL IKSITMKNFDQFPDHRPMFCKSVDPMLGEMLFIMDGERWKEHRNMLS
Haplotype_G      DAKYVGIFEFLTPVIMIRDLDL IKSITMKNFDQFPDHRPMFCKSVDPMLGEMLFIMDGERWKEHRNMLS

NCBI_CYP9Q2      PTFTSSKIKTMFVHMSECAKRFAH HLSKLPKEDRETEMKALLTRYTNDVIAACIYGVNVDSIKEPRNVF207
Haplotype_H      PTFTSSKIKTMFVHMSECAKRFAH HLSKLPKEDRETEMKALLTRYTNDVIAACIYGVNVDSIKEPRNVF
Haplotype_J      PTFTSSKIKTMFVHMSECAKRFAH HLSKLPKEDRETEMKALLTRYTNDVIAACIYGVNVDSIKEPRNVF
Haplotype_I      PTFTSSKIKTMFVHMSECAKRFAH HLSKLPKEDRETEMKALLTRYTNDVIAACIYGVNVDSIKEPRNVF
Haplotype_G      PTFTSSKIKTMFVHMSECAKRFAH HLSKLPKEDRETEMKALLTRYTNDVIAACIYGVNVDSIKEPRNVF

NCBI_CYP9Q2      YMYGRVGATLIGLKKNLKIMVHRNMPWLANLRLNL ILERHIAKFFTDLVVETVEERERNGTTNSDLIQL276
Haplotype_H      YMYGRVGATLIGLKKNLKIMVHRNMPWLANLRLNL ILERHIAKFFTDLVVETVEERERNGTTNSDLIQL
Haplotype_J      YMYGRVGATLIGLKKNLKIMVHRNMPWLANLRLNL ILERHIAKFFTDLVVETVEERERNGTTNSDLIQL
Haplotype_I      YMYGRVGATLIGLKKNLKIMVHRNMPWLANLRLNL ILERHIAKFFTDLVVETVEERERNGTTNSDLIQL
Haplotype_G      YMYGRVGATLIGLKKNLKIMVHRNMPWLANLRLNL ILERHIAKFFTDLVVETVEERERNGTTNSDLIQL

NCBI_CYP9Q2      MMDTRNKKESGKKNLTVQNMANHAFSFFFGGFDTVSSQTCVLLHMLVENPEVQQR LQOEIDETLESNNG345
Haplotype_H      MMDTRNKKESGKKNLTVQNMANHAFSFFFGGFDTVSSQTCVLLHMLVENPEVQQR LQOEIDETLESNNG
Haplotype_J      MMDTRNKKESGKKNLTVQNMANHAFSFFFGGFDTVSSQTCVLLHMLVENPEVQQR LQOEIDETLESNNG
Haplotype_I      MMDTRNKKESGKKNLTVQNMANHAFSFFFGGFDTVSSQTCVLLHMLVENPEVQQR LQOEIDETLESNNG
Haplotype_G      MMDTRNKKESGKKNLTVQNMANHAFSFFFGGFDTVSSQTCVLLHMLVENPEVQQR LQOEIDETLESNNG

NCBI_CYP9Q2      QLSYDV IQEMRYLDAVINEILRLHP IAVFIDRM CVKSFELPPALPGDVPFTVKPGMNVWI PVKAIHHDP414
Haplotype_H      QLSYDV IQEMRYLDAVINEILRLHP IAVFIDRM CVKSFELPPALPGDVPFTVKPGMNVWI PVKAIHHDP
Haplotype_J      QLSYDV IQEMRYLDAVINEILRLHP IAVFIDRM CVKSFELPPALPGDVPFTVKPGMNVWI PVKAIHHDP
Haplotype_I      QLSYDV IQEMRYLDAVINEILRLHP IAVFIDRM CVKSFELPPALPGDVPFTVKPGMNVWI PVKAIHHDP
Haplotype_G      QLSYDV IQEMRYLDAVINEILRLHP IAVFIDRM CVKSFELPPALPGDVPFTVKPGMNVWI PVKAIHHDP

NCBI_CYP9Q2      RYYDEPEKFKPERFLDNGKNIIGSGAYFPFGIGPRICIGNRFAL IEMKVLVCH IILAVCDIKAGARTGIP483
Haplotype_H      RYYDEPEKFKPERFLDNGKNIIGSGAYFPFGIGPRICIGNRFAL IEMKVLVCH IILAVCDIKAGARTGIP
Haplotype_J      RYYDEPEKFKPERFLDNGKNIIGSGAYFPFGIGPRICIGNRFAL IEMKVLVCH IILAVCDIKAGARTGIP
Haplotype_I      RYYDEPEKFKPERFLDNGKNIIGSGAYFPFGIGPRICIGNRFAL IEMKVLVCH IILAVCDIKAGARTGIP
Haplotype_G      RYYDEPEKFKPERFLDNGKNIIGSGAYFPFGIGPRICIGNRFAL IEMKVLVCH IILAVCDIKAGARTGIP

NCBI_CYP9Q2      LEFEKGVFNATAKTGFWLKIEPRKYSYHSGQINGLVNNH VINGACKTGI532
Haplotype_H      LEFEKGVFNATAKTGFWLKIEPRKYSYHSGQINGLVNNH -----
Haplotype_J      LEFEKGVFNATAKTGFWLKIEPRKYSYHSGQINGLVNNH -----
Haplotype_I      LEFEKGVFNATAKTGFWLKIEPRKYSYHSGQINGLVNNH -----
Haplotype_G      LEFEKGVFNATAKTGFWLKIEPRKYSYHSGQINGLVNNH -----
```

Figure S 4.3

NCBI_CYP9Q3	MDYLTIISLSLITVFVAVYYLATRNNDFFKKHGI PHVPPVPFPLGNMGSLVRQKSNLHDVIDR TY NLDPGA ⁶⁹
Haplotype_O	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR MY NLDPGA
Haplotype_K	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR MY NLDPGA
Haplotype_Q	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR TY NLDPGA
Haplotype_M	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR MY NLDPGA
Haplotype_N	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR MY NLDPGA
Haplotype_L	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR MY NLDPGA
Haplotype_P	-----HGIPHVPPVPFPLGNMGSLVRQKSNLHDVIDR TY NLDPGA
NCBI_CYP9Q3	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT ¹³⁸
Haplotype_O	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
Haplotype_K	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
Haplotype_Q	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
Haplotype_M	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
Haplotype_N	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
Haplotype_L	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
Haplotype_P	KYVGIYEFTTPI IILRDLDLIKTITMKYLDHFPDHRSFAYEGADPVFGSMLFAMKGERWKEHRNMLTPT
NCBI_CYP9Q3	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY ²⁰⁷
Haplotype_O	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
Haplotype_K	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
Haplotype_Q	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
Haplotype_M	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
Haplotype_N	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
Haplotype_L	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
Haplotype_P	LTSSKIKGMFKLMT TE CAVRFADFLSVLPENERETEMKALLSRYANDVIASCVYGVSVDSINDPKNIFVY
NCBI_CYP9Q3	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM ²⁷⁶
Haplotype_O	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
Haplotype_K	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
Haplotype_Q	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
Haplotype_M	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
Haplotype_N	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
Haplotype_L	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
Haplotype_P	YGRGTNVVGLKKSMEFVLIHRNMPWLAKL FL RFLEKHVQKFFYDLVYETIESREKLGTRNSDVLQLLM
NCBI_CYP9Q3	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG ³⁴⁵
Haplotype_O	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
Haplotype_K	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
Haplotype_Q	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
Haplotype_M	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
Haplotype_N	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
Haplotype_L	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
Haplotype_P	DIRKANSSGKMTTMTVENVAIHAF TF FFGGFDSITSVTTLLTQMLAEHPDVQARLQOEIDETLRSDNG
NCBI_CYP9Q3	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC VE SFQL PA PVPGGQPF TL LRPGMNVWIPLAAIGRDP ⁴¹⁴
Haplotype_O	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC VE SFQL PA PVPGGQPF TL LRPGMNVWIPLAAIGRDP
Haplotype_K	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC VE SFQL PA PVPGGQPF TL LRPGMNVWIPLAAIGRDP
Haplotype_Q	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC VE SFQL PA PVPGGQPF TL LRPGMNVWIPLAAIGRDP
Haplotype_M	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC AE SFQL PP PVPGGQPF TL LRPGMNVWIPLAAIGRDP
Haplotype_N	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC AE SFQL PP PVPGGQPF TL LRPGMNVWIPLAAIGRDP
Haplotype_L	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC AE SFQL PA PVPGGQPF TL LRPGMNVWIPLAAIGRDP
Haplotype_P	VLT Y DAVHGMYMDAVINETMRFCVLPFLDRMC VE SFQL PA PVPGGQPF TL LRPGMNVWIPLAAIGRDP
NCBI_CYP9Q3	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP ⁴⁸³
Haplotype_O	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
Haplotype_K	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
Haplotype_Q	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
Haplotype_M	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
Haplotype_N	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
Haplotype_L	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
Haplotype_P	EYFEDPKFDPDRFLNPEAGIKNSGAHFFPGLGQRKCIGERFAMMEMKVLLCYVLAACNVRIGSKTTVP
NCBI_CYP9Q3	MKLEKGLINANVKGFWLKIEPRKVTYNSSRSN ⁵¹⁷
Haplotype_O	MKLEKGLINAN-----
Haplotype_K	MKLEKGLINAN-----
Haplotype_Q	MKLEKGLINAN-----
Haplotype_M	MKLEKGLINAN-----
Haplotype_N	MKLEKGLINAN-----
Haplotype_L	MKLEKGLINAN-----
Haplotype_P	MKLEKGLINAN-----

Table 4.1. Percent of deviance explained in survival at different levels of analysis and estimates of broad-sense heritability

	% deviance explained	Broad sense heritability
Between Colonies	2.0	
Between Patriline within Colonies	18.9	37.8%
Between bees within Patriline within Colonies	79.1	

Table S 4.1. The list of primers used to discern the patriline of the tested worker bees

F: Forward primer, R: Reverse primer. Italic text denoted the name of the fluorophore dye.

Loci	Primer Sequence (5' – 3')
HB-SEX-02	F: <i>HEX</i> -ACGCATTGAAGGATATTATGA R: AATTTGAACATTTCGATCACC
HB-THE-03	F: <i>FAM</i> -TAACTGGTCGTCGGTGTT R: CACGTAGAGAATCCCATTGT
AC006	F: <i>PET</i> -GATCGTGGAACCGCGAC R: CACGGCCTCGTAACGGTC
HB-C16-05	F: <i>NED</i> -ATTTTATGCGCGTTTCGTA R: CATGGCTCCTCCATTAAATC
HB-C16-01	F: <i>HEX</i> -AAAATGCGATTCTAATCTGG R: TTGCCTAAAATGCTTGCTAT
A024	F: <i>FAM</i> -CACAAGTTCCAACAATGC R: CACATTGAGGATGAGCG
A107	F: <i>NED</i> -CCGTGGGAGGTTTATTGTCG R: CCTTCGTAACGGATGACACC
A007	F: <i>PET</i> -GTTAGTGCCCTCCTCTTGC R: CCCTTCCTCTTTCATCTTCC
A079	F: <i>HEX</i> -CGAAGGTTGCGGAGTCCTC R: GTCGTCGGACCGATGCG
A113	F: <i>NED</i> -CTCGAATCGTGGCGTCC R: CCTGTATTTTGCAACCTCGC
HB-THE-02	F: <i>FAM</i> -GGGAAAGATATTAGGGAGGA R: CGACGAAAAATTACAAGGAC

Table S 4.2. The list of primers used to sequence the three CYP9Q genes

F: Forward primer, R: Reverse primer.

Gene	Primer sequence (5' – 3')
CYP9Q1_A	F: ACCTGTCCACGAGGAATCAC R: TCTTTCCTCCTCGATTGCAT
CYP9Q1_B	F: GACACGATCGAGACGAGGA R: CAAGAATTCTCCCTGCGTTC
CYP9Q2_A	F: TTATCGTTGGCTCTCGTCCT R: ACCCGATTCTTCTTGTTC
CYP9Q2_B	F: CGAGGAACAAGAAGGAATCG R: ACACGCGCCGTTAATAACAT
CYP9Q3_A	F: CGTATTCGTGGCCGTTTATT R: TTCGCCTTGTCCCTTATGTC
CYP9Q3_B	F: CTCGAGAAACACGTGCAAAA R: ACCTTCCTAGGCTCGATCTTC

N. Tsvetkov and A. Zayed³

Introduction

There is a large body of literature showing sometimes contradicting effects of neonicotinoid (NNI) insecticides on the health of honey bees (Lawrence et al. 2016, Carreck and Ratnieks 2014, Alkassab and Kirchner 2017). The discrepancy in the literature has caused an academic debate regarding the impact of NNIs on bees and the possible biases that may lead to these inconsistencies (Carreck and Ratnieks 2014, Blacquiere et al. 2012, Cutler and Scott-Dupree 2016, Cutler, Scott-Dupree, and Drexler 2014, Benuszak, Laurent, and Chauzat 2017). Perhaps more importantly, it has also caused different regulatory agencies to reach contradicting conclusions regarding the safety of NNIs (Canada 2017, Department for Environment 2017).

Part of the discrepancy in the literature can be explained by basic study methodology (e.g. field vs. laboratory conditions; (Carreck and Ratnieks 2014)), yearly fluctuations in colony phenotypes, even within the same study (Osterman et al. 2019), or interactions between NNI exposure and the surrounding environment (Woodcock et al. 2017). Moreover, researchers typically focus their efforts on a few phenotypes that they can quantify. Sublethal effects, which are defined as physiological and/or behavioral effects after an exposure to a non-lethal dose (Alkassab and Kirchner 2017), by their definition, are endless in scope. Practically, when researchers focus on a few traits studies will miss any sublethal effects on unstudied phenotypes. Moreover, when researchers pick a phenotype to assess, they may introduce bias by choosing phenotypes that are a priori predicted to change (or perhaps not change) following exposure. These issues collectively lead to a ‘streetlight effect’, which occurs when people search for missing objects (knowledge in our case!) in the place where it is easiest to look – a problem that is common in many scientific fields (e.g. Battaglia and Atkinson 2015).

³ This submitted manuscript has been reprinted with the permission of its co-authors from the original submitted manuscript: Tsvetkov, N. and Zayed, A. (2020) Searching beyond the streetlight: neonicotinoid exposure alters the neurogenomic state of worker honey bees. [submitted]

One way to reduce the ‘streetlight effect’ in ecotoxicological studies is to quantify a very large number of phenotypes – a type of enquiry that is suitable for the application of genomics. For example, RNA sequencing (RNAseq) provides a feasible and objective way to simultaneously query transcript abundance for thousands of genes and quantify how gene expression changes in response to pesticide exposure (Grozinger and Zayed 2020, Lozier and Zayed 2017). With this approach we can observe thousands of phenotypes, i.e. expression of thousands of genes. Moreover, given the large body of knowledge linking genes with biological process and molecular functions in insects (Kanehisa and Goto 2000, Ashburner et al. 2000, Consortium 2019), it is possible to use transcriptomics to provide insight about the typical traits, molecular and physiological processes that are impacted by exposure to pesticides.

In honey bees, previous research has clearly demonstrated a very strong relationship between brain gene expression and typical worker behaviours (Zayed and Robinson 2012). For example, to our knowledge, all distinct honey bee behavioural states studied to date (e.g. aggression, scouting, foraging, communication, and learning) appear to be associated with a specific pattern of brain gene expression (i.e. neurogenomic state) involving tens to thousands of genes (Zayed and Robinson 2012, Wang et al. 2013). In many cases, the close relationship between neurogenic state and behavioural state is causal, where shifts in neurogenomic states lead to shifts in worker behaviour (Zayed and Robinson 2012). As such, analysis of brain transcriptomes of bees exposed to pesticides is, in theory, capable of highlighting how pesticides impact bee behaviour.

Although a few studies explored the effects of NNIs on the honey bee transcriptome (Wu, Luo, et al. 2017, Li et al. 2019, Christen et al. 2018, Morfin et al. 2019), none have done so for worker bees that were naturally exposed in the field. This is important because De Smet et al. (2017) demonstrated that there is a significant difference in gene expression response to NNIs between bees exposed in the hive versus those exposed in laboratory cages. Additionally, it is not clear how NNI exposure influences gene expression in the different honey bee adult worker castes, such as nurses and foragers (Winston 1991).

In this study, we analyzed the brain transcriptomes of worker honey bees exposed to NNIs under field conditions and worker honey bees that were experimentally exposed to clothianidin through a spiked pollen patty placed inside the hive (Tsvetkov et al. 2017). Briefly, we first conducted a season-long field study that utilized colonies kept near and far from corn in Canada to determine typical routes and exposure to NNIs. Corn production represents the largest use of arable land in North America (Hamel and Dorff 2014) and almost all corn is grown from NNI-treated seeds (Stewart and Baute 2013). We collected honey bee foragers and nurses from these field colonies right after corn plantation in late May for gene expression analysis, allowing us to explore how natural exposure to the myriads of agrochemicals, including NNIs, influences the typical neurogenomic state of worker bees. We then performed a controlled experiment mimicking the NNI exposure we found in the field (Tsvetkov et al. 2017) by feeding honey bee colonies clothianidin-infused pollen patties. We collected foragers and nurses 30 days after the start of the experiment allowing us to investigate how chronic sublethal exposure to clothianidin influences the neurogenomic state of worker bees.

Material and Methods

Honey bees

Honey bees were collected from colonies described in detail by Tsvetkov et al. (2017). Briefly, in the 2014 field study, 55 bee colonies were randomly allocated to five apiaries located near (<500m) NNI treated corn ('exposed sites') or to six apiaries located at least 3km from agriculture ('unexposed sites'). For the current study, bees were collected from two different sites studied by (Tsvetkov et al. 2017), one near corn (Wellington 2) and one at least 3km away from corn (Toronto 2). Each site had five colonies and we collected five foragers and five nurses from each colony. We identified foragers as those bees returning to the hive with pollen loads and nurses as those bees who inserted their heads into a cell with larvae (Winston 1991). The bees were collected right after corn plantation on May 30th, 2014 due to previous reports that corn planting coincided with bee mortality in Canada (Canada 2012). The samples were transported to the laboratory on dry ice and later stored at -80°C until analysis. Pesticide analysis of the pollen from the Wellington county site '2' site found 6.6ppb of clothianidin and 2.0ppb of

thiamethoxam on May 5th, 2014 and 11.5ppb of clothianidin and 4.0ppb of thiamethoxam on May 30th, 2014 (Figure S 5.1A, (Tsvetkov et al. 2017)). Pesticide analysis of the pollen in the colonies from the Toronto site '2' found no NNI residues in early or late May (clothianidin limit of detection (LOD): 1.4ppb; imidacloprid LOD: 0.7ppb; thiamethoxam LOD: 0.7ppb; acetamiprid LOD: 1.0ppb; thiacloprid LOD: 0.9ppb; nitenpyram LOD: 0.8ppb; dinotefuran LOD: 0.6ppb)(Tsvetkov et al. 2017). We also found coumaphos (miticide), carboxin (fungicide), Imazlil (fungicide), and Diuron (herbicide) in the Toronto 2 site and coumaphos and Pyraclostrobin (fungicide) in the Wellington 2 site (Tsvetkov et al. 2017).

In 2015, we carried out an experiment mimicking the NNI field exposure found in 2014 (Tsvetkov et al. 2017). Ten colonies in a single apiary received artificial pollen for a 12-week period. Half of the colonies received a pollen patty spiked with clothianidin; the most commonly found NNI in the 2014 field study. The exposure started with 4.9ppb of clothianidin in week 1 and was lowered each week, until the dose reached 2.0ppb at week 5 and stayed the same until week 12 (Figure S 5.1B). Five nurses and five foragers were collected from each queen-right colony 30 days after the start of our experiment. All of the bees were placed on dry ice immediately after collection and then stored at -80°C.

RNA Extraction and Analysis

We performed the brain dissections on dry ice in cold ethanol. The hypopharyngeal gland was removed as well as the ocelli. For the 2014 bees, five nurse brains and five forager brains were pooled into two samples per colony, while for the 2015 bees each brain was extracted and analyzed individually. We extracted RNA from the brains using RNeasy Mini Kit (Qiagen) following manufacturing instructions.

The samples were sent to G enome Qu ebec's Innovation Center for library preparations and Illumina RNA sequencing with HiSeq4000 PE. We used Trimmomatic (Bolger, Lohse, and Usadel 2014) to remove adapters, low quality bases (leading and trailing 20), and low quality reads (reads shorter than 50 bases and when the average quality per base drops below 25 for a 20 base window). After filtering, the average across samples was 41.7 million sequences in 98.4 basepair length. Then, we used a FastQC (Bioinformatics 2011) to perform quality control

checks on the following criteria: mean quality score, per sequence quality score, per base sequence content (ATGC), per sequence GC content, per base N content, sequence length distribution, and adapter content. The data successfully passed the quality check in all relevant areas.

The RNA sequences were aligned and gene expression quantified with the *amel* 4.5 *Apis mellifera* genome assembly and Official Gene Set v3.2 (Weinstock et al. 2006, Elsik et al. 2014, Elsik et al. 2015) using Spliced Transcripts Alignment to a Reference (STAR) (Dobin et al. 2013). The gene expression counts were then processed using EdgeR (Robinson, McCarthy, and Smyth 2010, McCarthy, Chen, and Smyth 2012) in R version 3.6.3 (Team 2005). Any genes that were only expressed in one sample were filtered out. We detected the following number of expressed genes after filtering: 2014 foragers: 10,976; 2014 nurses: 10,852; 2015 foragers: 11,381; 2015 nurses: 11, 271. The remaining counts were normalized, the dispersions of the model estimated, and a generalized linear model ran. Genes were denoted as differentially expressed if the p-value was below 0.05 after a Benjamini adjustment (Benjamini and Hochberg 1995).

Functional Annotation Analysis

In order to gain insight into the function of the differentially expressed genes, we converted the honey bee genes into *Drosophila melanogaster* homologues using HymenopteraMine (Elsik et al. 2016). We mapped the following number of genes onto *D. melanogaster*: 2014 foragers: 7,974; 2014 nurses: 7,954; 2015 foragers: 8,097; 2015 nurses: 8,075. Then we analyzed the differentially expressed genes using DAVID 6.8 (Huang, Sherman, and Lempicki 2008b, a). We used the following options: Biological Process All, Molecular Function All, Cellular Component All, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis and Keywords. The p-values for the enrichment analyses were corrected using the Benjamini adjustment (Benjamini and Hochberg 1995). Illustrations of the enrichment analysis were performed using the online tool GOrilla (Eden et al. 2009, Eden et al. 2007) using the *D. melanogaster* homologues.

Comparisons

We compared our gene lists with previously published research on the effects of NNIs on honey bee gene expression (Shi et al. 2017, Wu, Chang, et al. 2017, Wu, Luo, et al. 2017, Christen et al. 2018, Derecka et al. 2013, Li et al. 2019, Morfin et al. 2019). Previously published differentially expressed gene lists were obtained from the supplementary data provided by the authors with each publication. Where required, the gene names were converted into the current iteration of the honey bee genome annotation using HymenopteraMine (Elsik et al. 2016). These gene lists were compared to the differentially expressed genes found in our study and to the background gene lists from our study. Then a hypergeometric test (Johnson, Kemp, and Kotz 2005) was conducted in order to determine if the overlap was statistically different from chance and a p-adjustment was done using the Holm-Bonferroni method (Holm 1979). These tests were performed in R version 3.6.3 (Team 2005).

Results

2014 Field Study - Foragers

We detected 278 differentially expressed genes (DEGs) with 251 of them being up regulated in field exposed foragers. A GO analysis showed a significant enrichment in regulation of several biological processes, including regulation of transcription (Figure S 5.2A, $p < 0.05$), as well as an array of post-embryonic development processes, including eye development (Figure S 5.2B, $p < 0.05$). We also examined if specific annotation clusters were enriched among DEGs and found that the top three clusters contained terms associated with post-embryonic development (Enrichment score 8.35), tissue development (Enrichment score 5.27), and brain development (Enrichment score 3.93). Other notable enriched clusters were neuron development, learning and memory, and regulation of glucose metabolic process.

Due to the large transcriptional signal we detected, we compared our honey bee DEGs with the transcriptional factors identified in the honey bee transcriptional regulatory network discovered by Chandrasekaran et al. (2011). We found 16 overlapping transcriptional factors, which represented a statistically significant overlap (Table S 5.1; hypergeometric test, $p < 0.001$).

Twelve of these 16 transcription factors are involved in behavioural maturation of honey bee workers, while four of them are involved in foraging behaviours (Chandrasekaran et al. 2011). Two of the transcription factors were down regulated in foragers located near corn. One was kruppel homologue 1 (*Kr-h1*) and the other zinc finger protein 578 (*LOC411780*).

Several detoxification genes were also differentially expressed. They were: CYP9Q1 (*LOC410492*; logFC: 0.70), cytochrome P450 314A1 (*Cyp314a1*; also known as *shade*; logFC: 0.62), carboxylesterase (*LOC726134*; logFC: 1.10), and NADPH-cytochrome P450 reductase (*LOC724870*; logFC= -0.45).

2014 Field Study - Nurses

In contrast to the large number of DEGs found in foragers, we only detected 9 differentially expressed genes in nurses, most of which (7 genes) were downregulated in the naturally exposed bees. A GO analysis revealed an enrichment of the annotation term ‘Signal’ (p<0.008). The KEGG pathway starch and sucrose metabolism was marginally enriched among the DEGs (p=0.054). The largest log-Fold change was observed for alpha-amylase (*LOC406114*; logFC: -5.62), chymotrypsin inhibitor (*LOC725114*; logFC: -5.09), and for pancreatic triacylglycerol lipase (*LOC551268*; logFC: -4.09). Out of the two upregulated genes in exposed nurses, cuticular protein 5 (*CRP5*) had the highest logFC change (1.90), while the other was uncharacterized (*LOC100578072*; logFC= 1.88).

2015 Apiary Experiment - Foragers

We discovered 45 genes to be differentially expressed between the experimentally exposed foragers, of which 38 were upregulated in the exposed bees relative to controls. A GO analysis revealed no enriched processes after a Benjamini correction (p>0.05).

Myosin heavy chain, non-muscle (*LOC100576096*) had one of the largest fold-changes (3.47) and was also differentially expressed in 2014 foragers and 2015 nurses. A transcription factor, brachyury protein (*LOC412976*) and unconventional myosin-IXb (*LOC551706*), an orthologue of *dachs*, were also upregulated.

Only seven genes were downregulated in the exposed foragers. The two most downregulated were a triacylglycerol lipase (*LOC551268*; logFC: -2.70), which was also differentially expressed in 2015 nurses, and fatty acid hydroxylase domain-containing protein 2 (*LOC409360*; logFC: -2.02). The other genes of note were *cyp6as5*, NADPH oxidase 5, and fatty acid hydroxylase domain-containing protein 2-like (*LOC727357*), all of which are involved in oxidoreductase and all were downregulated.

2015 Apiary Experiment - Nurses

We detected 63 DEGs, 41 of which were upregulated in the experimentally exposed nurses relative to controls. A GO analysis showed a significant enrichment of the cellular component extracellular region (p=0.005) and the annotation term ‘Signal’ (p=0.001). No other terms were statistically significant after a Benjamini correction.

Here, the highest log-fold change (8.51) was for a cuticle protein 18.7 (*LOC725804*) and AMP deaminase 2 (logFC: 6.26). One transcription factor was upregulated, a homeobox protein Hox-B1a (*LOC724422*). One of the most downregulated genes was TOX high mobility group box family member 3-like (*LOC107965368*; logFC: -2.15).

Overlapping DEGs

We compared the genes that were differentially expressed in our different experiments (Figure 5.1; Table S 5.2). We found a statistically significant overlap between the DEGs found in the 2014 field exposed bees and in the 2015 apiary exposed bees (forager DEGs: hypergeometric test, p=0.005; nurse DEGs: hypergeometric test, p=0.049) these genes may be associated with NNI related exposure. We also found a statistically significant overlap between the DEGs found in the 2015 apiary exposed nurses and foragers (hypergeometric test, p<0.001), suggesting that there may be a common set of genes associated with clothianidin exposure in both castes. The DEGs found in the 2014 field exposed foragers and nurses did not overlap.

We compared our data with previously published studies on the transcriptome response following NNI exposure (Table 5.1). We found a statistically significant overlap between the

DEGs of our 2014 field exposed nurses and the DEGs reported by Christen et al. (2018) for imidacloprid (3ppb: hypergeometric test $p=0.003$; 30ppb: hypergeometric test, $p=0.004$), and thiamethoxam (1ppb: hypergeometric test, $p=0.007$; 10ppb: hypergeometric test, $p<0.001$). We also found a statistically significant overlap between the DEGs found in our 2014 field exposed foragers and the DEGs found by Christen et al. (2018) in the 30ppb imidacloprid treatment group (hypergeometric test, $p=0.006$). Additionally, a statistically significant overlap was found between the DEGs of our 2014 field exposed nurses and those reported by Wu, Luo, et al. (2017)(hypergeometric test, $p<0.001$). We found no statistically significant overlap between the DEGs in our study and those in the clothianidin treatment group reported by Christen et al. (2018) (3ppb: 0DEGs, 30ppb: hypergeometric test, $p=0.0869$). Likewise, we found no statistically significant overlap between the DEGs of our study and those reported by Shi et al. (2017), those reported by Li et al. (2019), and those reported by Morfin et al. (2019)(hypergeometric test, $p>0.1$).

We also compared our study to the transcriptome studies that exposed larvae to NNIs (Table 5.1). We found a statistically significant overlap between our field exposed nurse DEGs and the DEGs reported by Wu, Chang, et al. (2017) and Derecka et al. (2013). No other overlap was statistically significant (hypergeometric test, $p>0.1$).

Discussion

In our study we sought to use a transcriptomic analysis to provide an unbiased snapshot of the effects of field realistic exposure to NNIs on honey bees. First, we performed RNAseq analysis on the brains of foragers and nurses that were collected from colonies located near NNI treated corn fields and away from such fields. Second, we performed a similar transcriptomic analysis on the brains of foragers and nurses collected from colonies that were experimentally exposed to field realistic levels of clothianidin spiked into pollen patties relative to a control group. Across both the natural and experimental exposure, the neurogenomic states of exposed bees were clearly distinct from those of the unexposed bees. As such, our study unequivocally shows that exposure to sublethal doses of NNIs is sufficient to alter the neurogenomic state of honey bees.

In honey bees, individual neurogenomic states are associated with many behaviours that impact colony fitness, such as aggression, scouting, foraging, communication (Zayed and Robinson 2012) and learning and memory (Wang et al. 2013). This close relationship between brain gene expression and behaviour is often causal (Zayed and Robinson 2012), suggesting that NNI induced changes in the neurogenomic state of exposed bees may influence important behaviours related to behavioural maturation, learning and memory, and foraging. Indeed, previous studies have demonstrated that exposure to NNIs can alter learning and memory (Tison et al. 2019, Zhang and Nieh 2015) and navigation in honey bees (Fischer et al. 2014).

One important finding of our study is that nurses and foragers appear to respond differently to NNIs. This indicates that it is crucial for any phenotypic assay to distinguish between these two worker castes, which contribute to colony fitness in different ways. The 2014 RNAseq study strongly suggests that exposure influences phenotypes that are critical for resource gathering such as learning and memory, locomotion, and vision in foragers and phenotypes associated with carbohydrate metabolism in nurses. The latter may impact the ability of nurses to produce high quality brood food (Eischen, Rothenbuhler, and Kulinčević 1984).

We believe that our data shows that field realistic exposure to NNIs affects the developmental process, which manifests itself in different ways during the life cycle of the bee. In nurses of both years, DEGs were associated with cuticle and chitin. Chitin is an amino polysaccharide, which is an essential component of insect cuticle and the peritrophic matrix (Merzendorfer and Zimoch 2003). Additionally, GO terms related to chitin and cuticle were found in the exposed foragers of both years. We also found unconventional myosin-IXb (*LOC551706*) to be upregulated in exposed foragers in both years. It is an orthologue to *dachs* in *D. melanogaster*, which influences growth through interactions with *Warts*, positive regulation of Hippo signaling, and participates in Dachsous-Fat signaling (Misra and Irvine, 2016; Saavedra et al. 2016; Zhang et al. 2016). *LOC100576096* was upregulated in exposed foragers in both years and in nurses in 2015. It is described as myosin heavy chain, non-muscle-like, which are required for morphogenesis and cytokinesis (Sechi et al. 2014).

Our 2014 field exposed foragers had 16 transcription factors that were differentially expressed. Most of these transcription factors are known to play a role in the maturation process as a bee transfers from being a nurse to being a forager (Chandrasekaran et al. 2011). However, we compared our exposed foragers with other foragers, thus it is unlikely that these transcription factors are the result of the maturation process. Half of the transcription factors are known to be involved in neural development or differentiation (Sup. Table 3). NNIs are known to affect learning and memory in honey bees (e.g. Tison et al. 2019) as well as navigation (Fischer et al. 2014, Tison et al. 2016).

Two of the transcription factors were down regulated in the field exposed foragers: *Kr-h1* and *LOC411780*. *LOC411780* is a homologue to *D. melanogaster's crol*, which links the Ecdysone steroid hormone pathway and the Wingless signalling pathway. It is required for cell cycle progression and reducing *crol* reduces wing size (Mitchell et al. 2008). *Kr-h1* is expressed at a lower levels in nurses relative to foragers (Grozinger and Robinson 2007) and increasing cyclic guanosine monophosphate (cGMP) levels increase *Kr-h1* expression (Fussnecker and Grozinger 2008). Higher levels of cGMP also improves honey bee's spatial memory (Tsvetkov et al. 2018). Therefore, it is possible that lower expression of *Kr-h1* is an indication of a poorer spatial memory and thus navigation, which would be consistent with previous findings about NNIs (Tison et al. 2016, Fischer et al. 2014).

Several lines of evidence point to carbohydrate metabolism being impacted by NNI exposure. First, the DEGs that overlapped between our 2014 field exposed nurses and those published by Christen et al. (2018) and Wu, Luo, et al. (2017) are involved in starch and sucrose metabolism. Second, GO terms related to glucose metabolism were found in our 2014 field exposed foragers and GO terms related to amino-sugar metabolism were found in both the 2015 apiary exposed foragers and in the nurses. These overlaps, despite the varied exposure methods used in the studies, strongly suggest that these metabolic processes are part of the honey bee's physiological response to NNIs.

A substantial proportion of our DEGs (113/395) had no orthologues or homologs in the fruit fly, which limits our ability to study the molecular functional and biological process

associated with these genes. Although it limits the current functional analysis, the lack of *D. melanogaster* homologues indicates that our DEGs contain a large number of taxonomically restricted genes, which tend to be associated with social behaviours (Johnson and Tsutsui 2011). It is possible that NNI exposure may target genes involved in regulating social behaviour in honey bees. Indeed, a recent study found that NNI exposure impairs nursing and alters social dynamics within the nest (Crall et al. 2018).

We found no statistically significant overlap between the DEGs found in our study and those found in three previously published studies (Li et al. 2019, Shi et al. 2017, Morfin et al. 2019). In addition, the DEGs we found in our 2015 apiary exposed bees did not significantly overlap with any of the six previously published transcriptome studies. Some of the discrepancy could be attributed to the differences in the duration exposure, dosage, and type of NNI used (Christen, Mittner, and Fent 2016). However, given that we found statistically significant overlaps between the DEGs in our field exposed bees and our apiary exposed bees, we believe that the primary reason for the lack of overlap with previously published research is that honey bees have a different transcriptomic response to NNI exposure in cages versus NNI exposure in a colony (De Smet et al. 2017).

Summary

We investigated the effects of field realistic NNI exposure on the honey bee brain gene expression. We found that the DEGs are associated with development, particularly in bees exposed under field conditions. We also found no to minimal overlap in the DEGs in our study and in previously published research, which further demonstrates the importance of conducting honey bee research under colony conditions. Our study offers novel insights into the effects of field realistic NNI exposure on honey bee nurse and forager gene expression. Future research could build on this work by exploring the effects of NNIs on nurse bees metabolic pathways involved in chitin and on the effects of NNIs on the maturation process.

Acknowledgement

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Figure Captions

Figure 5.1. A Venn Diagram of the number of overlapping DEGs

Foragers collected in the 2014 field study (F2014), nurses collected in the 2014 field study (N2014), foragers collected in the 2015 apiary experiment (F2015), and nurses collected in the 2015 apiary experiment (N2015). Statistically significant overlap was found between F2014-F2015, N2014-N2015, and N2015-F2015 (hypergeometric test, $p < 0.05$).

Figure S 5.1. The amount of NNIs honey bees experiences in 2014 and 2015

The amount of neonicotinoids found in (A) the pollen of the colonies located near corn in the 2014 field study and (B) in the pollen supplements given to the exposed colonies in the 2015 Apiary Experiment. Bees for the RNA analysis were collected on May 30th in 2014 and at day 30 from the start of the experiment in 2015 (Tsvetkov et al. 2017).

Figure S 5.2. Biological Process of the differentially expressed genes in the 2014 field exposed foragers

Separated into (A) “biological regulation” and (B) “Others” for clarity. Colored cells represent biological processes that were statistically significantly enriched after a Benjamini correction ($p < 0.05$). The darker the color the lower the p value.

Figure 5.1

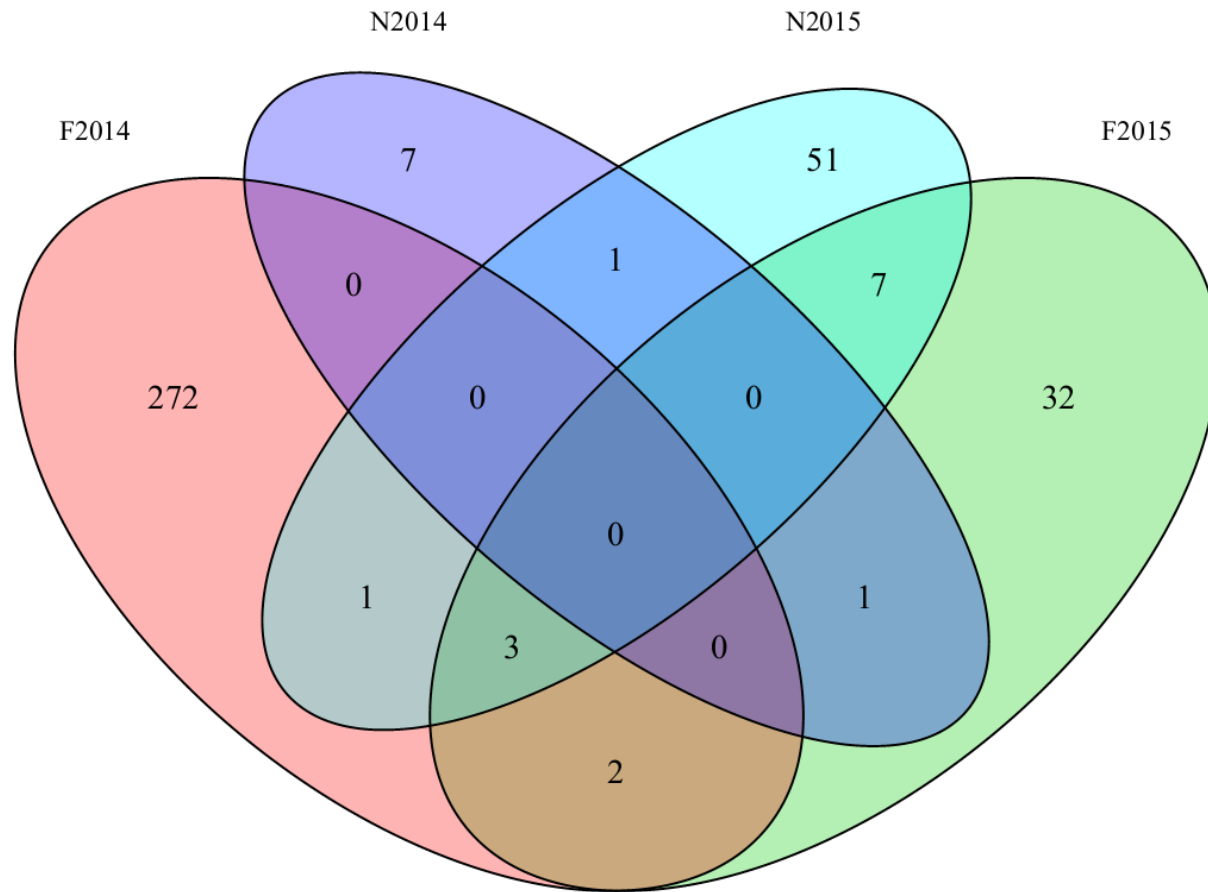


Figure S 5.1

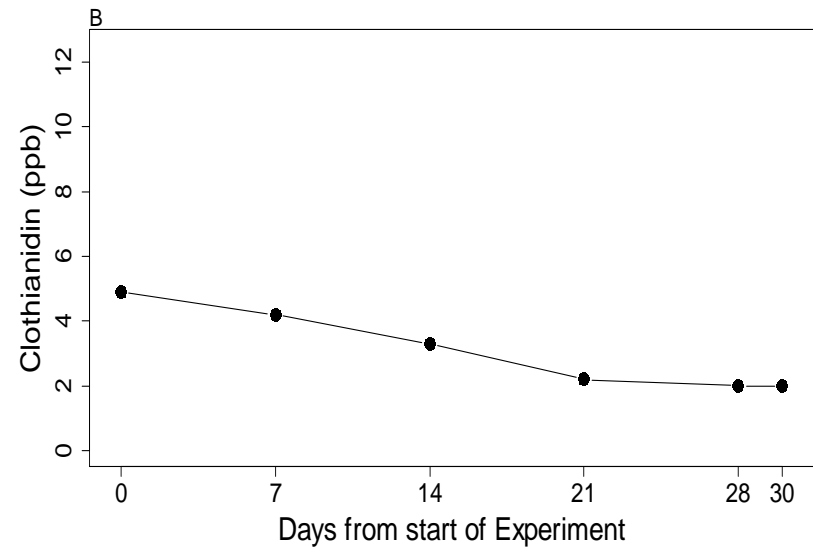
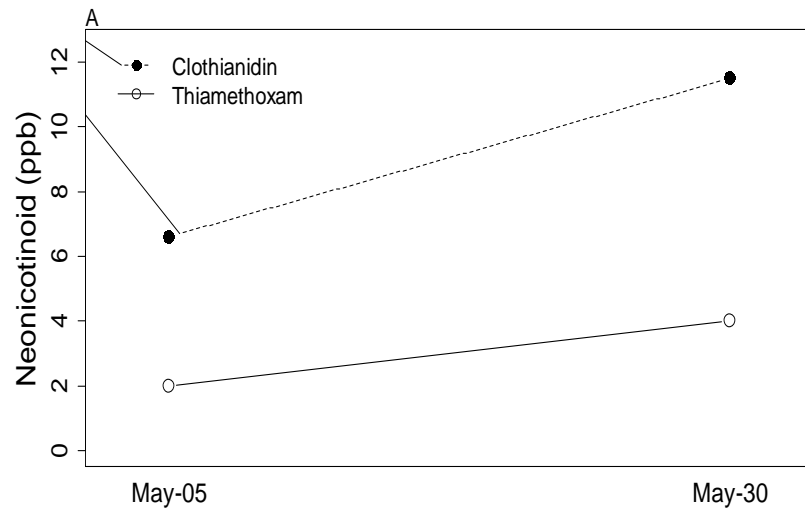


Table 5.1. Summary of previously published papers on the effects of NNIs on the honey bee transcriptomes and the number of differentially expressed genes (DEGs) overlapping with the current study

Statistically significant overlaps are marked with * and bolded (hypergeometric test, $p < 0.05$). The numbers denoted in the parenthesis are the number of DEGs found in the current study. F2014 stands for field exposed foragers in 2014; N2014 stands for field exposed nurses in 2014; F2015 stands for apiary exposed foragers in 2015; N2015 stands for apiary exposed nurses in 2015. HPG stands for hypopharyngeal gland. TMX = Thiamethoxam; IMD = Imidacloprid; CLO = Clothianidin

Reference	Study Design	Exposure Duration	NNI: Dose (ppb)	Sample Analyzed	DEGs	Overlapping DEGs			
						F2014 (278)	N2014 (9)	F2015 (45)	N2015 (63)
(Shi et al. 2017)	4 day old workers taken from the hive and exposed in cages	10 days	TMX: 10	Whole Bees	609	9	2	3	7
(Wu, Luo, et al. 2017)	Newly emerged workers raised in cages for one week and then exposed in cages	Up to 8 days	IMD: 10	Pools of whole bees collected after 1day, 2 days, 4 days, and 8 days of exposure	509	6	4*	2	3
(Christen et al. 2018)	Mixed aged bees collected from the hive and exposed in cages	48 hours	CLO: 3	Bee Brains+HPG	18	0	0	0	0
			CLO: 30		244	6	1	2	1
			IMD: 3	Bee Brains+HPG	26	1	2*	0	0
			IMD: 30		113	7*	2*	0	0
			TMX: 1	Bee Brains+HPG	6	0	1*	0	0
			TMX: 10		25	1	2*	0	0

(Morfin et al. 2019)	Newly emerged bees fed in cages	7 days	CLO: 10	Bee brains	298	1	2	1	3
(Li et al. 2019)	11 day old workers taken from the hive and then exposed in cages	11 days	IMD: 20	Bee Brains	131	6	0	0	2
(Derecka et al. 2013)	Colonies received sugar syrup with NNI	15 days	IMD: 2	6-9 day old larvae	300	3	4*	1	4
(Wu, Chang, et al. 2017)	NNI solution was pipetted into the larvae cell in a colony	4 days	IMD: 500	Bee heads from newly emerged bees	578	13	4*	4	4

Table S 5.1. Differentially expressed transcription factors found in the 2014 field exposed foragers

Fly (*Drosophila melanogaster*) homologues and their summaries from FlyBase (version FB2020_03) (Thurmond et al. 2019). logFC = log fold change

Gene ID	Symbol	logFC	Names	Fly homologue and its summary
GB43022	Kr-h1	-0.604	kruppel homolog 1	Kruppel homolog 1 (Kr-h1) encodes a transcriptional regulator involved in axon pathfinding, neurite and axon remodeling as well as pupal photoreceptor maturation. In all cases, the function of the product of Kr-h1 is linked to that of the 20-hydroxyecdysone hormone
GB49050	LOC411780	-0.378	zinc finger protein 578	crooked legs (crol) encodes a zinc finger transcription factor induced by 20-hydroxyecdysone at the onset of metamorphosis. It regulates wg transcription and cell cycle progression in the wing. Its over-expression in the eye accelerates the cell cycle and de-represses silenced genes.
GB45157	LOC410347	0.526	protein big brother	Big brother (Bgb) encodes a beta-subunit of the transcription factor complex core binding factor, which is involved in transcription regulation. It regulates hemocyte proliferation and acts redundantly with the product of Bro in embryonic segmentation
GB49611	LOC724983	0.551	retinal homeobox protein Rx1	Retinal Homeobox (Rx) encodes a homeodomain transcription factor required for processes of brain development. It is involved in growth regulation, proliferation and cell survival.
GB52109	LOC726709	0.555	uncharacterized LOC726709	klarsicht (klar) encodes a member of the Nesprin family that links microtubule motors and various cellular structures. It controls the migration and positioning of nuclei in photoreceptors and muscles. It also regulates the motion of RNP granules in oocytes and lipid droplets in embryos.
GB44976	LOC410253	0.617	ataxin-2 homolog	vriple (vri) encodes a bZIP transcription factor acting as an enhancer of decapentaplegic phenotypes both in embryo and in wing. It is involved in hair and cell growth and in tracheal development. Vri is a clock-controlled gene acting as a repressor of the products of Clk and cry.

GB40150	Foxp	0.626	FoxP protein	Forkhead box P (FoxP) encodes a transcription factor expressed in the nervous system. It is involved in locomotion, operant self-learning and courtship behavior.
GB47799	LOC410468	0.633	protein hairy	Hairy (h) encodes a bHLH transcriptional repressor that recruits the corepressor encoded by gro to target promoters. It is a pair-rule gene that contributes to embryonic segmentation and peripheral neurogenesis.
GB47057	LOC113218618	0.645	protein tramtrack, beta isoform-like	tramtrack (ttk) - zinc finger - represses neural cell fate in the peripheral nervous system - a master repressor of enteroendocrine cell specification in intestinal stem cell lineages - regulates morphogenetic events during tracheal development. <i>tramtrack</i> is expressed downstream of Notch in the peripheral nervous system.
GB55387	LOC100577139	0.665	Lilliputian	Lilliputian (lilli) - transcription factor - Fragile X mental retardation 2 (Fmr2) family - Functions in MAPK and Dpp signaling pathways - affects growth, a function associated with the insulin pathway - affects the cytoskeleton early in development.
GB49751	LOC551364	0.708	homeobox protein SIX3	Optix (Optix) encodes a homeobox containing DNA binding protein and a member of the SIX class of proteins. It functions as a repressor via interaction with the transcriptional co-repressor encoded by gro. It is involved in eye formation and morphogenetic furrow movement.
GB50686	LOC408411	0.784	transcription factor Sox-2	SoxNeuro (SoxN) encodes an HMG-domain transcription factor. In early embryos it specifies neural progenitors in the central nervous system, while in later embryos it negatively regulates Wg signaling and controls expression of genes required for denticle construction with the product of ovo.
GB45063	LOC726415	0.789	LIM/homeobox protein Lhx9	tailup (tup) encodes a transcription factor that regulates neuronal sub-type identity, including motor, serotonergic and dopaminergic neuron identity. It regulates germ band retraction, dorsal closure, muscle and heart development.

GB44042	LOC411009	0.861	dachshund homolog 2	dachshund (dac) encodes a transcriptional cofactor that physically interacts with several other retinal determination proteins, including those encoded by eye and so, and regulates eye, leg, gonad, and brain development
GB50048	Mblk-1	0.882	transcription factor mblk-1-like	Ecdysone-induced protein 93F (Eip93F) - Rho-type guanine nucleotide exchange factor (Eip93F) encodes a DNA binding protein that plays an >> important role as an adult determinant during fly metamorphosis.
GB44229	LOC724740	0.909	fork head domain transcription factor slp2	sloppy paired 2 (slp2) encodes a transcription factor of the fork-head family. Together with the product of slp1, it regulates a wide variety of developmental processes including embryonic segmentation, ventral fate specification in the retina, and temporal patterning of the neuroblasts that produce medulla neurons.

Table S 5.2. The overlapping DEGs between the different groups within this study

F2014 stands for field exposed foragers in 2014; N2014 stands for field exposed nurses in 2014; F2015 stands for apiary exposed foragers in 2015; N2015 stands for apiary exposed nurses in 2015. + = was differentially expressed; - = was not differentially expressed.

F2014	N2014	F2015	N2015	Symbol	Description
+	-	+	+	LOC102654154	uncharacterized LOC102654154
+	-	+	+	LOC100576096	myosin heavy chain, non-muscle
+	-	+	+	LOC102656247	dynein-1-beta heavy chain, flagellar inner arm II complex
+	-	+	-	LOC551782	bestrophin-4
+	-	+	-	LOC551706	unconventional myosin-IXb
+	-	-	+	LOC113218918	putative mediator of RNA polymerase II transcription subunit 26
-	-	+	+	LOC413693	cilia- and flagella-associated protein 251
-	-	+	+	LOC726981	circadian clock-controlled protein
-	-	+	+	LOC100576287	uncharacterized LOC100576287
-	-	+	+	LOC551347	UPF0605 protein CG18335
-	-	+	+	LOC410606	protein Skeletor, isoforms B/C
-	-	+	+	LOC411651	Bardet-Biedl syndrome 7 protein homolog
-	-	+	+	LOC100577541	uncharacterized LOC100577541
-	+	+	-	LOC551268	pancreatic triacylglycerol lipase
-	+	-	+	CPR5	cuticular protein 5

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Introduction

Bees play a significant role in pollination of agricultural crops (Rucker, Thurman, and Burgett 2012) and non-agricultural plants (Potts et al. 2016). Bumble bees are particularly important due to their ability to “buzz” pollinate and their ability to fly in cool temperatures (Plowright and Lavery 1984), which makes them better pollinators than honey bees for certain plants (Banda and Paxton 1990). However, many bumble bee species are experiencing steep population declines (Cameron et al. 2011, Williams and Osborne 2009, Colla and Packer 2008, Colla et al. 2012), threatening both food security and critical ecosystem services (Steffan-Dewenter, Potts, and Packer 2005, Klein et al. 2007). Determining the causes of pollinator decline is critical for the conservation of bees in general, and bumble bees in particular (Potts et al. 2016). Conservation genomics – the use of high throughput genomics to study species targeted for conservation – is an emerging field that promises to revolutionize conservation biology (Grozinger and Zayed 2020, Lozier and Zayed 2017, Trapp, McAfee, and Foster 2017). This approach is especially useful for the conservation of native bee species, as it allows us to see ‘the unseen’ stressors that impact bee populations (Grozinger and Zayed 2020).

Bombus terricola, Kirby 1837 is native to North America (Williams et al. 2014) and has experienced major declines in its relative abundance in the last two decades (Cameron et al. 2011, Colla and Packer 2008). It is now extirpated in Illinois (Grixti et al. 2009) and has experienced significant range declines (Richardson et al. 2019, Jacobson et al. 2018), especially in its southern range (Bartomeus et al. 2013). Currently, it is on the IUCN Red List as vulnerable (Hatfield et al. 2015). As with other bumble bees, several hypotheses for the decline

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have been proposed. These include habitat loss, climate change, pesticide use, and pathogen spillover – the introduction of novel pathogens from commercial honey bee and bumble bee colonies to native species (Goulson et al. 2015, Kerr et al. 2015, Szabo et al. 2012, Kent et al. 2018, Grixti et al. 2009). Kent et al. (2018) recently sequenced the genome of *B. terricola* in an effort to understand the factors underlying its decline. Our first population genomic analysis revealed high inbreeding and a low effective population size for *B. terricola*. Moreover, several genes related to immunity exhibited evidence of recent positive selection (Kent et al. 2018), perhaps reflecting an adaptive response to ameliorate stress from pathogens (Colla et al. 2006, Szabo et al. 2012).

Here, we apply a transcriptomic approach to investigate transcriptional signs of stress in *B. terricola*. RNA sequencing can detect changes in genome-wide gene expression associated with a wide variety of stressors, including pathogens, pesticide exposure, and nutritional stress (Grozingler and Zayed 2020). We set up a comparison of *B. terricola* workers collected near agricultural crops and away from agriculture. Agriculture exposes bees to multiple stressors including habitat degradation, pesticides, and pathogens (Colla et al. 2006, Otterstatter and Thomson 2008, Sachman-Ruiz, Narváez-Padilla, and Reynaud 2015). We used the transcriptomic dataset to test several hypotheses; if the decline of *B. terricola* is driven by exposure to pathogens, then we would expect to see patterns of differential expression for genes related to immunity. If the decline is related to pesticide exposure, then we would expect to see changes in the expression of genes involved in detoxification. Finally, if the decline is related to nutritional stress, then we would expect metabolism-related genes to show evidence of differential expression. Of course, if bees are experiencing multiple stressors simultaneously, we would expect to see a combination of these signatures. In addition to the analysis of *B. terricola* genes, we also examined unaligned sequencing reads to directly test for specific pathogens that may have infected the sampled *B. terricola* workers using a database of common bumble bee pathogens (Hernández-Jarguín et al. 2018, Alger et al. 2019, Parmentier et al. 2016). Our study sheds light on the stressors acting on *B. terricola* populations, in addition to demonstrating the utility of genomics in conservation biology.

Methods

Bee Collections

Bombus workers were collected by net between July 19th and August 22nd, 2016 in southern Ontario, Canada; those that were identified as *Bombus terricola* in the field were immediately frozen in liquid nitrogen. Each collection location was qualified as agricultural or non-agricultural (Figure 6.1) based on a 500-1000 meter radius land use (Osborne et al. 2008, Greenleaf et al. 2007) from point of collection using GlobCover 2009 (Bontemps et al. 2011). Locations that had no agricultural land use within 500 meters and less than 10% agricultural land use within 1000 meters were designated non-agricultural. While our sample size is small, we note that we are still able to meet minimum sample size requirements for RNA seq analyses (Conesa et al. 2016).

RNA Extraction and Analysis

RNA was extracted from the abdomens of three worker bees from each of the ten sites (N=30) using the Qiagen RNease Mini kit. We used abdomens as it is the location most likely to detect gene expression related to detoxification (Mao, Schuler, and Berenbaum 2013), nutrition (Alaux et al. 2011), and pathogens (Aufauvre et al. 2014), as well as response to stressors involving hormones and ovary activation (Wang et al. 2012). The samples were then sent to Gènome Québec's Innovation Center for NextGen RNA sequencing using Illumina HiSeq4000 PE 100pb.

We used Trimmomatic (Bolger, Lohse, and Usadel 2014) to remove adapters, low quality bases, and low quality reads. An average of 23,263,068 reads per sample survived the filtering. Quality check was performed using FastQC (Bioinformatics 2011). The data successfully passed the quality check in all relevant areas.

We then aligned the RNA sequences to the *B. terricola* genome (Kent et al. 2018) using the Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al. 2013) and generated gene counts. The gene expression counts were then processed using EdgeR (Robinson,

McCarthy, and Smyth 2010, McCarthy, Chen, and Smyth 2012) in R version 3.2.2 (Team 2005). Any genes that were only expressed in one sample were filtered out, and then the remaining counts were normalized. Differentially expressed genes (DEGs) were calculated based on an Exact Test using a Bonferroni-Hochberg (Benjamini and Hochberg 1995) p-adjustment in order to account for multiple testing.

Reads that were not mapped onto the *B. terrecola* genome were used to investigate the presence of RNA viruses and other pathogens (Batty et al. 2013, Razzauti et al. 2015, Hernández-Jarguín et al. 2018). We aligned and counted the unmapped reads using STAR (Dobin et al. 2013) onto the genomes of common bumble bee pathogens (Supp. Table 1;(Alger et al. 2019, Parmentier et al. 2016). To ensure specificity, we aligned the unmapped reads onto multiple genomes simultaneously, which ensures that ambiguous or multimapped reads were not counted. The gene counts were processed using Edge R (Robinson, McCarthy, and Smyth 2010, McCarthy, Chen, and Smyth 2012) in R version 3.2.2 (Team 2005). Any genes that were only expressed in one sample were filtered out. We used a generalized linear model (GLM) (Nelder and Wedderburn 1972) with a binomial family structure to analyze the prevalence data.

Gene Ontology Analysis

Using a best-match Blastx (Camacho et al. 2009, Boratyn et al. 2012) we mapped all of the *B. terrecola* genes onto the *Drosophila melanogaster* (fruit fly) genome v.6.16 (Myers et al. 2000, Adams et al. 2000, Hoskins et al. 2015) and *Apis mellifera* (honey bee) genome v.4.5 (Consortium 2006, Elsik et al. 2014). We found 7845 *D. melanogaster* homologues, out of which 54 were DEGs, and 8495 *A. mellifera* homologues, out which 54 were DEGs. Gene ontology (GO) analysis was performed in DAVID 6.8 (Huang, Sherman, and Lempicki 2008b, a) using the *D. melanogaster* homologues. We selected the following annotation databases for the analysis: ‘GO Biological Process All’, ‘GO Molecular Function All’, ‘GO Cellular Component All’, as well as Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway and Keywords. We used the Benjamini p-value correction (Benjamini and Hochberg 1995).

The *A. mellifera* homologues were used to compare this study with previously published research on that species. We chose studies that used whole bees or abdomens for their gene

expression analyses, as gene expression profiles can differ between tissues. Where required, the gene names were converted into the current iteration of the honey bee genome using HymenopteraMine (Elsik et al. 2016). These gene lists were compared to our study. We used a hypergeometric test (Johnson, Kemp, and Kotz 2005) to determine if the overlap between published gene lists and our gene lists were statistically different from chance. The a p-adjustment was done using the Holm-Bonferroni method (Holm 1979). These tests were performed in R version 3.6.3 (Team 2005).

Results

We were able to quantify the expression of 9,455 expressed genes in the abdomens of *Bombus terreicola* workers. We found 61 differentially expressed genes (DEGs), 36 of which were upregulated in bees collected from agricultural areas. A GO analysis revealed a statistically significant enrichment in biological processes related to myofibril and muscle cell development ($p < 0.05$, Table S 6.2). Keyword enrichment was found for muscle protein ($p < 0.001$, Table S 6.2) and alternative splicing ($p = 0.035$, Table S 6.2). The KEGG pathway for biosynthesis of antibiotics was also significantly enriched ($p = 0.006$, Table S 6.2). No GO term enrichment was found to be statistically significant for the downregulated genes ($p > 0.1$, Table S 6.2).

In order to compare our study to previously published research, we converted our *B. terreicola* genes to *A. mellifera* homologues. We focused on studies that exposed honey bees to various stressors and looked at global transcriptomic response in either the abdomen or whole bees. We found statistically significant overlap between the DEGs in our study and those found in a study of common immune responses (Doublet et al. 2017)(Table 6.1, hypergeometric test, $p < 0.001$). Furthermore, we found statistically significant overlap with a study that exposed honey bees to *Lotmaria passim*, a trypanosomatid parasite (hypergeometric test, $p = 0.003$; (Liu et al. 2020) and an overlap with bees exposed to sacbrood virus (SBV) and deformed wing virus (DWV; hypergeometric test, $p < 0.001$), but not DWV alone (hypergeometric test, $p = 1$; (Ryabov et al. 2016).

We also found a statistically significant overlap with three studies that exposed honey bees to insecticides. Two exposed the bees to neonicotinoids (Shi et al. (2017): hypergeometric

test, $p=0.029$; Wu, Luo, et al. (2017): hypergeometric test, $p=0.003$) and the third to fipronil (Aufauvre et al. 2014)(hypergeometric test, $p=0.023$). We found no statistically significant overlap when the study exposed honey bees to *Nosema ceranae*, certain viruses, or poor diet (Table 6.1).

We crossed referenced unaligned transcriptomic reads to a database of sixteen bumble bee pathogens, and discovered five matches (Figure 6.2). We found that bumble bees from agricultural areas had a marginally higher prevalence of sacbrood virus (SBV; GLM, $z\text{-value}=-1.754$, $p=0.0795$; prevalence in Agr: 0.667, NonAgr: 0.333), while bumble bees from non-agricultural areas had a marginally higher prevalence of *Lotmaria passim* (GLM, $z\text{-value}=1.832$, $p=0.0670$; Agr:0.111, NonAgr: 0.417). The other detected pathogens did not have a statistically significant difference in their prevalence near or away from agriculture (*Nosema ceranae*: GLM, $z\text{-value}=0.006$, $p=0.9953$; Agr: 0.778, NonAgr: 1.0; *Crithidia bombi*: GLM, $z\text{-value}=0.607$, $p=0.544$; Agr: 0.556, NonAgr: 0.667; black queen cell virus (BQCV): GLM, $z\text{-value}=-1.548$, $p=0.1216$, Agr: 0.778, NonAgr: 0.500).

Discussion

In the present study, we used transcriptomics to gain a better understanding of the stressors experienced by *B. terricola* in the field. We found 61 DEGs in abdomens of workers collected in agricultural and non-agricultural areas. The genes that were upregulated in the bees collected in agricultural areas were related to muscle function and development, as well as biosynthesis of antibiotics. We then compared our DEGs to previously published studies on transcriptomic responses of honey bees to various stressors. We found statistically significant overlaps with studies that exposed bees to pesticides and certain pathogens. These results point to pesticides as a relevant stressor affecting bumble bees near agriculture.

Pesticides have been previously implicated in bumble bee declines (Gill, Ramos-Rodriguez, and Raine 2012, Whitehorn, O'Connor, et al. 2012b). However, whether a link is present between pesticide use and bee decline in the field tends to depend on the bee species and the geographical location (Szabo et al. 2012, Woodcock et al. 2017, Rundlof et al. 2015). Tsvetkov et al. (2017) showed that pollen collected by bees from May to August near

agricultural areas in Ontario contains neonicotinoid pesticides. It is therefore, probable that *B. terricola* workers are also exposed to pesticides during these months in Ontario. Our gene expression data supports this. First, we have statistically significant overlap in DEGs with three separate studies that exposed honey bees to pesticides. Second, two of those overlapping genes are cytochrome P450 genes (LOC551223, LOC413833), which are detoxification genes in honey bees and bumble bees (Berenbaum and Johnson 2015, Manjon et al. 2018). Finally, the enrichment of muscle development GO terms could be related to neonicotinoid exposure, as these pesticides cause hyperactivity (Boily et al. 2013, Suchail, Guez, and Belzunces 2001), locomotor deficits (Charreton et al. 2015), and impact foraging ability (Yang et al. 2008, Henry et al. 2012).

We also found that pathogens are likely a major stress for *B. terricola* workers in agricultural areas. This is supported by the enrichment of genes responsible for the biosynthesis of antibiotics and by the overlap with previous studies focused on immune challenges. We also detected five pathogens in the *B. terricola*: two trypanosomatid parasites, *Crithidia bombi* and *Lotmaria passim* (Lipa and Triggiani 1988, Schwarz et al. 2015), a microsporidian parasite *Nosema ceranae* (Higes, Martín, and Meana 2006), and two RNA viruses black queen cell virus (BQCV) and sacbrood virus (SBV) (Chen and Siede 2007). SBV was more prevalent in bees collected from agricultural areas.

That said, it appears that bees in both agricultural and non-agricultural areas experience pathogen stress. We found that *L. passim* infection was more prevalent in non-agricultural areas and in both group of bees, we found high prevalence of BQCV, *N. ceranae*, and *C. bombi*. SBV, BQCV, and *N. ceranae* are common pathogens in honey bee and managed bumble bee colonies (Chen and Siede 2007, Chen et al. 2008, Graystock, Goulson, and Hughes 2014, Bushmann et al. 2012). These findings lend further support to the pathogen spillover hypothesis (Colla et al. 2006, Szabo et al. 2012, Kent et al. 2018).

We compared our bumble bee DEGs with DEGs from honey bees challenged with different stressors. We did this because of the availability of literature on honey bee gene expression is much greater than that on bumble bees (Trapp, McAfee, and Foster 2017).

However, we think these contrasts between *Bombus* and *Apis* are justified because many of the stress response pathways, such as detoxification and immunity, are strikingly similar between bumble bees and honey bees (Sadd et al. 2015, Barribeau et al. 2015). Additionally, honey bees and bumble bees are often exposed to the same stressors in the field (Woodcock et al. 2017, Rundlöf et al. 2015), including bumble bees being exposed to honey bee pathogens (Furst et al. 2014, McMahon et al. 2015).

While our work highlighted pesticides and pathogens as important stressors acting on current *B. terricola* populations, our study is limited in that we were only able to test for a small subset of stressors. First, we can only detect ‘signatures’ of stressors that were explored in previously published research. Second, we cannot detect stressors that would affect bumble bees in the same manner in both agricultural and non-agricultural sites, such as climate change (Kerr et al. 2015); these would not lead to differentially expressed genes in our analysis. Finally, we cannot detect stressors that exert their effects on queens, males, or during larval development (McFrederick and LeBuhn 2006). However, despite these limitations, we believe that the transcriptomic approach we used here does provide a valuable insight into the probable stressors acting on *B. terricola*. Moreover, the diagnostic power of conservation genomics will only improve as more transcriptomic literature becomes available for bees.

Conclusion

Like several other bumble bee species, *B. terricola* is declining rapidly in North America (Colla and Packer 2008, Cameron et al. 2011). Using a transcriptomic approach, we found that *B. terricola* workers in agricultural areas exhibit transcriptional signatures of exposure to pesticides and pathogens. Pathogens have been implicated in *B. terricola* previously (Kent et al. 2018, Szabo et al. 2012), but, here, we were able to detect several specific pathogens that may be contributing to *B. terricola*’s decline. We also presented the first evidence that *B. terricola* workers are experiencing xenobiotic stressors in the field. This is significant, because pesticides are known to impact important colony development and function (Whitehorn, O’connor, et al. 2012a, Rundlöf et al. 2015), and impact the individual immune response of workers (T O’Neal, Anderson, and Wu-Smart 2018). In this study, we demonstrated that transcriptomics is a valuable

tool for conservation, which allows us to capture a real-time snapshot of the stressors bees experience under natural conditions.

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Figure Legends

Figure 6.1. Map of the collected *Bombus terricola* workers

Workers from agricultural (yellow) and non-agricultural (green) locations in Ontario, Canada. Three worker abdomens were analyzed from each location.

Figure 6.2. Detected pathogens in *B. terricola* workers

We found a marginally increased prevalence in *L. passim* in bees collected from non-agricultural areas (GLM, z-value=1.832, p=0.0670) and increased sacbrood virus (SBV) prevalence in bees collected from agricultural areas (GLM, z-value=-1.754, p=0.0795). ns= not significant, ‡ p<0.1

Figure 6.1

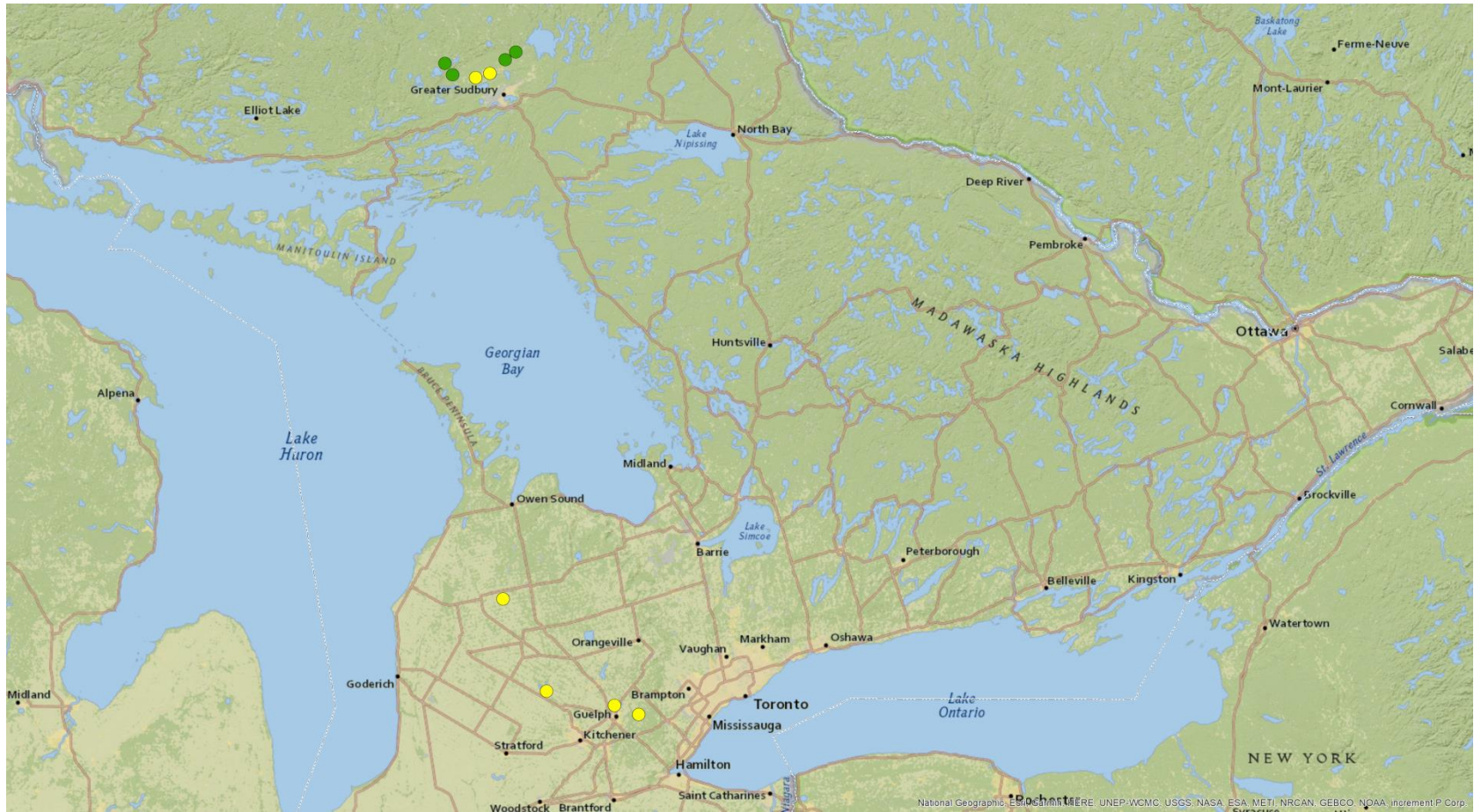


Figure 6.2

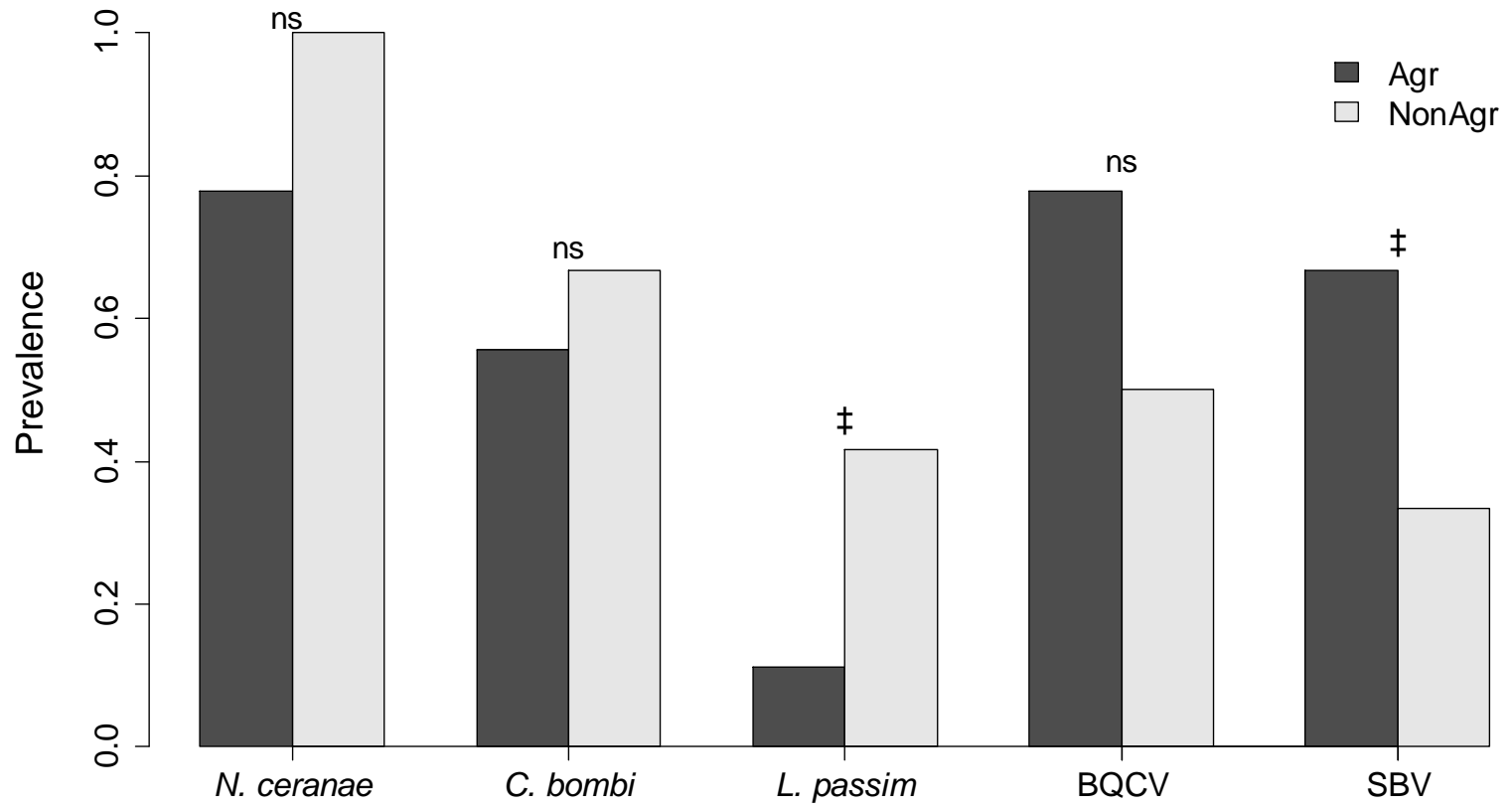


Table 6.1. Number of DEGs overlapping between our study and previously published transcriptomic studies

Statistically significant overlaps are denoted with *(hypergeometric test, $p < 0.05$).

Stressor Type	Stress	DEG Overlap	p-value
Pesticide (Shi et al. 2017)	Thiamethoxam	8	0.032*
Pesticide (Wu, Luo, et al. 2017)	Imidacloprid	7	0.003*
Pesticide (Aufauvre et al. 2014)	Fipronil	2	0.025*
Pathogen (Doublet et al. 2017)	Immune challenge†	10	<0.001*
Pathogen (Liu et al. 2020)	<i>Lotmaria passim</i>	10	0.003*
Pathogen (Ryabov et al. 2016)	Sacbrood virus + deformed wing virus	24	<0.001*
Pathogen (Ryabov et al. 2016)	Deformed wing virus	0	1
Pathogen (Aufauvre et al. 2014)	<i>Nosema ceranae</i>	0	1
Pathogen (Badaoui et al. 2017)	<i>Nosema ceranae</i>	2	0.294
Pathogen (Brutscher, Daughenbaugh, and Flenniken 2017)	Sindbis virus	13	0.104
Pathogen (Brutscher, Daughenbaugh, and Flenniken 2017)	Double stranded RNA	3	1
Pathogen (Rutter et al. 2019)	Israeli acute paralysis virus	0	1
Nutrition (Rutter et al. 2019)	Chestnut vs Rockrose (less nutritious) pollen	17	0.186
Nutrition (Corby-Harris et al. 2014)	No pollen diet	1	0.188
Nutrition (Alaux et al. 2011)	No pollen diet	12	1
Nutrition (Wang et al. 2012)	High and low pollen-hoarding	13	0.553

†DEGs aggregated across multiple studies

Table S 6.1. The genomes of the common bumble bee pathogens used in this study

Name	GenBank Assembly
Acute bee paralysis virus	GCA_000856345.1
Black queen cell virus	GCA_000851425.1
Big sioux river virus	GCA_002219505.1
Deformed wing virus	GCA_000852585.1
Israeli acute paralysis virus	GCA_000870485.1
Lake Sinai virus	GCA_001925995.1
Slow bee paralysis virus	GCA_000887395.1
Sacbrood virus	GCA_000847625.1
<i>Ascospaera apis</i>	GCA_001636715.1
<i>Crithidia bombi</i>	GCA_900240985.1*
<i>Lotmaria passim</i>	GCA_000635995.1*
<i>Melissococcus plutonius</i>	GCA_003966875.1
<i>Nosema apis</i>	GCA_000447185.1
<i>Nosema bombycis</i>	GCA_000383075.1
<i>Nosema ceranae</i>	GCF_000988165.1
<i>Paenibacillus larvae subsp. larvae</i>	GCF_002003265.1

*These genomes did not contain annotations. Therefore, we annotated the downloaded genome using the parasite genome annotation pipeline with *Leishmania major Friedlin* as reference organism (Steinbiss et al. 2016, Liu et al. 2020).

Table S 6.2. Gene ontology analysis of the upregulated genes in agricultural *B. terricola*

Only statistically significant terms after a Benjamini p-value correction are shown. CELL COMP = Cellular Component; BIO PROC = Biological Processes.

Category	Term	p-value
KEYWORDS	Muscle protein	0.00000005
CELL COMP	actin cytoskeleton	0.000087
CELL COMP	contractile fiber	0.001000
CELL COMP	myofibril	0.001200
KEGG PATHWAY	Biosynthesis of antibiotics	0.005500
CELL COMP	cytoskeletal part	0.006700
CELL COMP	sarcomere	0.009000
BIO PROC	myofibril assembly	0.009300
CELL COMP	contractile fiber part	0.009600
CELL COMP	cytoskeleton	0.009700
BIO PROC	muscle cell development	0.009800
BIO PROC	striated muscle cell development	0.014000
BIO PROC	muscle system process	0.018000
BIO PROC	sarcomere organization	0.022000
BIO PROC	actomyosin structure organization	0.023000
BIO PROC	striated muscle cell differentiation	0.034000
BIO PROC	cellular component assembly involved in morphogenesis	0.035000
KEYWORDS	Alternative splicing	0.035000
BIO PROC	muscle cell differentiation	0.047000

Chapter 7 Conclusions and future forward

Bee declines have been gaining more attention over the past two decades (Cameron and Sadd 2020). Pin pointing the reasons behind the declines have proved difficult, even in the most studied bee species, the honey bee (Potts et al. 2010). However, several drivers were identified, among them neonicotinoids (NNIs)(Potts et al. 2010, Cameron and Sadd 2020), the most commonly used insecticides in the world (Goulson 2013). Despite the attention NNIs received from bee researchers (Cameron and Sadd 2020) critical knowledge gaps precluded the findings from being conclusive. In particular, information regarding the dosage and duration of NNI exposure bees experience in the field was incomplete (Carreck and Ratnieks 2014).

Therefore, in this dissertation, I first set out to fill in this gap by conducting a season long study on honey bee colonies placed near NNI treated corn crops in Ontario and Quebec (Chapters 2 and 3). I found that honey bees were exposed to NNIs for an average of three months and to a sublethal dose that is comparable to many laboratory studies, which found negative impacts of the NNI exposure on honey bee health. I also conducted an apiary experiment in order to determine what kind of an effect this dosage and duration has on honey bee colonies. I exposed colonies to a decreasing dose of clothianidin, starting from 4.9ppb, which was a conservative dose in comparison to the exposure honey bees experienced near corn crops. . I found that NNI exposure reduced the bees' hygienic behaviour and their ability to maintain a laying queen (Chapter 2). I also found that this kind of NNI exposure had a subtle impact on the honey bees' immune system (Chapter 3). This work illustrated the importance of conducting season long studies, as many of the impacts of NNIs were only noticeable after a few months of exposure.

Besides NNIs, I found other agrochemicals inside honey bee colonies, including herbicides and fungicides (Chapter 2). I set out to see whether field levels of these agrochemicals interacted with NNIs and found that the fungicide boscalid at a field natural dose, doubled NNI toxicity in honey bees. Although there are some efforts, interactions between chemical groups is rarely studied (Glavan and Bozic 2013), even though field realistic exposure is characterized by an

exposure to many chemicals at a time and the patent for NNI seed-dressing calls for the presence of at least three fungicides (Schneidersmann and Stypa 2003).

I set out to examine the effects honey bee genetics can have on NNI toxicity (Chapter 4). Rinkevich et al. (2015) showed that the dose of NNIs needed to kill 50% of the honey bees (LD_{50}) varied based on honey bee background genetics (stocks). Therefore, I focused on survival after 24 hours of exposure as a proxy for NNI sensitivity. I found that patriline has a significant effect on whether a worker survived and was able to estimate the broad sense heritability (H^2) as 37.8%. I then sequenced the primary NNI detoxification genes (Manjon et al. 2018) and found that certain polymorphisms in CYP9Q3 and CYP9Q1 were associated with a higher survival rate. This study quantified the genetic component behind the variation to NNI susceptibility and strongly suggests that polymorphisms in two genes affect NNI metabolism. Further research is being conducted to look into the gene expression pattern in workers exposed to a sublethal dose of NNI from low and high surviving patrilines. Additionally, molecular modeling can shed light on whether the polymorphisms found in my study affect how CYP9Q3 and CYP9Q1 interact with NNIs (Mao, Schuler, and Berenbaum 2011). Finally, more work needs to be done in order to determine how genetics plays a role in the sublethal and chronic NNI toxicity honey bees experience in the field (Tsvetkov et al. 2017).

One challenge with measuring sublethal toxicity is that the types of traits we are able to measure accurately are limited and the number of traits we are able to measure in a single study is also limited. This leads to a ‘streetlight effect’, where people focus on traits that are easiest to measure, which limits our understanding (Battaglia and Atkinson 2015). However, with newer tools, such as transcriptomics, we can overcome the ‘streetlight effect’ (Grozingler and Zayed 2020, Lozier and Zayed 2017). RNA sequencing allows us to quantify gene expression for thousands of genes simultaneously. By combining it with the existing body of knowledge, which links gene expression to biological processes and molecular functions in insects (Kanehisa and Goto 2000, Ashburner et al. 2000, Consortium 2019), we can effectively quantify thousands of traits simultaneously.

I applied this paradigm to honey bees collected from the experiments described in Chapter 2 (Chapter 4). I found that after exposure to NNIs in the field, foragers, who collect pollen and nectar for the hive, had gene expression associated with traits that are important in navigation, such as learning and memory and vision. While nurses, who feed the brood inside the hive, had gene expression associated with metabolic changes. This study provided critical insight into how field and field realistic exposure affects foragers and nurses differently. Additionally, comparison with previously published studies showed little to no overlap in the shift of gene expression pattern following NNI exposure between bees exposed under laboratory conditions and under field and apiary conditions. This illustrates another ‘streetlight effect’ – studies on honey bees isolated from the hive are easier to conduct, but might miss important information regarding honey bees’ physiology (Alaux et al. 2009, Grozinger et al. 2003).

Transcriptomics of field collected bees can also be useful as a conservation tool, although it is rarely applied (Grozinger and Zayed 2020, Lozier and Zayed 2017). It allows us to examine several hypotheses at the same time and can be done with relatively few sample collections (Conesa et al. 2016). Thus, I looked into the gene expression of *Bombus terricola* workers, a declining bumble bee (Cameron et al. 2011, Colla and Packer 2008). I found gene expression patterns that were associated with stress from pesticide exposure and pathogens. Additionally, I was able to detect specific pathogens in these workers, further pointing to pathogens as a source of stress. Conservation genomics allowed me to gain insight into the possible reasons behind the decline of *B. terricola*. This allows for future work to focus on specific hypotheses based on evidence and allows for conservation efforts to be better targeted for this species.

In the beginning, I set out to quantify the NNI exposure honey bees experience near corn fields and how this kind of exposure affects their health. I learned that honey bees experience a cocktail of agrochemicals in the field for most of their active season. These agrochemicals can act synergistically with each other or with other environmental factors, such as pathogens, to reduce the bees’ health. NNIs also interact with honey bees’ genetics, which have a large effect on NNI’s toxicity. Given the large number of possible interaction effects and traits NNIs can affect, I used transcriptomics to, effectively, look at thousands of traits simultaneously. I found that the natural hive environment and the duties bees perform also interact with NNIs. Finally, I

applied what I learned in honey bees to a native bumble bee. There too I found signs of multiple stressors working simultaneously. Overall, my dissertation demonstrates that field studies are indispensable when studying bees and that conservation genomics is a practical tool for the conservation efforts of wild bees.

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Appendix A: Statement on contributions

Chapter 1:

The overview chapter was written by NT

Chapter 2:

Tsvetkov N, Samson-Robert O, Sood K, Patel HS, Malena DA, Gajiwala PH, Maciukiewicz P, Fournier V, Zayed A. 2017. “Chronic exposure to neonicotinoids reduces honey bee health near corn crops.” *Science*. No. 356(6345):1395-7.

NT designed the study, carried out the sample collection, data analysis, drafted the manuscript, and coordinated the study. OSR participated in the sample collection, in the study design, and provided feedback on the manuscript. KS participated in data collection. DAM participated in data collection. PHG participated in data collection. PM participated in data collection. VF provided funding, participated in the study design, coordination, and provided feedback on the manuscript. AZ designed the study, provided funding, coordinated the study, and critically revised the manuscript.

Chapter 3:

N. Tsvetkov, H. S. Patel, O. Samson-Robert, V. Fournier, and A. Zayed. Field realistic exposure to neonicotinoids has a subtle impact on the honey bees’ immune system and pathosphere. submitted.

NT designed the study, carried out the sample collection, data analysis, wrote the manuscript, and coordinated the study. HSP performed the molecular lab work and participated in the sample collection. OSR participated in the sample collection, in the study design, and provided feedback on the manuscript. VF provided funding, participated in the study design, coordination, and provided feedback on the manuscript. AZ provided funding, conceived of the study, designed the study, coordinated the study and critically revised the manuscript.

Chapter 4:

N. Tsvetkov and A. Zayed. Natural polymorphisms in CYP9Q genes help explain large variation in the heritable honey bee sensitivity to neonicotinoid insecticides.

NT designed the study, carried out the data collection, data analysis, and wrote the manuscript. AZ participated in study design, provided funding, and critically revised the manuscript.

Chapter 5:

N. Tsvetkov and A. Zayed. Searching beyond the streetlight: neonicotinoid exposure alters the neurogenomic state of worker honey bees. submitted

NT designed the study, carried out sample collection, molecular work, data analysis, and wrote the manuscript. AZ provided funding, designed the study, and critically revised the manuscript.

Chapter 6:

N. Tsvetkov, V.J. MacPahil, S. R. Colla, and A. Zayed. Conservation genomics reveals pesticide and pathogen stress in the declining bumble bee *Bombus terricola*. To be submitted.

NT designed the study, carried out the molecular work, data analysis, and wrote the manuscript. VJM participated in study design and carried out the field collections. SRC participated in study design and provided funding. AZ provided funding and critically revised the manuscript.

Chapter 7:

Conclusions and future forward was written by NT.

Sincerely,

Nadejda Tsvetkov

Approved by,

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Associate Professor

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