Chronic exposure of imidacloprid and clothianidin reduce queen survival, foraging and nectar storing in colonies of *Bombus impatiens*

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Abstract

The 20 year research focus on residue levels below 10 ppb of neonicotinyl insecticides found in nectar and pollen of seed-treated crops (corn, canola, and sunflower) has not demonstrated a reduction in bee colony health in most field studies. However, the label rate of neonicotinyl use on crops and landscape plants is much higher than seed treatments. In addition, crops and flowers can be retreated multiple times a season which can contribute to chronic exposure to bees at higher residue levels. In an 11 week greenhouse cage study with queenright colonies of Bombus impatiens Cresson, provided 0, 10, 20, 50 and 100 ppb imidacloprid or clothianidin in sugar syrup, neither neonicotinyl reduced production of brood, workers, and queens. Male production decreased in 10-100 ppb imidacloprid and 50-100 ppb clothianidin treatments. However, starting at 6 weeks queen mortality was significantly higher in 20-100 ppb imidacloprid or clothinaidin. The largest impact was the reduction in worker movement, consumption, number of syrup filled wax pots, and the addition of wax to the colony, which resulted in reduced colony weight. Queens and nest bees fed on the sugar syrup stored in wax pots that were filled prior to the start of the experiment. Foraging bees did not return sugar syrup to the nest, but remained on the floor of the flight box. We argue that queen mortality at 20, 50, and 100 ppb was related to lack of syrup in storage pots. We speculate that as queens started to die at week 6, workers in 20-100 ppb treatments produced fewer males and instead provisioned cells to produce new queens, since queen production was not reduced at higher doses, but male production was reduced. Since neonicotinyls in this and other studies were shown to reduce food consumption and foraging, wild bumblebee colonies that depend on workers to forage will be negatively affected by exposure to imidacloprid above 20 ppb. Solitary bees will be greatly impacted as the foraging queens solely provision the larvae.

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Chapter 1: Stressors affecting bumblebee populations

Bees as important pollinators

Globally, there are 352,000 species of flowering plants. Among these, 87.5% rely on or benefit from animal pollination services to complete their reproductive life cycles (Ollerton et al. 2011). While animal pollinator diversity includes birds and bats, the insects Coleoptera (beetles), Diptera (flies), Lepidoptera (butterflies and moths), and Hymenoptera (bees and wasps) account for the majority of pollinators (Klein et al. 2007, Hopwood et al. 2012). Of these, bees are the most economically important pollinators, due to a long co-evolution with flowering plants, selecting for beneficial morphological, physiological, and behavioral adaptations (Tepedino 1979).

Currently 300 plants are commonly used as a food source for humans (McGregor 1976). Of these, cereal crops make up most of the human diet (Delaplane and Mayer 2000). The majority of cereal crops are wind pollinated and therefore do not provide bees with forage or nesting sites (Corbert 1991). Despite this, 35% of crops benefit from pollinator services, including 100 crop species in North America alone (Klein et al. 2007, Vaughan and Black 2007). Many of these crops are fruits, vegetables, nuts, and seed crops (Corbert et al. 1991). Although the number of plants directly used by humans is a small proportion of the total plants that have mutualistic relationships with pollinators, it is important to note that there are indirect human benefits gained by plant communities sustained through pollination but not directly linked to our food supply (Kremen et al. 2007). These indirect benefits including aesthetic value of flowers, reduction of soil erosion, food and forage for innumerable animals, and maintenance of temperate forest, grassland, and desert ecological dynamics (McGregor 1976).

There are a disproportionate number of studies documenting the benefits and stressors affecting honey bee (*Apis mellifera*) pollination compared to bumblebees (*Bombus* spp.) and solitary bees. This is because honey bees are commercially managed, easy to obtain, most commonly used for pollination of economically important crops, and have preexisting experimental protocols (Hopwood et al. 2012). This review aims to highlight the known information on bumblebees but honey bee studies are cited in the absence of known effects on bumblebees.

Honey bees, a non-native European species, have historically been used to meet pollination needs of large scale agriculture operations, but populations have been declining over the last 50 years (Vaughan and Black 2007). Of the economically important plant crops humans rely upon, honey bees provide approximately $16.4 billion dollars worth of pollinator services (Morse and Calderone 2000, Losey and Vaughan 2006). A later estimate separated monetary value gained from honey bees verses wild bees and found that $3.07 billion dollars or 18.7% of pollinator services are contributed by wild bees (Losey and Vaughan 2006). With the stability of honey bee pollinator services declining, reliance on wild pollinators for agriculture pollination services may increase in the future (Vaughan and Black 2007). Ricketts et al. (2008) demonstrated that diverse pollinator communities buffer against population fluctuations over time, providing more consistent pollination services. In addition, a recent study showed wild bee pollinators increased fruit set in 41 crops, while honey bees increased fruit set in only 14% of these crops (Garibaldi et al. 2013). Garibaldi's work further quantified pollination services by comparing the fruit set of flowers visited by wild pollinators verses honey bees and showed wild pollinators enhanced fruit set twice as much as honey bees (Garibaldi et al. 2013).

Pollination advantages of bumblebees

Honey bees and many wild bees have different methods of performing pollination services. All bees visit flowers for pollen and nectar. During this process they passively transfer pollen between flowers when their body contacts a flower's pistil during pollen or nectar collection. Some wild bees, such as bumblebees, perform buzz pollination: the process of sonicating a flower by biting or curling the bee's abdomen around an anther and vibrating indirect flight muscles to facilitate the release of pollen. The result is that bumblebees can gather pollen 400 times faster than honey bees (Winter et al. 2006). This type of pollination is especially beneficial for Ericaceae (blueberries, cranberries) and Solanaceae (eggplant, pepper, tomato) plants (Michener 2007). In addition to the ability to perform buzz pollination, bumblebees have a large variation in body size and proboscis length. These morphological characteristics allow bumblebees to forage at lower light and temperature conditions compared to honey bees and to utilize a wide variety of

plants, making them critical pollinators in temperate climates (Winter et al. 2006). These attributes have led to commercial rearing programs of bumblebees as an economic tool. Commercial rearing started in 1987 and has increased to the production of approximately one million colonies annually worldwide. The majority of commercially reared bumblebees are either *B. terrestris*, used in Eurasia, or *B. impatiens*, used in North America (Velthuis and van Doorn 2006).

Causes of decline in bees

While the pollination contributions and utility of wild bees have been documented, many bee species are in decline. Our knowledge and understanding of factors contributing to bee decline and even which species are in decline are impeded by a lack of historical base line data (Grixti et al. 2009). Of the 54 species of bumblebees in North America, the following two studies document 10 species in decline (Winter et al. 2006). A state wide survey of bumblebee species in Illinois concluded that *B. affinis*, *B. borealis*, *B. fraternus*, *B. pensylvanicus*, *B. ternarius*, *B. terricola*, *B. vagans*, and *B. variabilis* had been either extirpated or were declining over the last century compared to historical information (Grixti et al. 2009). A larger nationwide historical comparison by Cameron et al. (2011) surveyed *Bombus* spp. at 382 locations across the United States. Findings indicated substantial range reduction of two western species, *B. franklini* and *B. occidentalis*, and three eastern species, *B. affinis*, *B. pensylvanicus*, and *B. terricola*.

While bumblebees are considered generalists, foraging on a wide variety of plant species, they have experienced the largest loss of forb-bee interactions (Burkle et al. 2013).

A study in 1888 by Charles Robertson documented 532 interactions between forbs and bees. When interactions were quantified in 2009, only 125 of the original interactions were observed. Although 121 new interactions were documented, overall 46% fewer interactions occur today among forbs and bees compared to 125 years ago. Of these lost interactions, 34% of had been performed by 55 wild bee species that could no longer be found at study sites (Burkle et al. 2013). Historically, forb-bee interactions were redundant, with many species pollinating the same plant. Over time, these networks have degraded due to fewer species present. For example, Burkle et al. (2013) documented the

diversity of bees visiting *Claytonia virginica* (Virginia spring beauty) in Illinois and found a 50% reduction in the number of bee species compared to 1971. With fewer bees visiting plants, the likelihood of plants achieving optimal pollination or sustaining viable populations decreases (Delaplane and Mayer 2000, Biesmeijer et al. 2006).

The causes of managed and wild bee decline are numerable and often additive. These stressors include habitat loss and fragmentation, parasites, pathogens, temporal changes, and pesticide use.

Habitat fragmentation

Wild bees initiate new nests in meadows, grasslands, fallow farmland, and forest edges. Agricultural intensification between 1900-1970 and an increase in urbanization has reduced suitable habitat for bees (Corbet et al. 1991, Grixti et al. 2009). Agricultural intensification may decrease floral resources and nesting sites and increase the amount of insecticide used (Potts et al. 2010). While some fields provide pollen and nectar for bees when a crop is in bloom, they often do not provide continuous floral resources needed to sustain the life-histories of social and solitary bees (Corbet et al. 1991). Grixti et al. (2009) reported that bumblebee populations declined sharply in Illinois between 1940 and 1960, a time period coinciding with conversion of natural habitats to agricultural areas. Current agricultural practices often rely on large plantings of monoculture crops. Averill (2011) studied how landscape complexity (homogeneity versus heterogeneity) effects *B. impatiens* homing by releasing foraging bees at varying distances from nesting sites and found that with increasing homogeneity of a landscape, homing efficiency decreased. In turn, landscape homogeneity may increase distances between suitable nesting habitat for wild bees and their forage. Furthermore, Ricketts et al. (2008) showed that wild bee visitation to flowers dropped 50% at a distance of 668 meters away from natural, undisturbed, bee habitat, which may be a consequence of wild bees having short foraging ranges. For example, in bumblebees, the foraging distances of two dissimilar bumblebee species, *B. pascuorum* and *B. terrestris*, were shown to be between 312-449 meters and 625-758 meters, respectively (Darvill et al. 2004, Knight et al. 2005). To increase pollinator services by bumblebees with short foraging ranges, a shift in cultural practices is needed. Vaughan and Black (2007) suggested the use of refuges near

agricultural sites to support alternative bee friendly forage and nesting sites. Morandin and Winston (2006) determined that if 30% of land within 750 feet of canola fields was uncultivated, there was a significantly larger abundance of bees within agricultural fields, resulting in larger seed set. In California, stability and quantity of pollinator services by wild bees significantly increased when oak woodland and chaparral habitat could be found within 1-2.5 kilometers of fields (Kremen 2004).

In addition to effects of agricultural intensification, fragmentation limits accessible floral resources and nesting sites and has been implicated in reducing genetic diversity of bees through local population extinction. Fragmentation may lead to isolation between intra-specific populations and therefore reduce genetic out-crossing and diversity; this would reduce an isolated population's genetic phenotypes and increase its susceptibility to future perturbations. Cameron et al. (2011) tested genetic diversity of bumblebee populations and found that declining species had significantly lower genetic variation. Genetic shifts occurring during hybridization could also lead to changes in plant-pollinator relationships if commercially reared gynes (daughter queens) become naturalized outside of their native ranges (Winter et al. 2006).

Bumblebee pathogens and parasites

Little is known about many of the lethal and sublethal implications associated with bumblebees and the pathogens and parasites they can carry (Otterstatter and Whidden 2004). The majority of research has focused on the spread of pathogens and parasites from commercially produced bumblebees into wild populations. Up to 73% of commercial bumblebees used in greenhouse pollination leave greenhouses to forage on nearby flowering plants, which may lead to non-native bumblebee species becoming naturalized or horizontal transmission of pathogens and parasites through shared floral resources (Winter et al. 2006, Colla et al. 2006, Durrer and Schmid-Hempel 1994). This could be especially problematic for wild bee populations because commercially reared bumblebees often carry higher loads of intestinal microsporidians (e.g. *Crithardi bombi* and *Nosema bombi*) and tracheal mites, *Locustacarus buchneri* (Colla et al. 2006). Wild bumblebee populations near greenhouses had *C. bombi* in 27% of bees sampled and *N. bombi* in 15-25% of bees sampled. In contrast, wild bee populations away from

greenhouses showed infection rates of <4% *C. bombi* and *N. bombi* (Colla et al. 2006). A study conducted in southwest Alberta showed <10% of wild bumblebees sampled were parasitized by *L. buchneri*, although some *Bombus* spp. in mid-summer may have infection rates up to 50% (Otterstatter and Whidden 2004). Pathogens are often expressed in bumblebees as sublethal effects (Winter et al. 2006). *Crithidia bombi* is known to impair bumblebees' ability to perceive color rewards associated with flowers, thereby reducing foraging efficiency (Gegear et al. 2006). A laboratory study using *B. terrestris* showed that colonies infected with *N. bombi* had five times the mortality rate of uninfected colonies (Winter et al. 2006). Cameron et al. (2011) corroborated these laboratory results, showing that *N. bombi* was significantly higher in the declining species of *B. occidentalis* (37%) and *B. pensylvanicus* (15.2%) captured during field surveys. *Bombus* spp. are also known to harbor the brood parasitoids *Melittobia acasta* and *Melittobia chalybii*, although little is known about the effect these parasites have on colony health (Winter et al. 2006).

Resent research has focused on the transmission and effects of viruses on bumblebees. Honey bees and bumblebees visit 90% of the same floral resources (Potts et al. 2010). Morkeski and Averill (2010) tested wild bumblebee populations for known honey bee pathogens and found acute bee paralysis virus (ABPV), deformed wing virus (DWV), kashmir bee virus (KBV), and black queen cell virus (BQCV). Further testing of commercially obtained *B. impatiens* colonies revealed the presence of DWV and BQCV. Not only are these viruses able to be transmitted between hosts, but DWV may be more prevalent in bumblebees than its original honey bee host (Potts et al. 2010). The consequences of bumblebees having these viruses, i.e., whether they have an impact on bumblebee health and survivorship, have yet to be fully understood (Morkeski and Averill 2010).

Effects of climate change

Further shifts in habitat fragmentation and in virulence of pathogens and pests may be influenced by climate change (Potts et al. 2010). Climatic temperature shifts have been linked to decline of several *Bombus* spp. in Britain indicating the susceptibility of some species with narrow climatic ranges (Williams et al. 2007). Species with longer

development times and shorter foraging ranges may be more at risk for decline (Biesmeijer et al. 2006). A large genera of bees, *Andrena* spp., often emerge early in the spring. Burkle et al. (2013), observed bees at historical survey sites on the plant *Claytonia virginica* and showed that *Andrena* spp. have three times lower fidelity to this floral resource than 120 years ago. Observations like these give evidence to phenological mismatch (peak bee activity occurring earlier than peak plant flowering) and especially affects species emerging in early spring (Burkle et al. 2013).

Pesticide use and pollinators

Conventional, modern agricultural practices began following the end of World War II, when the development of effective herbicides and synthetic insecticides became widely available and utilized (Johansen 1977). Herbicides are often applied for field preparation and along field margins, reducing native plant flora used by bees (Vaughan and Black 2007). Potts et al. (2010) compared bee diversity in fallow land bordering conventional and organic fields and found that unused land next to organic farms supported higher bee diversity. In addition to implementing the use of herbicides, the synthesis of conventional insecticides became commercially viable and insecticides began to be commonly applied to large areas of crops, rangelands and forests (Johansen 1977). These synthesized insecticide classes included organophosphates, carbamates, pyrethroids, and neonicotinoids (Grixti et al. 2009). All are considered broad-spectrum insecticides, meaning that these insecticides affect target pest insects and any beneficial insects that come in contact with the chemical (e.g. pollinators or natural biological control agents). Factors affecting the efficacy of these broad-spectrum insecticides toward target or non-target insects include timing of application (morning verses evening, bloom verses non-bloom), and application method (e.g. seed treatment, soil drench, foliar spray, plant injection).

In addition, differences in life history and bee size influence effects of pesticides (Brittain and Potts 2011). Nest location may be one life history parameter considered before pesticide application, as different species of bees use specific plant materials for nest building (i.e. soil, leaves, plant hairs, or resin) (Vaughan and Black 2007). In addition, the size of bumblebees can vary eight to ten-fold within a colony. Smaller bees

have a higher surface area to volume ratio and therefore may have a lower lethal dose to 50% of a population (LD50) (Averill 2011).

Neonicotinyl use and uptake

To date, the most commonly used insecticides are neonicotinoids, originally synthesized in 1985 and first registered in France in 1991 for sugar beet (Sur and Stork 2003). Today, neonicotinoids are used in over 140 crops (Whitehorn et al. 2012). In 2009, Minnesota used 51,497 pounds of imidacloprid and 19,454 pounds of clothianidin in agriculture and urban landscapes (Pesticide Sales Database 2009). Imidacloprid and clothianidin, the most commonly used neonicotinoids, have also been shown to have long residual half-lives of up to 997 and 1,155 days in soil, respectively (Hopwood et al. 2012). In addition, neonicotinoids have a low mammalian toxicity but remain highly toxic to insects, binding to acetylcholinesterase receptors. Neonicotinoid use has increased because these chemicals pose relatively low risk to humans and have a long active period in plants so a reduced number of applications are needed to control pest insects.

Neonicotinoids are systemic insecticides, taken up through soil or leaves and translocated through the xylem to all parts of a plant. For bees, this means that neonicotinyl accumulation in pollen and nectar is of concern. Bumblebees and other wild bee larvae may consume higher amounts of neonicotinoid residue compared to honey bee larvae because bumblebees and other bees are fed unprocessed pollen (Fisher and Moriarty 2011). Neonicotinoid use can be separated into three treatment categories based on concentrations applied: seed treatments, field applications, and landscape or ornamental treatments. The amount of active ingredient (AI) found in the pollen and nectar is generally correlated with amount of AI applied to the plant, although expression of neonicotinyl residues in pollen and nectar are influenced by environmental factors (i.e. heat and moisture) (Dively and Kamal 2012). A common imidacloprid seed treatment, Gaucho, is applied at $\leq$1.5 mg AI/ft^2 depending on the crop (EFSA 2013). For field crops a formulation of imidacloprid, Admire 2, can be applied at five times the rate of a seed treatment, up to 5.2 mg AI/ft^2. Another formulation of imidacloprid used in nurseries for ornamentals, Marathon, can be applied at 300 times the rate of a seed treatment, up to

300 mg AI/3 gallon pot. Table 1 shows imidacloprid expression in flowers for seed, field, and ornamental treatments.

Neonicotinoids in bees

Understanding how the amount of AI applied to a plant effects the amount of AI found in pollen and nectar is important to gauge which treatment rates will cause harm to bees. Neonicotinoid chemicals contain either cyano or nitro substituted functional groups. Those containing nitro groups (NNO_2), imidacloprid, clothianidin, dinotefuran, and thiamethoxam, have been shown to be more toxic to honey bees than neonicotinoids containing cyano groups (NCN), thiacloprid and acetamiprid (Decourtye and Devillers 2010). Neonicotinoids act on the central nervous system by targeting nicotinic acetylcholine receptor. Typically, acetylcholine is released by a presynaptic neuron and travels through the synapse where acetylcholine binds to an acetylcholine receptor on the postsynaptic neuron, thereby continuing the electrical signal (Casida and Durkin2013). This signal is continued until acetylcholinesterase hydrolyzes acetylcholine from the receptor (Tomizawa and Casida 2003). Neonicotinoids disrupt this process by acting as a high affinity acetylcholine mimic. Like acetylcholine, neonicotinoids can bind to nicotinic acetylcholine receptors, but once bound, acetylcholinesterase has a harder time hydrolyzing the neonicotinoid. This causes the continued firing of neurons and results in trembling, uncoordinated or hyperactive movement, and potential death (Suchail et al. 2000, 2001). Table 2 shows the range of neonicotinoid LD50 values for bumblebees. Within 4.5-5 hours, half of the neonicotinoids molecules bound to the acetylcholine receptors will have degraded into metabolites (Suchail et al. 2003).

In addition to potential lethal effects of neonicotinoids, imidacloprid is known to cause sublethal effects on mushroom body formation in bees (Tome et al. 2012). Mushroom bodies are dense groups of neurons located in the brain and are important for memory, learning and orientation (Rossler and Groh 2012). Neonicotinoids have been shown to reduce long-term memory formation when using an olfactory learning test, proboscis extension reflex (PER) (Gauthier et al. 2006).

Neonicotinoid effects on bumblebee foraging

Neonicotinoid sublethal affects toward mushroom body formation (i.e. memory, learning, and orientation) are thought to influence foraging ability and efficacy. Appendix 1 reviews literature on imidacloprid and clothianidin and neonicotinoid interactive effects on foraging *Bombus* spp. To date, the majority of studies look at neonicotinoid concentrations common in seed treatments. Recent research using radio frequency identification tags (RFID) by Gill et al. (2012) showed foragers challenged with 10 ppb imidacloprid return to colonies with smaller pollen loads and therefore recruited 1.5 times as many workers to forage. Conflicting results toward forager recruitment are presented by Gels et al. (2002), who showed that non-irrigated tall fescue sprayed with imidacloprid reduced the number of foraging bees. In addition to foragers collecting smaller pollen loads, studies show increased foraging and flower handling time (Gill et al. 2012, Morandin and Winston 2003). Foragers also showed increased homing failure, indicating affects on orientation and learning (Gill et al. 2012, Averill 2011). Further observations by Averill (2011) noted that smaller bees had increased homing failure, probably because of higher surface area to volume ratio. While these results often manifest as sublethal effects initially, there is potential for lethal toxicity to occur. A caged greenhouse study evaluated foraging effects of imidacloprid at 10 and 20 ppb and found significantly higher worker mortality (Mommaerts et al. 2010). The above significant effects were also shown to be true when a combination of 10 ppb imidacloprid and 37,500 ppb λ-cyhalothrin, a pyrethroid were given to bees (Gill et al. 2012).

Neonicotinoid effects on bumblebee colony health

Neonicotinoid contaminated pollen and nectar reduces foraging efficacy and may also affect *Bombus* spp. colony health. This section discusses effects of residues commonly found in neonicotinoid treated seeds and field crops. Appendix 2 reviews literature on imidacloprid and clothianidin and neonicotinoid interactive effects on full *Bombus* spp. colonies with queens.

As previously discussed, field studies showed foragers exposed to neonicotinoids carried reduced pollen loads. In contrast, three greenhouse studies failed to show an effect on syrup and pollen consumption (i.e. foraging) after exposure to neonicotinoid

concentrations up to 36 ppb (Mommaerts et al. 2010, Morandin and Winston 2003, Franklin et al. 2004). However, one field study showed reduced amount of sugar syrup stored within the colony (Gels et al. 2002). Neonicotinoid contaminated pollen and nectar brought back to the colony may affect the life span and behavior of individuals who consume it. Worker and queen mortality were quantified in greenhouse and field studies, and significant effects on worker mortality occurred at or above 10 ppb (Mommaerts et al. 2010, Gill et al. 2012). While no increased queen mortality was observed at these residue concentrations, reduced queen fecundity (number of eggs laid) was observed at or above 10 ppb (Mommaerts et al. 2010). Immature bees (brood) are fed syrup and pollen collected by foraging bees. Reduced brood numbers were shown at 10 ppb imidacloprid and in colonies foraging on non-irrigated white clover treated with imidacloprid. These changes in the amount of brood resulted in significantly fewer workers produced (Gill et al. 2012, Gels et al. 2002). Another study quantifying the adult bee population, showed reduced queen production when exposed to pollen contaminated with 6 ppb or more of imidacloprid (Whitehorn et al. 2012). In contrast, five other studies showed no effects to the number of queens reared (Tasei et al. 2001, Mommaerts et al. 2010, Morandin and Winston 2003, Franklin et al. 2004, Gels et al. 2002, Thompson et al. 2013). While reduced brood numbers were observed, reflecting the reduced fecundity in queens, brood that was produced was not ejected at 2 ppb (Mommaerts et al. 2010). No studies observed affects on the number of males produced (Whitehorn et al. 2012, Morandin and Winston 2003, Franklin et al. 2004, Gels et al. 2002). In addition, some studies measured weight of bees produced, although only one study showed reduced weights (Gels et al. 2002). The reduced bee weight shown by Gels et al. (2002) could be an artifact of the natural variation in bee size, which can vary between eight and tenfold Averill (2011), or could indicate change in nurse bee management of brood. In another measurement of colony health, colony weight, two studies showed significantly lower weights of treated colonies (Whitehorn et al. 2012, Gels et al. 2002).

Another set of studies looking at colony health used micro-colonies (queenless colonies consisting of three to five female *B. terrestris* workers that were not allowed to forage). Appendix 3 reviews literature on the effects of imidacloprid to *B. terrestris*

micro-colonies. Notable differences between full colony studies and micro-colony studies are discussed here. Micro-colonies consumed significantly less pollen and syrup at a range of doses as low as 1.27 ppb imidacloprid (Laycock et al. 2012). In contrast, Tasei et al. (2000) showed no affect on pollen and syrup consumption at treatments above 6 ppb. Similar to full colony studies, significant increases in worker mortality were observed in micro-colony studies (Tasei et al. 2000, Mommaerts et al. 2010). Because micro-colonies consist only of workers, only unfertilized eggs can be laid, resulting in males. The amount of brood produced was significantly reduced starting at 1.27 ppb, a lower concentration than was observed in full colony studies (Tasei et al. 2000; Laycock et al. 2012). Further work by Laycock et al. (2012), showed worker fecundity was reduced as much as 42% at 1.27 ppb. Despite these results, the number of workers with mature oocytes and the size of oocytes were only significantly different from controls at 159 ppb, indicating a behavioral rather than a physiological effect (Laycock et al. 2012). In contrast to full colony study results, the number of males produced was significantly affected starting at 10 ppb (Mommaerts et al. 2010). Further effects on brood rearing were quantified by Tasei et al. (2001), who showed that treated micro-colonies do not eject as many larvae as control colonies, potentially indicating altered behavior of nurse bees.

The gap in knowledge

The use of neonicotinoid insecticides have become ubiquitous within agriculture and landscapes. While the effects of neonicotinoid use have been well documented for their effects on bumblebees within agriculture, little information is available for the effects of neonicotinoid use in landscapes on bumblebees. The objectives of my research were to address the knowledge gap for neonicotinyl residue commonly found in landscapes and to quantify the effects on *B. impatiens* health. My study evaluated four concentrations (10, 20, 50, 100 ppb) of the two most commonly used neonicotinyl insecticides, imidacloprid and clothianidin. During an 11 week greenhouse study, effects on queens, colony health, and worker behavior were quantified. I found that imidacloprid and clothianidin reduced colony consumption for all treatments and significantly reduced worker movement between 20-100 ppb. Reduced worker efficacy lead to workers storing

less syrup and adding less biomass to colonies. This caused nest bees (queen and young workers) to rely on syrup stored prior to neonicotinoid treatment and eventually resulted in premature queen mortality due to lack of stored syrup. Nutrient limitations and early queen death also resulted in fewer eggs and males produced.

This is the first study to use full queenright bumblebee colonies and observe the effects of imidacloprid and clothianidin at a range of realistic residues found in agriculture and landscapes over a colonies life cycle. Understanding the mechanism by which chronic exposure of neonicotinoids effects bumblebee colonies is an important information gap which may be used by government organizations and stakeholders to make informed decision on acceptable neonicotinoid application rates and uses. Through this process we may be able to reduce the harm to wild and managed bees and improve pollination and yield of the many plants that are associated with bees.

Chapter 2: Chronic exposure of imidacloprid and clothianidin reduce queen survival, foraging and nectar storing in colonies of *Bombus impatiens*.

Introduction

Honey bees, bumblebees, and other native bees pollinate 30% of the plants that produce the vegetables, fruits, and nuts that we consume (Klein et al. 2007). According to the Department of Agriculture (USDA), more than 100 crops in North America require pollinators (Vaughan and Black 2007). Pollination contributes approximately $15 billion worth of additional crop yields (Morse and Calderone 2000), and wild bees contribute substantially to crop production (McGregor 1976, Garibaldi et al. 2013).

In 2007, there were 49.5% fewer managed honey bees (*Apis mellifera*) colonies in North America than in 1961 (vanEngelsdorp and Meixner 2010). Managed honey bee colony mortality was estimated as 30% since 2007 (USDA 2011, vanEngelsdorp et al. 2013). Colony stressors include habitat loss, nutrient deficiencies, *Nosema* pathogens (Higes et al. 2007, 2009), viruses (Cox-Foster et al. 2007), *Varroa* mites (vanEngelsdorp et al. 2013), pesticide exposure (Johnson et al. 2010, Frazier et al. 2008, 2011), interactions between disease and insecticides (Alaux et al. 2010, Petits et al. 2012), and *Nosema* and fipronil (Vidau et al. 2011, Aufauvre et al. 2012). Additionally, North American bumblebee species *Bombus occidentalis B. pensylvanicus* and B. *affinus* are in

decline. These species had significantly higher *N. bombi* loads and lower genetic diversity compared to healthy populations (Winter et al. 2006,Cameron et al. 2010). A combination of factors is most likely to contribute to bee losses (Fraiser et al. 2011, Blacquiere et al. 2012).

The neonicotinyl insecticides, imidacloprid, thiamethoxam, clothianidin, and dinotefuran, were implicated in the decline of bees as they accumulate in pollen and nectar, are systemic, and are expressed up to years from a single application (Doering 2004, 2005, EPA 2007, Blacquiere et al. 2012). Neonicotinyls are applied in various ways (seed treatments, soil drenches, foliar sprays, irrigation systems, tree injections) on agricultural and landscape plants. Most genetically modified crops (corn, canola, and soybeans) use seed treatments of imidacloprid (Gaucho), clothianidin (Poncho), or thiamethoxam (Crusier) (Baldwin 2003). The annual market for neonicotinyl insecticides is in the billions of dollars due to their low mammalian toxicity, systemic nature, and extended efficacy (Aliouane et al. 2009). In the U.S., at least 143 million acres of the total 442 million acres of cropland are treated with over 2 million pounds of imidacloprid, clothianidin, and thiamethoxam (Pilatic 2012). In 2009 in Minnesota, where most crops use seed treatments, corn, soybeans, potatoes and canola used 46,766 pounds of imidacloprid and 19,347 pounds of clothianidin (MDA 2012).

Residue levels of neonicotinoids in pollen and nectar differ depending on application method in crops and landscapes. From these seed treatments, neonicotinyl insecticide residues occur in pollen and nectar. Gaucho, an imidacloprid seed treatment of ≤1.0 mg AI/seed depending on the crop (Bonmatin et al. 2005, Girolami et al. 2009) resulted in 4.4-7.6 ppb imidacloprid residue in canola pollen, 3 ppb in sunflower pollen, and 3.3 ppb in maize pollen (Scott-Dupree and Spivak 2001, Bonmatin et al. 2005, EFSA 2012). The field crop pumpkin treated with an imidacloprid soil drench, resulted in 122 ppb in pollen and 18 ppb in nectar (Dively and Kamal 2012). In fact, landscape applications of imidacloprid result in much higher levels of residue in nectar and pollen. A homeowners' formulation of imidacloprid, Bayer Advanced Tree and Shrub, or professional Marathon 1% G permits 270-300 mg AI to be applied to a 3 gallon pot, resulting in a 400 times higher application rate compared to Gaucho treated corn of 0.675

mg AI/seed. Doering et al. (2005) found 1,038–2,816 ppb in *Cornus* spp., dogwood flowers, 17 months after application. Thus, the potential for neonicotinyl insecticides to impact bee health through chronic exposure may be underestimated if residue levels are higher than reported for seed treatments.

Neonicotinyl insecticides are neurotoxins that affect mechanosensory stimuli, vision, olfaction, learning, and memory (Gauthie 2010, Tome et al. 2012). Additionally, neonicotinoids bind to mushroom bodies in bee brains (Tome 2012) which are particularly large in social bees compared to other insects, comprising over 40% of the neurons in the honeybee brains (Rossler and Groh 2012). At 2.5 ppb imidacloprid or clothianidin affected the Kenyon Cells (KC), excitability and inhibited action potential firing, which impaired mushroom body function (Palmer et al. 2013). The effects of cholinergic pesticides on KCs are expected to lead to significant impairment of all cognitive functions that depend on this higher-order brain region, including multisensory integration, associative learning and memory, and spatial orientation.

Neonicotinoids are able to affect behavioral performance in honey bees (Lambin et al. 2001, Decourtye et al. 2003, 2004). Sublethal exposure of honey bees to neonicotinoids significantly impairs olfactory learning in laboratory-based studies (Decourtye et al. 2003, 2004) and adversely affects navigation and foraging behavior in the field (Iwasa et al. 2004, Blacquiere et al. 2012, Henry et al. 2012, Gill et al. 2012, Schneider 2012). A recent paper by Willamson and Wright (2013) found that bees fed 13 ppb or 23 ppb imidacloprid were less likely to form long-term memory and had reduced learning. Eiri and Nieh (2012) determined that foragers fed 0.21 ng/bee or 24 ppb imidacloprid produced significantly fewer waggle dance circuits (10.5- and 4.5-fold fewer for 50% and 30% sucrose solutions, respectively) 24 h later as compared to 0 ppb treatments. Waggle dancing can significantly increase colony food intake, and a sublethal dose may impair colony fitness.

Field studies on the effects of residue in pollen and nectar from seed treatments usually showed no effects on colony health of honey bees and bumblebees. A study on queenright colonies of *B. terrestris* for 4 weeks in the field near imidacloprid seed-treated sunflowers found no difference in worker or queen production (Tasei et al. 2001).

Queenright colonies of *B. terrestris* for 9 weeks in the field near untreated and imidacloprid and clothinaidin seed-treated canola showed no effects on queen mortality or colony weight (Thompson 2013). Honey bees exposed to flowering canola (maximum of 2.24 ppb in nectar and 2.59 ppb in pollen) *Brassica napus*, grown from clothianidin-treated seed for 4 months showed no differences in mortality, worker longevity, or brood development from controls (Cutler and Scott-Dupree 2007).

Field and cage studies that exposed bees to higher amounts of neonicotinyl-treated sugar syrup have been repeatedly shown to reduce colony health and bee foraging. A 4 week study with queenright *B. terrestris* found that 10 ppb imidacloprid in sugar syrup reduced brood production by 23% and worker production by 27%, but did not increase queen or worker mortality or reduce colony weight. However, 50% of the workers got lost when foraging and were less efficient pollen collectors (Gill et al. 2012). In a 2 week study in the greenhouse in flight cages with queenright colonies of *B. terrestris,* bumblebees were fed 10 ppb and 20 ppb imidacloprid in sugar syrup which reduced worker survival by 62% and 95%, respectively, and produced no brood (Mommaerts et al. 2010). Whitehorn (2012) showed that queenright colonies of *B. terrestris* fed 0.7 and 1.4 ppb imidacloprid in sugar water for 2 weeks and then monitored in the field for 6 weeks, could not recover from imidacloprid effects, colony weight was lower by 8% and 12% and queen production by 85% and 90% respectively compared to controls. Foraging was reduced at 10 ppb imidacloprid for *B. terrestris* (Mommaerts et al. 2010, Gill et al. 2012) and 30 ppb imidacloprid for *B. impatiens* (Morandin and Winston 2003). Honeybee foraging was reduced at 15 ppb imidacloprid, 5 ppb clothianidin (Schenider et al. 2012), and 67 ppb thiamethoxam (Henry et al. 2012).

The objectives of this study were to investigate the effects of higher residues of imidacloprid and clothianidin, similar to those found in some crops and landscape plants, on individual behavior and colony health of the American bumblebee, *Bombus impatiens* Cresson by monitoring: 1) queen health (mortality and movement), 2) worker behavior (worker movement, colony and bee consumption of sugar syrup), 3) colony health (colony weight, weight and number of wax pots containing stored sugar syrup, dead and alive brood, bees produced by caste, bees on nest, and worker bee weight).

Methods

Bumblebee colonies

We obtained commercially-reared *Bombus impatiens* (research grade A colonies) consisting of a queen with 30-50 workers housed in a 25.4 X 22.9 X 12.7 cm plastic brood box from Koppert Biological Systems (Howell, MI). Colonies were fed Bee Happy sugar syrup (Koppert Biological Systems, Howell, MI) in the brood box. Once received, we assessed colonies for the presence of the queen and number of workers by placing the plastic brood box into a 2-sleeve BioQuip (Rancho Dominguez, CA) rearing cage 35.6 X 35.6 X 61 cm under 2-100 watt red lights (Industrial Performance, Lenexa, KS) as bees cannot see in this wavelength. In addition, 15 psi CO_2 (20 pound carbon dioxide tank) was applied through a hose directly onto the colony, further reducing movement. We then removed all bees from the colony with a forceps (wide tip featherweight, BioQuip) and placed them into 30 mL wide mouth plastic vials and weighed the colony to the nearest gram (Taylor 3839 Glass Digital Diet Scale). The bees and nest were placed into a modified brood box with a Plexiglas lid (21.6 X 17.8 X 0.6 cm), which allowed for weekly photographs of the colony. The brood box was connected to a 29 cm square flight box (Bug Dorm 1, Bio Quip, Rancho Dominguez, CA) by a 1.9 x 30.5 cm plastic tube.

Colonies were established on benches in the greenhouse with temperature controlled to 22 C (Wadsworth Control System, STEP 50A) and humidity controlled to 60% (Aqua Fog Turbo XE). Additional environmental adjustments were made manually to temperature using fans to increase air circulation, and to humidity using a garden soaker hose placed underneath a greenhouse bench. Temperature and humidity were monitored with two data loggers (EL USB -1, Omega Engineering, Stamford, CT).

Supplemental pollen rolls were placed on the floor of the brood boxes once a week. The supplemental pollen was collected in 2010 from honey bee colonies and stored in a -20°C freezer. It was mixed with Bee Happy to create a paste which could be molded into 7 X 1 cm rolls and coated with bees wax (Revlon Paraffin Spa RVS1213) and stored at -20°C.

In the flight box, colonies were fed 50% sugar syrup from 118 ml round containers (Gladware) with a lid that was modified with a 2 cm hole through which a

Koppert polyester wick was threaded. Bees were fed untreated sugar syrup for 2 weeks prior to the start of the study. The syrup was replaced 3 times per week.

Experimental design

Colonies were provided imidacloprid or clothianidin in 50% sugar syrup for 5 treatments (0, 10, 20, 50, and 100 ppb) for 11 weeks. The experiment was performed twice for each neonicotinyl for a total of n = 8 or 9 colonies/dose/neonicotinyl (imidacloprid: July 6 to September 15, 2011 and September 14 to November 23, 2011; clothianidin: January 18 to March 30, 2012 and March 12 to May 25, 2012).

Sugar syrup (50%) was made by adding granulated beet sugar (1000g) (Cargill, Renville, MN) to 1000 mL deionized water. Analytical grade imidacloprid and clothianidin (Fischer Scientific, West Chester, PA) (PS-2086, Lot no: 446-128B, 99.5 percent and PS-2261, Lot no:463-125A, 98.4 percent, respectively) were made into a 100,000 ppb stock solution by adding 0.02 grams (Sartorius ED323-CW milligram balance) into 200 mL of the sucrose solution (Fisher Scientific stirring hotplate 18 X 18 cm). Dilutions of 10, 20, 50, 100 ppb were made by pipetting 33.5, 67, 167.5, and 335 µL stock solutions (20-200 µL VWR Signature™ Ergonomic High Performance Single-Channel Variable Volume Pipettor) into bottles (PYREX Low Actinic 1L Round Media Storage Bottles) filled with 335 mL of 50% sugar syrup solution and stored at 5.5° C. Stock solutions were made every 3 weeks and sugar syrup solutions were made weekly.

Residue analysis: Validation of imidacloprid and clothianidin in sugar syrup and stored nectar

Treated sugar syrup (0, 10, 20, 50, 100, and 100,000 ppb (stock)) samples were stored in 20 mL glass scintillation vials. Stored syrup extracted from all the wax pots in one colony was weighed and stored in 2 X 5 mm (2 ml) plastic microcentrifuge tubes. Both stock and extracted syrup samples were kept at -80°C until shipped on dry ice to USDA, AMS, Gastonia, NC. Treatments of sugar syrup samples from two dates (imidacloprid, n = 12, August 26, 2011 and October 5, 2012; clothianidin n = 12, October 5, 2012 and April 5, 2012) and stored sugar syrup in wax pots from three different colonies per treatment (n = 13 imidacloprid and n =15 clothinaidin) were analyzed using the standard USDA NSL method (USDA,AMS,Gastonia, NC).

Effect of chronic dose on queen mortality and movement

Once a week, queen status (alive, dead, or absent) was recorded. Activity within each brood box was video recorded twice for 30 mins during weeks 4 and 8 (Bullet camera, Sony micro 550, NS 03-BU 4000HB, 12v, Recorder PV 1000, Lawmate, Stunt Camera, Grand Rapids, Michigan). From these videos, the movement of five workers and the queen were quantified by counting the number of seconds each bee moved in 300 seconds.

Effect of chronic dose on worker behavior

Every week (0-11), syrup consumption per colony was measured three times a week by pouring the remaining sugar syrup into a graduated cylinder. Individual bee consumption was estimated by dividing the mean weekly consumption by the number of bees on the nest.

Effect of chronic dose on colony health

At week 11, colony weights were taken with a digital scale and colonies were dissected. The number of wax pots containing stored sugar syrup was counted, and the syrup was transferred into 2 ml microcentrifuge tubes, weighed, and stored at -80C. Every week (0-11), a picture was taken of each colony (Nikon D100 camera, AF Nikon 28-105 mm macro lens) and pictures were analyzed for the number of wax pots containing stored sugar syrup and the number of bees on the nest (Microsoft Windows Paint, Windows 7 Enterprise). The number of stored sugar syrup pots added during the experiment was determined by subtracting the number of pots at week 0 from week 11.

When the queen died or at week 11 the colonies were dissected. The brood (eggs, larvae and pupae) was counted and categorized as dead or alive according to color; brood was considered alive if white and firm and dead if discolored. The original queen and daughter queens were differentiated from workers by size (Cnaani et al. 2002). Male bees were identified by the presence of a patch of yellow hair on the frons. At weeks 4, 6, and 8 bee weight was quantified by removing 20 foragers from the flight box of each colony. Bees were individually placed into 37 mL clear plastic solo cups on ice, individually weighed, painted on the dorsal thoracic sclerite, to ensure that a bee was not reweighted,

and replaced into the flight box. Every other week dead bees were removed from the flight box, identified to caste, and frozen.

Statistical analyses

Cumulative queen mortality and number of wax sugar syrup pots was assessed with a Kruskal-Wallis, nonparametric Chi-Square test and a Wilcoxon nonparametric multiple comparison test (JMP Pro9, SAS Institute, 2012). Colony consumption, individual bee consumption, bees on nest, and bee weight were analyzed in Proc Mixed (SAS, version 9.2, SAS Institute, 2012) for treatment, week, and interactions, tested for homogeneity with a Levine test, transformed if needed, and assessed for treatment differences with a Tukey-Kramer HSD. If there was as significant interaction in Proc Mixed, then the data was analyzed with ANOVA's by week. Also, parameters for which data were available only at week 0 and week 11 were analyzed using ANOVA (JMP Pro9, SAS Institute, 2012), and analyzed with tests listed previously. If the Levene's test was significant after transformation, a Welch's test was used to correct for unequal variance.

Results

Residue

Residue in 100,000 ppb stock solution for imidacloprid (I) (I, 13% greater) and clothianidin (C) (C, 3% greater) were found to be slightly higher than what we calculated and also, varied among the 10-100 ppb stock treatments for unknown reasons (Table 3, 4). The neonicotinyl residue in stored syrup for 0 ppb treatments was measured as 0 ppb, but 20-100 ppb treatments imidacloprid and clothianidin, had 50-100% less residue than expected (mean of the stored syrup residue in the wax pots/mean of the residue in the original sugar syrup, % less residue: I: 10 ppb is 17% lower; 20 ppb is 51% lower; 50 ppb is 57% lower; 100 ppb is 100% lower; and C: 10 ppb is 42% higher; 20 ppb is 56% lower; 50 ppb and 100 ppb are 100% lower (Table 3, 4).

Effect of chronic dose on queen mortality and queen movement

Imidacloprid and clothianidin treatments did not demonstrate immediate toxicity to queens, but by week 6, an increase in queen mortality was observed. By week 11 for both imidacloprid and clothianidin, queen mortality was significantly lower in 0 ppb and

10 ppb treatments compared to 20 ppb (63% I, 44% C), 50 ppb (87% I, 75% C), and 100 ppb (100% I, C) treatments (Figure 1, Kruskal-Wallis, Wilcoxon Test, SAS, JMP, 2012). For both neonicotinyls, videos of queen movement revealed no significant differences among treatments (I, $F = 1.70$, $DF = 4, 21$, $p = 0.188$; C, $F = 1.55$, $DF = 4, 6$, $p = 0.298$, ANOVA, SAS JMP, 2012).

Effect of chronic dose on worker behavior

Videos of the nest box provided direct evidence that neonicotinyls reduced worker movement in the nest. For imidacloprid, bees in 0 ppb moved significantly faster than those in 20 ppb (47% slower) and 50 ppb (59% slower) treatments while 100 ppb was not different from 0 ppb. The lack of movement inhibition in 100 ppb workers may be because the few bees remaining in the colony had not been exposed to imidacloprid treatment. For clothianidin, bees in 0 ppb moved significantly faster than those in the 50 ppb (73% slower) and 100 ppb (68% slower) treatments (I: $F = 6.27$, $DF = 3, 25$, $p = 0.003$; C: $F = 5.60$, $DF = 4, 15$, $p = 0.006$, ANOVA, SAS JMP, 2012).

Colony consumption for imidacloprid and clothianidin, when analyzed by showed a significant interaction of week and treatment (Table 5, Figure 2, Proc Mixed, Tukey Kramer HSD, interaction effects, SAS, 2012). When colony consumption was analyzed by week (ANOVA, SAS JMP, 2012), significantly more sugar syrup was consumed each week in 0 ppb compared to 10-100 ppb imidacloprid or clothianidin treatments (I: week 2: 10-100 ppb consumed 32%, 64%, 86%, and 90% less, respectively; week 4: 20-100 ppb consumed 45%, 82%, and 89% less, respectively; week 6: 10-100 ppb consumed 45%, 64%, 71%, and 89% less, respectively; week 8: 10-50 ppb consumed 50%, 61%, and 88% less, respectively; C: week 2: 10-100 ppb consumed 26%, 60%, 79%, and 82% less, respectively; week 4: 10-100 ppb consumed 24%, 63%, 86% and 94% less, respectively; week 6: 10-100 ppb consumed 29%, 70%, 89%, and 93% less, respectively; week 8: 10-100 ppb consumed 40%, 80%, 92%, and 95% less, respectively).

Individual bee consumption was determined by dividing consumption per colony by the number of bees on the nest. For imidacloprid, individual bee consumption was not different between 0 ppb and all treatments (Table 5, Figures 3, Proc Mixed, Tukey Kramer HSD, treatment effects, SAS, 2012). When comparing weeks, week 6 had

significantly more consumption compared to weeks 2 and 4 (Proc Mixed, Tukey Kramer HSD, week effects, SAS, 2012). However, when individual bee consumption was analyzed individually by week (ANOVA, SAS JMP, 2012), week 2 had significantly more sugar syrup consumed in 0 ppb compared 10-100 ppb imidacloprid treatments (50%, 64%, 86%, and 86% less, respectively) and 20-100 ppb clothianidin treatments (61%, 80%, and 83% less, respectively). Week 4 had significantly more sugar syrup consumed in 0 and 10 ppb compared 20-100 ppb imidacloprid treatments (42%, 67%, and 100% less, respectively) and 20-100 ppb clothianidin treatments (51%, 78%, and 89% less, respectively).

Effect of chronic dose on colony health

Compared to 0 ppb, colony weight was reduced in 10-100 ppb imidacloprid and 20-100 ppb clothianidin treatments, weight of wax syrup pots was reduced in 50-100 ppb imidacloprid and 10-100 ppb clothianidin treatments, and number of wax syrup pots was reduced in 50-100 ppb imidacloprid and 10-100 ppb clothianidin treatments.

Colony weight at week 0 was the same for all treatments of imidacloprid or clothianidin. At week 11, colony weight was significantly greater in 0 ppb (350g) compared to 10-100 ppb imidacloprid treatments (23%, 35%, 47%, and 51% less, respectively) and was significantly greater in 0 ppb (412g) and 10 ppb (275g) compared to 20-100 ppb clothianidin treatments (69%, 74%, and 81% less, respectively) (Figure 4, ANOVA, SAS JMP, 2012).

Both neonicotinyls reduced weight of wax pots at week 11. The weight of stored syrup in wax pots was significantly greater in 0 ppb (11.3g), 10 ppb (7.6g), and 20 ppb (3.2g) compared to 50 and 100 ppb imidacloprid treatments (95% and 81% less, respectively) and was significantly greater in 0 ppb (53.3g) compared to 10-100 ppb clothianidin treatments (58%, 85%, 86%, and 96% less, respectively) (Figure 5, ANOVA, SAS JMP, 2012).

For imidacloprid the number of syrup pots added was significantly more in 0 (+1 pot) compared to 50 ppb (-19 pots, 2,000% less) and 100 ppb (-21 pots, 2,200 % less) treatments. For all clothianidin treatments the number of stored syrup pots added was significantly more in 0 ppb (173 pots) compared to 10 ppb (63 pots, 64% less), 20 ppb

(11pots, 94% less), 50 ppb (-8 pots, 105% less) and 100 ppb (-17 pots, 110% less) treatments (I: Chi-square test = 10.23, DF = 4, p = 0.0368; C: Chi-square test, F = 21.54, DF = 4, p<0.0002, Figure 6, Kruskal-Wallis, Wilcoxon Test, SAS JMP, 2012).

Neither neonicotinyl demonstrated toxicity to brood, as dead brood was not significantly different among treatments. However, at week 11 the number of alive brood was significantly greater in 0 ppb compared to 20-100 ppb imidacloprid treatments and 50-100 ppb clothinaidin treatments, reflecting premature queen mortality. Total brood (dead+alive) was significantly greater in 0 ppb compared to 50 and 100 ppb for both imidacloprid and clothianidin (Figure 7, ANOVA, SAS JMP, 2012).

For both neonicotinyls, worker and queen production were not significantly different among treatments. However the number of males produced was significantly greater in 0 ppb compared to all treatments of imidacloprid (0-100 ppb produced 135, 30, 23, 13, 4 males, respectively) and 50-100 ppb treatments of clothianidin (0-100 ppb produced 64, 48, 28, 3, 2 males, respectively) (Figure 8, ANOVA, SAS JMP, 2012).

For imidacloprid, the number of bees on nest was not significantly different among treatments, but number of bees on nest significantly decreased from weeks 2-6 (Table 5, Figure 9, Proc Mixed, Tukey Kramer HSD, week effects, SAS, 2012). However when weeks were individually analyzed, week 4 and 6 had significantly more bees on the nest in 0 ppb compared to 100 ppb treatments (ANOVA, SAS JMP, 2012). For clothianidin, the numbers of bees on nest when analyzed showed a significant interaction of week and treatment (Table 5, Figure 9, Proc Mixed, Tukey Kramer HSD, interaction effects, SAS, 2012). However, when weeks were individually analyzed only at week 6, were significantly more bees on the nest in 0 and10 ppb treatments compared 50 ppb and 100 ppb treatments (ANOVA, SAS JMP, 2012).

For imidacloprid, bee weight was not significantly related to treatment, but decreased significantly between week 6 and 8 (I: treatment: F = 2.20, DF = 4, 35, p = 0.0894; week: F = 8.76, DF = 2, 38, p = 0.0007, Table 5, Proc Mixed, Tukey Kramer HSD, week effects, SAS, 2012). For clothianidin, bee weight was significantly different by treatment (only 20 ppb was different than 0 ppb) and week (from week 4 to 6 weight decreased) (C: treatment: F = 5.58, DF = 4, 34, p = 0.0015; week: F = 4.53, DF = 2, 26, p

= 0.0161, Table 5, Proc Mixed, SAS, Tukey Kramer HSD, treatment and week effects, SAS, 2012).

Discussion

Neonicotinyl treatments used in this study ranged from the highest amount found in seed-treatments (10 ppb) to levels found in landscape plants (20-100 ppb). Our highest concentration of 100 ppb was below the estimated oral LC50 for honey bees of 185 ppb (CDPR 2009) or 192 ppb (Fischer and Chalmers 2007). Our study demonstrates that 20 ppb imidacloprid or clothianidin fed to queenright colonies of *B. impatiens* for 11 weeks increased queen mortality and reduced colony consumption, colony weight, and male production. Neither neonicotinyl decreased worker and queen production. At week 11 for both imidacloprid and clothianidin, queen mortality was significantly lower in 0 ppb and 10 ppb compared to 20-100 ppb. Both of these neonicotinyls had similar toxicity, as expected by their similar acute oral LD50s: for imidacloprid 40 ng/bee for honey bees (Decoutye and Devillers 2010, EFSA 2012) and 2 ng/bee for bumblebees (Van Der Steen 2008) and for clothianidin 22 ng/bee for honey bees (Iwasa et al. 2004, EFSA, 2012).

There is little data on the effects of neonicotinyl insecticides on queens, since most studies investigated the effects of neonicotinyls on queenless microcolonies containing only workers, but these studies found slight effects on worker survival. In 76 queenless microcolonies of *B. terrestris* exposed to imidacloprid at 10 doses from 0.08 ppb to 125 ppb, only one worker died in 125 ppb (Laycock et al. 2012). An 11 week study on *B. terrestris* in queenless microcolonies at 0 ppb, 10 ppb, 20 ppb and 200 ppb imidacloprid found that worker mortality was 0% at 0 ppb and 10 ppb, 50% at 20 ppb, and 100% at 200 ppb. Thiamethoxam at 0 ppb showed 0% compared to 85% worker mortality at 100 ppb (Mommaerts et al. 2010). Laboratory feeding tests with *B. terrestris* at 2 doses, 10 ppb in sugar syrup and 6 ppb in pollen, and 25 ppb in sugar syrup and 16 ppb in pollen, found that imidacloprid significantly reduced worker survival by 10% in 4 weeks at the 2 doses (Tasei et al. 2000).

In this 11 week study, colony health was quantified by the weight and number of wax pots containing stored sugar syrup and colony weight. In 0 ppb treatments, bees added wax to the colony to make sugar syrup pots, gathered sugar syrup from small

containers in the flight box, and filled the wax pots with sugar syrup, thereby increasing the number of stored syrup pots, the weight of the syrup pots, and the entire colony weight. In higher neonicotinyl treatments, the number of stored sugar syrup pots (50-100 ppb imidacloprid, 10-100 ppb clothianidin) and the total colony weight (10-100 ppb imidacloprid, 20-100 ppb clothianidin) were reduced. For both imidacloprid and clothianidin, the residue in stored syrup pots for 20-100 ppb was 50-100% less residue than the concentration in the syrup the bees were consuming (Table 3, 4), indicating that syrup was not being returned to the pots in higher treatments. In this study, lethargic behavior of flight box and nest bees resulted in reduced foraging and colony consumption. In higher neonicotinyl treatments, nest bees emptied the storage pots and did not fill old pots. Queen and nest bees fed on sugar syrup and Bee Happy stored prior to the start of the experiment. This is further supported by the reduction in colony consumption at 10-100 ppb. We speculate that nest bees that went into foraging boxes to collect neonicotinyl-treated sugar syrup were impaired as a result of ingesting and detoxifying the insecticides, fed less and returned less syrup to the colony.

Videos inside nest boxes showed that nest bees moved faster in 0 ppb imidacloprid compared to 20 and 50 ppb and in 0 ppb clothianidin compared to 50 ppb and 100 ppb. In addition to nest bees, older foraging bees at 20-100 ppb did not return sugar syrup to the nest, but sat impaired with little movement on the floor of the flight box and lived many weeks. In this study, individual foragers subjected to chronic, sublethal doses of neonicotinyls, showed toxicological symptoms, such as trembling and uncoordinated movement and did not store sugar syrup in pots. Our results demonstrated that above 20 ppb imidacloprid and clothianidin in sugar syrup consumption was reduced and at 50 ppb and 100 ppb worker movement on the nest was reduced. Reduced consumption and movement are factors associated with foraging. Other bumblebee greenhouse studies demonstrated reduction in foraging. When a bee consumes a neonicotinyl, symptoms such as knockdown, trembling, and uncoordinated and hyperactive movement occur quickly, before the insecticide is detoxified in 6 hours and the bee recovers or dies (Suchail et al. 2000, 2001, 2004). Thus, bees can recover from chronic, sublethal doses of neonicotinyl insecticides, feed, and start the syndrome again.

For honey bees, an imidacloprid dose of 5 ng/bee was transformed in 24 hrs into the metabolites 5-hydroxy-imidacloprid and olefin, before being detoxified by the bee (Suchail et al. 2000, 2001). For bumblebees, an imidacloprid dose of 4.8 ng/bee was transformed quickly so metabolites were not detected in the bee (Tassei et al. 2000).

Similar to our results, a greenhouse cage study on queenright microcolonies of *B. terrestris* provided imidacloprid-treated sugar syrup found that bees were lethargic and spent less time foraging. At 20 ppb, the workers stayed near the nectar and pollen, were apathetic, did not move or forage, and eventually died by the food, whereas at 10 ppb all dead workers were found inside the nests and at 2 ppb, there was no reduction in worker movement and no mortality (Mommaerts et al. 2010). Greenhouse cage studies with *B. terrestris* fed flowers from *Cucumis sativus* (cucumbers) sprayed with the 4 mg/sgft of imidacloprid found that the bees stopped foraging and sat still for several hours and recovered or died (Incerti 2003). In greenhouse cage studies, *B. impatiens* workers fed 30 ppb imidacloprid in 30% sugar syrup spent 43% more time accessing flowers and 28% more time foraging compared to 0 and 7 ppb (Morandin and Winston 2003). Tunnel studies with imidacloprid-treated sugar syrup at 6 ppb found reduced number of active honey bees, resulting in more inactive bees sitting at the feeders (Colin et al. 2004).

The reduction in bumblebee foraging due to neonicotinyl treated sugar syrup found in greenhouse studies was supported by field studies. Gill et al. (2012) found that bees fitted with RFID (radio frequency identification tags) and fed 10 ppb imidacloprid in sugar syrup for 4 weeks had significantly more workers (50%) that did not return to the colony and "got lost". Worker foraging performance, particularly pollen collecting efficiency, was significantly reduced which led to increased colony demand for food as shown by increased worker recruitment to forage and less time spend on brood care. Averill (2011) found that imidacloprid at 5 ng/bee impaired the ability of foragers to orient to landmarks when displaced away from their nests in the field. In the field, imidacloprid seed-treated sunflowers reduced *B. terretris* forager return by 10% (33% treated and 23% 0 ppb), although residue in pollen and nectar were unknown (Tassei et al. 2001). One of the few studies that used intact hives with queens or queenright colonies found that colonies of *B. terrestris* provided 6 ppb imidacloprid pollen plus 0.7 ppb

imidacloprid nectar and double the dose for 2 weeks then placed in the field for 6 weeks had reduced colony weights of 8% and 12% and reduced daughter queen production of 85% and 90%, respectively (Whitehorn et al. 2012). Reduced colony weight must be related to worker foraging and behavior.

In this study, *B. impatiens* did not show any differences in number of dead brood, indicating imidacloprid and clothianidin were not toxic to young bees, unless the brood were feeding on syrup stored before the start of the experiment. At week 11, significantly more total brood production was correlated to more alive brood at the end of the study due to greater queen mortality that occurred earlier in higher treatments. Daughter queen production was not significantly different among treatments (0 ppb produced 5.8 daughter queens and 100 ppb produced 4.1 daughter queens). However, the number of males produced was significantly lower in all imidacloprid treatments and 50 and 100 ppb clothianidin treatments. We argue that queen mortality at 20, 50, and 100 ppb was related to lack of syrup in storage pots. We speculate that as queens started to die at week 6, workers in 20-100 ppb treatments used their limited resources to produce new queens instead of males, since queen production was not reduced at higher doses, but male production was reduced. Others have considered a link between neonicotinyl insecticides and nutrition. Laycock et al. (2012) found that male production was negatively dose-dependent (0 to 125 ppb imidacloprid), but reduction in ovary development was found only at the highest dosage of 125 ppb imidacloprid. However, queenless microcolonies that consumed more syrup and pollen produced more brood. Higher imidacloprid doses reduced pollen and syrup feeding, so lack of nutrition was suspected as the mechanism behind reduced male production by workers (Laycock et al. 2012). Another greenhouse study on *B. terrestris* found similar effects of decreased feeding, increased foraging time, and increased drone production with neonicotinyl insecticides. Queenless microcolonies fed 0 ppb, 10 ppb, 20 ppb and 200 ppb imidacloprid had lower male production, workers feed and foraged less, and it took longer to fly between food and the nest (Mommaerts et al. 2010). Another study found that *Bombus impatiens* queenless microcolonies fed 19 ppb imidacloprid-treated pollen consumed significantly less pollen, had shorter worker

longevity, and produced no males compared to 0 ppb in the greenhouse (Gradish et al. 2010).

Our data provide strong support that above 20 ppb imidacloprid or clothianidin colony health are significantly reduced as a result of decreased consumption, movement, and storage of syrup, which supports other studies that demonstrate decreased foraging. Chronic effects of neonicotinyl insecticides occur as bees feed on treated crops and garden plants that flower throughout the growing season. Native, annual bee colonies are vulnerable to imidacloprid treatments at numerous times in their life history, such as when bumblebee queens fly in the spring and autumn and directly feed on treated plants rather than stored nectar in the nest. Reduction in consumption, foraging, memory, and navigation for a queen or worker can be fatal to the individual and the colony.

Tables:

Table 1: Imidacloprid residue found in seed, field, and ornamental treatments

Treatment	Crop	Residue (ppb)			Reference
		Nectar	Pollen	Flower	
seed treatment	maize		0.6		Bonmatin et al. 2005
seed treatment	sunflower	3.0	1.9		Bonmatin et al. 2005
seed treatment	sunflower		13		Laurent and Rathahao 2003
seed treatment	rapeseed	0.6-0.8	4.4-7.6		Bonmatin et al. 2005
soil drench, field	pumpkin	122	17.6		Dively and Kamal 2012
soil drench, ornamental	*Eucalyptus*	660			Paine et al. 2011
soil drench, ornamental	dogwood			1038-2816	Doering et al. 2005

Table 2: Neonicotinoid LD50 for honey bees and bumblebees

Chemical	Species	Exposure	LD50	Units	Reference
imidacloprid	*Apis mellifera*	Oral: Acute	41-81	ng / bee	Schmuck et al. 2001
imidacloprid	*Apis mellifera*	Oral: Acute	4-40	ng / bee	Decourtye and Devillers 2010
imidacloprid	*Apis mellifera*	Oral: Acute	4-41	ng / bee	Gervais et al. 2010
imidacloprid	*Apis mellifera*	Oral: Acute	18	ng / bee	Iwasa et al. 2004
imidacloprid	*Apis mellifera*	Oral w/Foraging	18.5	ng / bee	CDPR 2009
imidacloprid	*Apis mellifera*	Oral w/Foraging	19.2	ng / bee	Fischer and Chalmers 2007
clothianidin	*Apis mellifera*	Oral: Acute	3.8	ng / bee	EFSA 2012
clothianidin	*Apis mellifera*	Oral: Acute	22	ng / bee	Iwasa et al. 2004
thiamethoxam	*Apis mellifera*	Oral: Acute	5	ng / bee	EFSA 2012
thiamethoxam	*Apis mellifera*	Oral: Acute	30	ng / bee	Iwasa et al. 2004
imidacloprid	*Bombus terrestris*	Oral: Acute	2.3	ng / bee	Van der Steen 2008
imidacloprid	*Bombus terrestris*	Oral w/Foraging	2	ng / bee	Mommaerts et al. 2010
clothianidin	*Bombus terrestris*	Oral: Acute	NA	ng / bee	
thiamethoxam	*Bombus terrestris*	Oral: Acute	12	ng / bee	Mommaerts et al. 2010

Table 3: Imidacloprid residue (ppb) in sugar syrup stock and stored syrup in wax pots (USDA, AMS, Gastonia, NC). NA= no sample

	Imidacloprid							
	Sugar syrup stock (ppb) (% trt, syrup/ trt)			**Stored syrup in wax pots (ppb) (% syrup, pot/syrup)**				
Treatment (trt)	**8/26/12**	**10/05/12**	**mean**	**9/22/2012 to 12/02/2012**			**mean**	**percent changed in pots, mean pot/mean syrup**
0 ppb	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	no change
10 ppb	10 (100)	17 (170)	13.5 (135)	10.8 (108)	7.7 (57)	15 (111)	11.2 (83)	17% lower 11.2/13.5 ppb
20 ppb	20 (97)	11 (57)	15.5 (78)	6.1 (39)	10.7 (69)	6 (39)	7.6 (49)	1% lower 7.6/15.5 ppb
50 ppb	80 (161)	61 (122)	70.5 (141)	60.1 (85)	0 (0)	NA	30.5 (43)	57% lower 30.5/70.5 ppb
100 ppb	114 (114)	139 (139)	126.5 (127)	2.7 (2)	0 (0)	NA	1.4 (0)	99% lower 1.4/126.5 ppb
100,000 ppb	107000 (107)	118000 (118)	112500 (113)	-	-	-	-	

Table 4: Clothianidin residue (ppb) in sugar syrup stock and stored syrup in wax pots (USDA, AMS, Gastonia, NC). NA= no sample

	Clothianidin							
	Sugar syrup stock (ppb) (% trt, syrup/ trt)			**Stored syrup in wax pots (ppb) (% syrup, pot/ syrup)**				
treatment (trt)	**10/05/12**	**4/05/12**	**mean**	**3/15/12 to 6/05/12**			**mean**	**percent changed in pots, mean pot/mean syrup**
0 ppb	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	0 (100)	no change
10 ppb	0 (0)	10 (100)	5 (50)	7.5 (75)	5.8 (116)	7.9 (158)	7.1 (142)	42% higher 7.1/5
20 ppb	14 (69)	20 (98)	17 (85)	0 (0)	10.1 (59)	12.3 (72)	7.5 (44)	56% lower 7.5/17
50 ppb	34 (68)	43 (85)	38.5 (77)	0 (0)	0 (0)	0 (0)	0 (0)	100% lower 0/38.5
100 ppb	67 (67)	85 (85)	76 (76)	0 (0)	0 (0)	0 (0)	0 (0)	100% lower 0/76
100,000 ppb	968000 (97)	110000 (110)	103400 (103)	-	-	-	-	

Table 5: SAS, ProcMixed week, treatment, and interaction effects.

Chemical	Measurement	Assessment weeks	Week F (df), P	Treatment F (df), P	Interaction F (df), P	
Imidacloprid	**Colony consumption**	2, 4, 6, 8	1.91 (3,77), 0.1356	32.40 (4,35), <0.0001	2.35 (11,77), 0.0148	
	Individual bee consumption	2, 4, 6, 8	8.52 (3,76), <0.0001	1.59 (4,35), 0.1998	0.87 (11,76), 0.5698	
	Bee weight	4, 6, 8	8.76 (2,38), 0.0007	2.20 (4,35), 0.0894	0.41 (8,38), 0.9096	
	Bees on nest	0, 2, 4, 6, 8	21.43 (4,112), <0.0001	3.67 (4,35), 0.0135	1.34 (15,112), 0.1910	
	Fixed Effect	Treatment (ppb)	n	Mean	Standard error	Tukey Kramer
	Treatment	0	39	50.49	10.05	A
		10	40	41.90	9.39	A
		20	35	36.91	7.60	A
		50	29	39.07	8.37	A
		100	28	26.04	6.83	-
Clothianidin	**Colony consumption**	2, 4, 6, 8	1.72 (3,85), 0.1689	85.70 (4,36), <0.0001	2.76 (12,85),0.0032	
	Individual bee consumption	2, 4, 6, 8	3.53 (3,84), 0.0183	14.13(4,36), <0.0001	0.96 (12,84), 0.4918	
	Fixed Effect	Treatment (ppb)	n	Mean	Standard error	Tukey Kramer
	Treatment	0	36	1.13	1.36	A
		10	31	0.73	1.24	B
		20	31	0.44	1.00	BC
		50	23	0.24	0.60	C
		100	19	0.16	0.54	C
	Bee weight	4, 6, 8	4.53 (2,46), 0.0161	5.58 (4,34), 0.0015	1.96 (7,46), 0.0807	
	Fixed Effect	Treatment (ppb)	n	Mean	Standard error	Tukey Kramer
	Treatment	0	27	0.12	0.69	A
		10	24	0.13	0.75	AB
		20	23	0.15	0.91	B
		50	13	0.16	0.70	AB
		100	7	0.11	0.56	-
	Bees on nest	0, 2, 4, 6, 8	26.95(4,120), <0.0001	2.95 (4,37), 0.0328	3.99(16,120),<0.0001	

Figures:

Fig. 1 Cumulative queen mortality

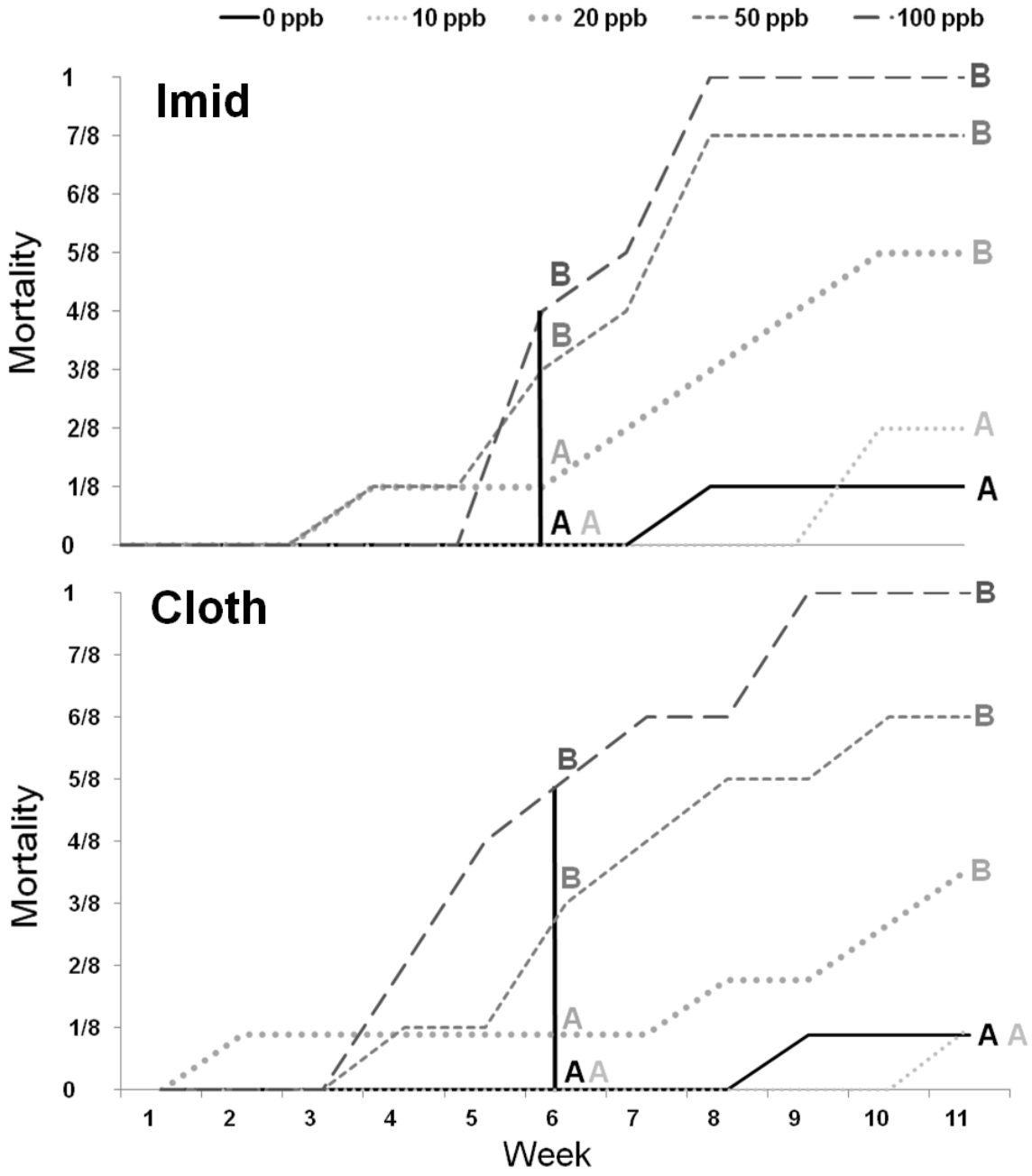

Fig. 2 Colony consumption

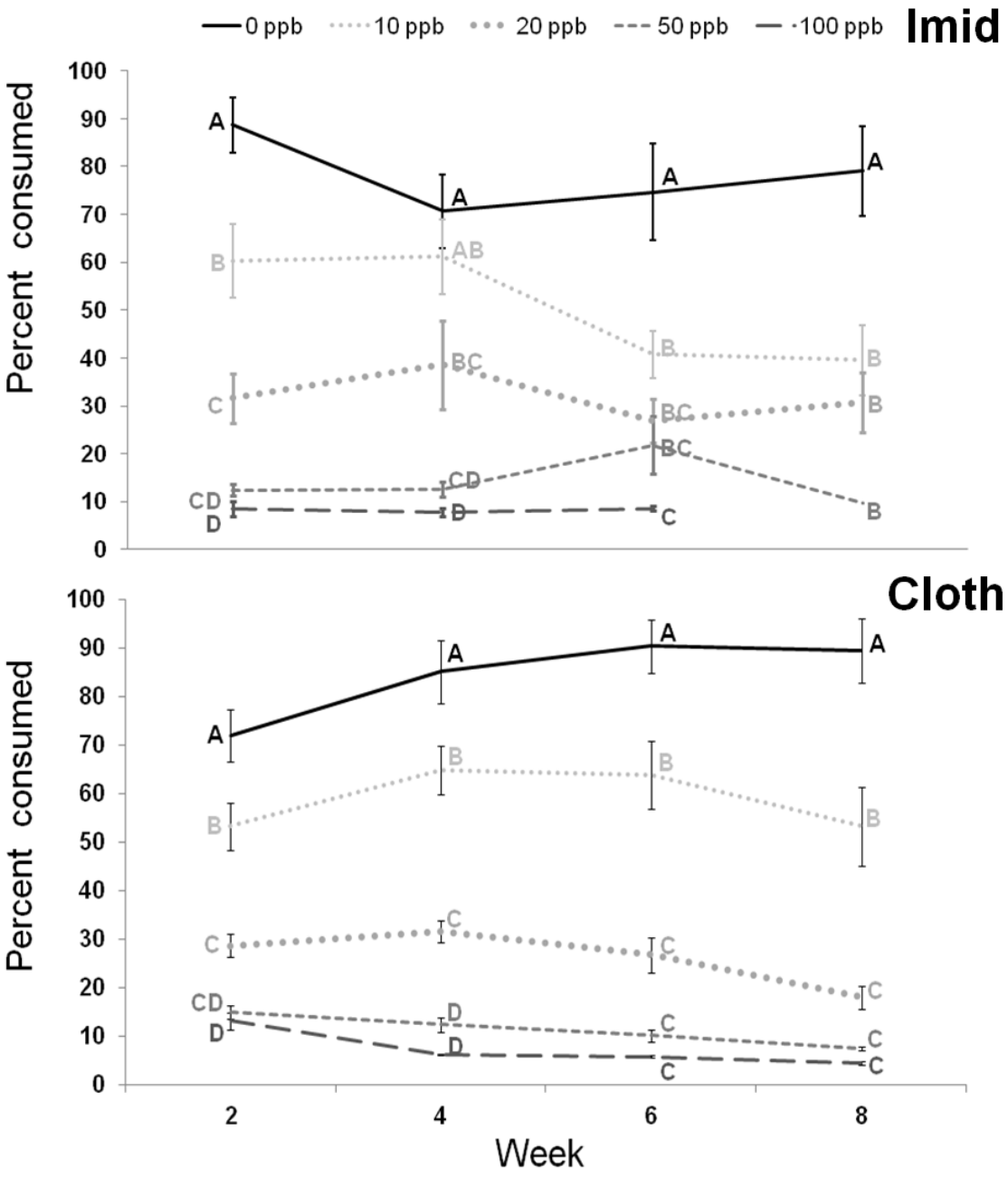

Fig. 3 Bee consumption

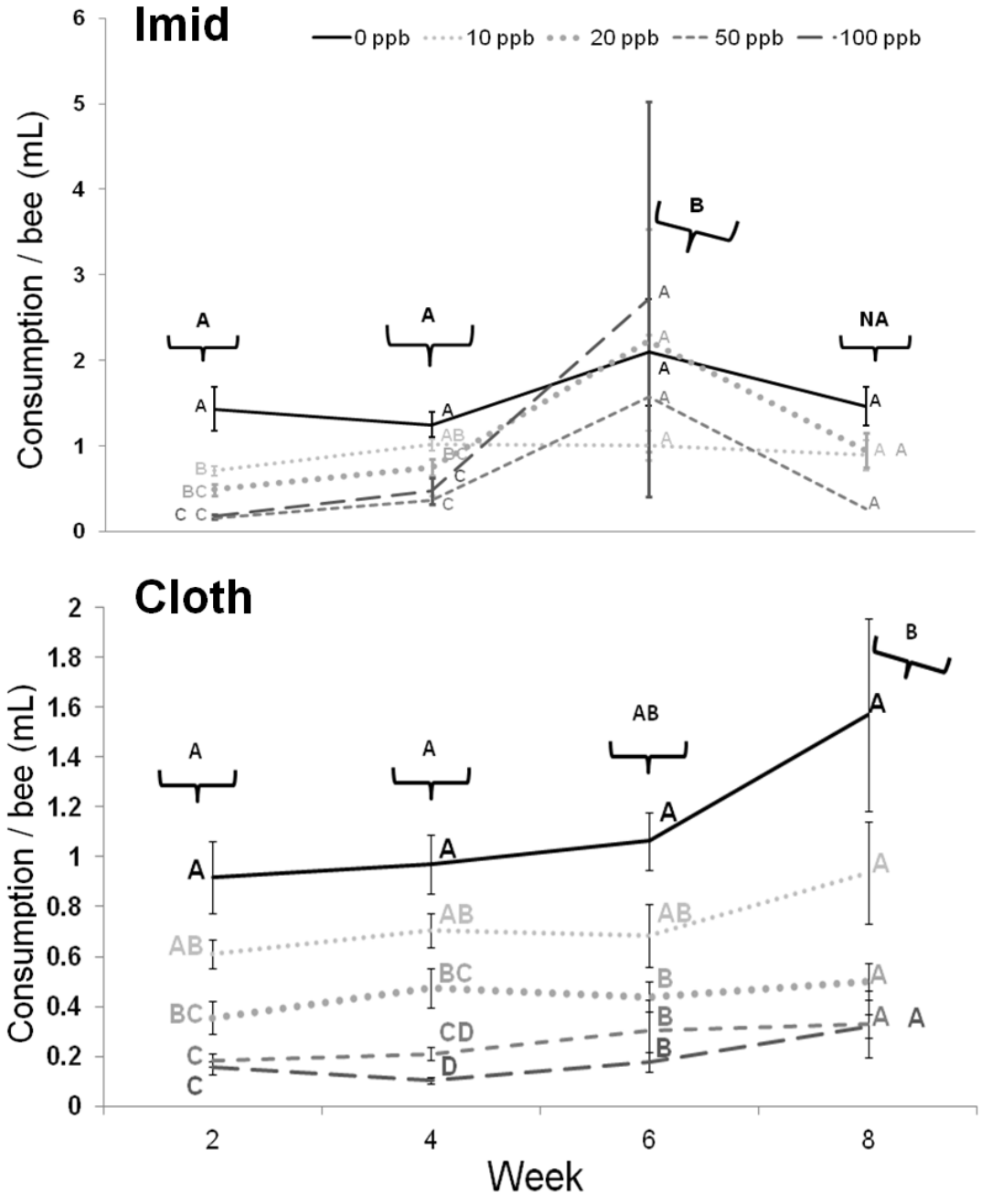

Fig. 4 Colony weight

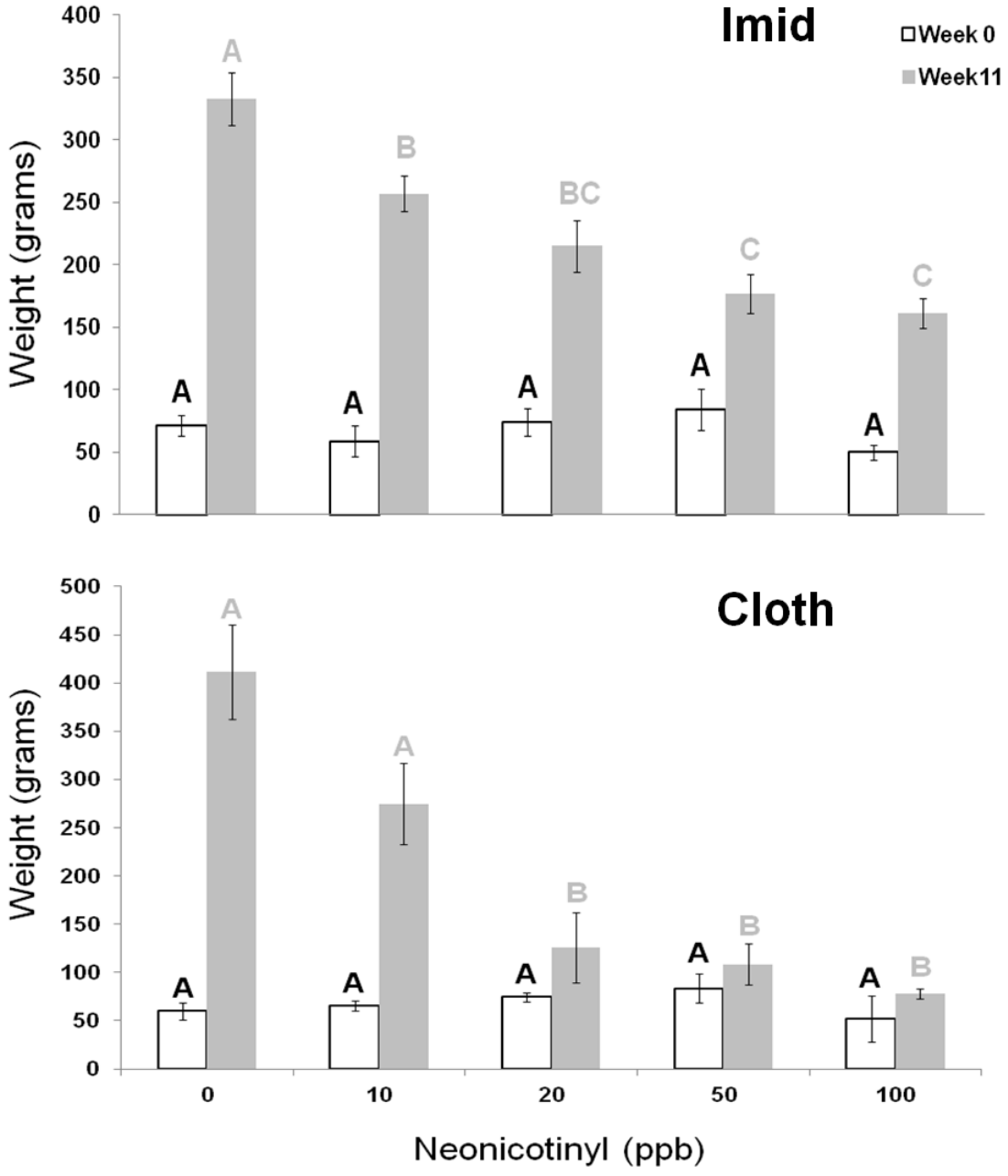

Fig. 5 Stored syrup weight

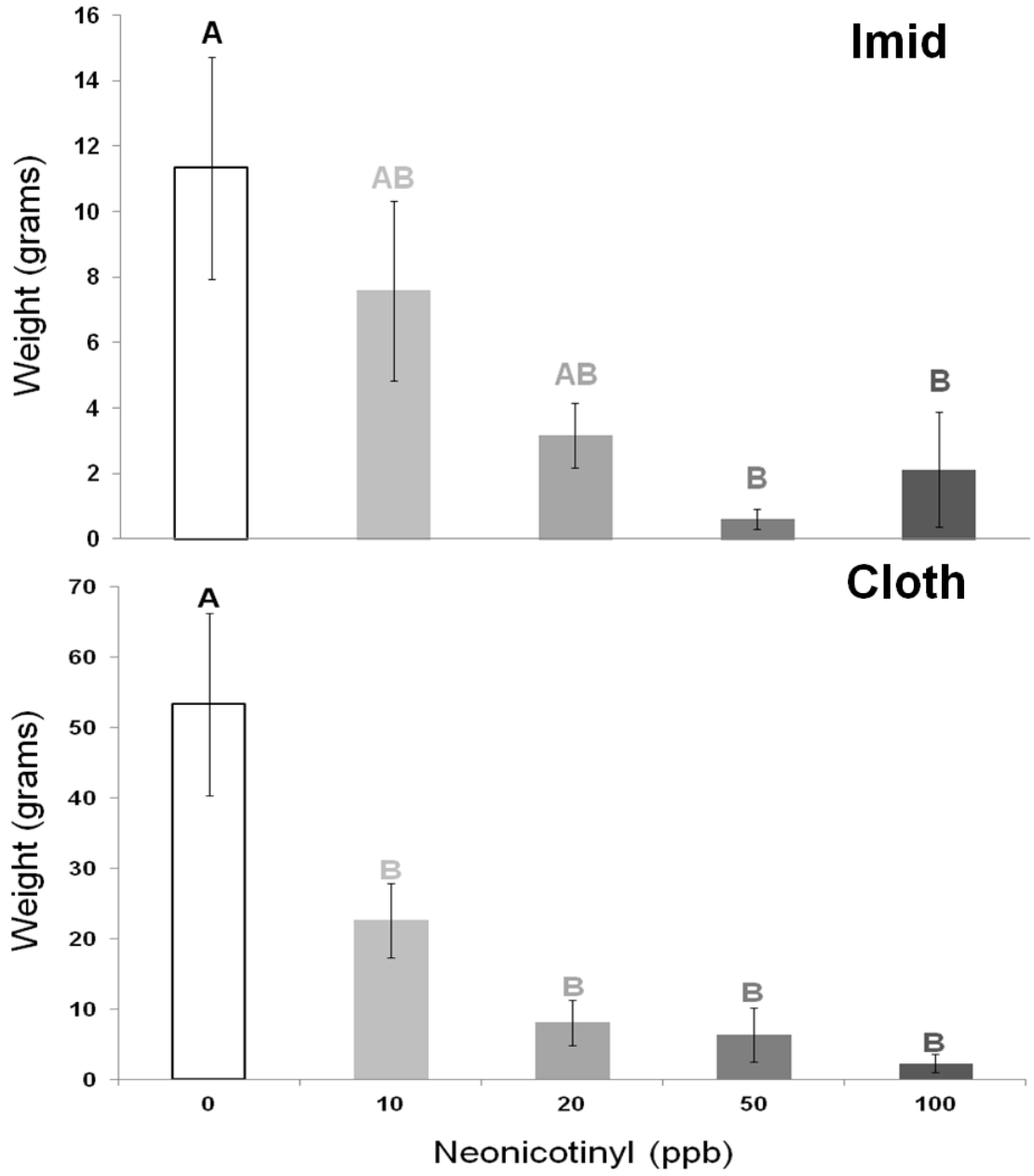

Fig. 6 Syrup pots added

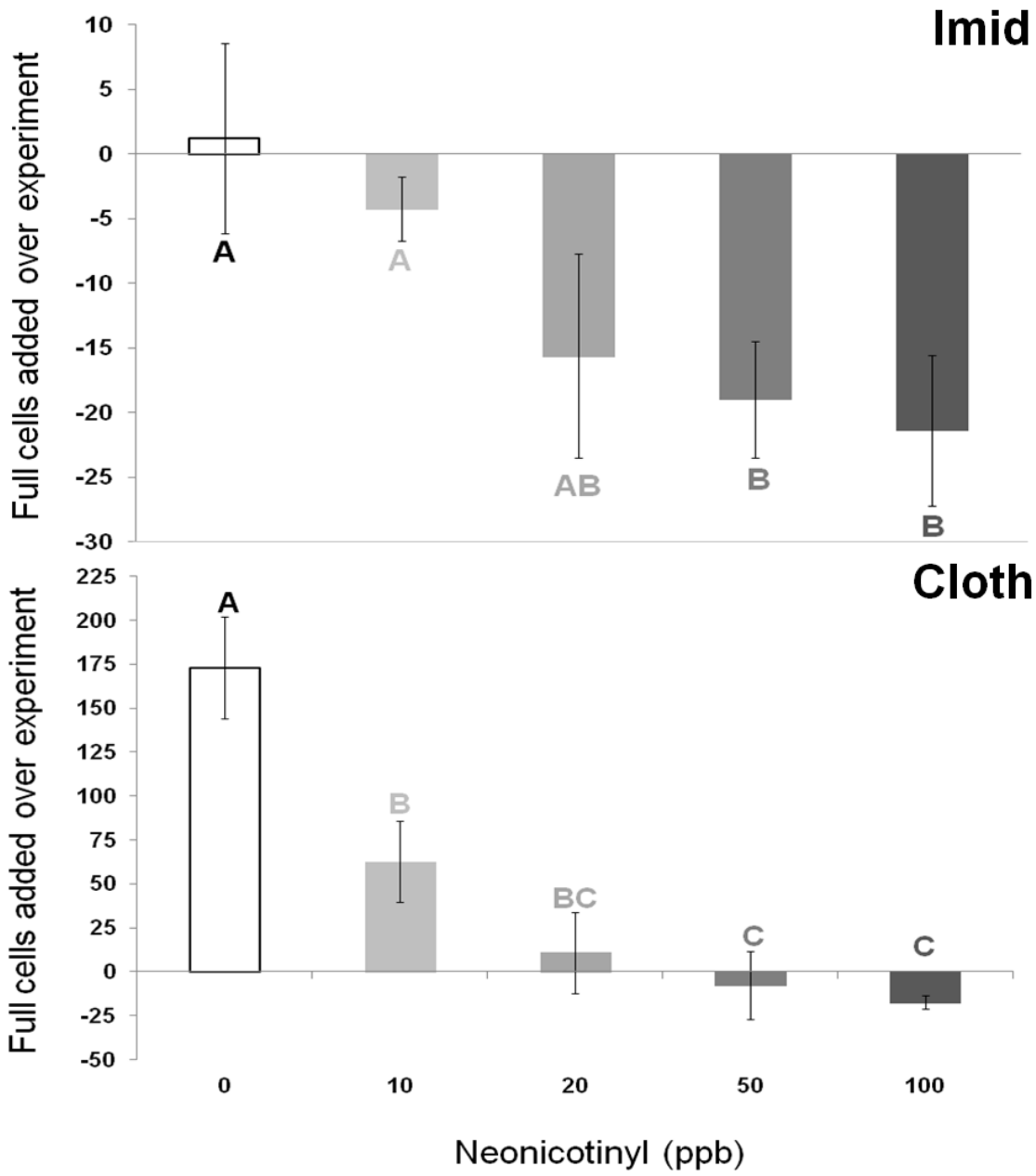

Fig. 7 Total, dead, and alive brood

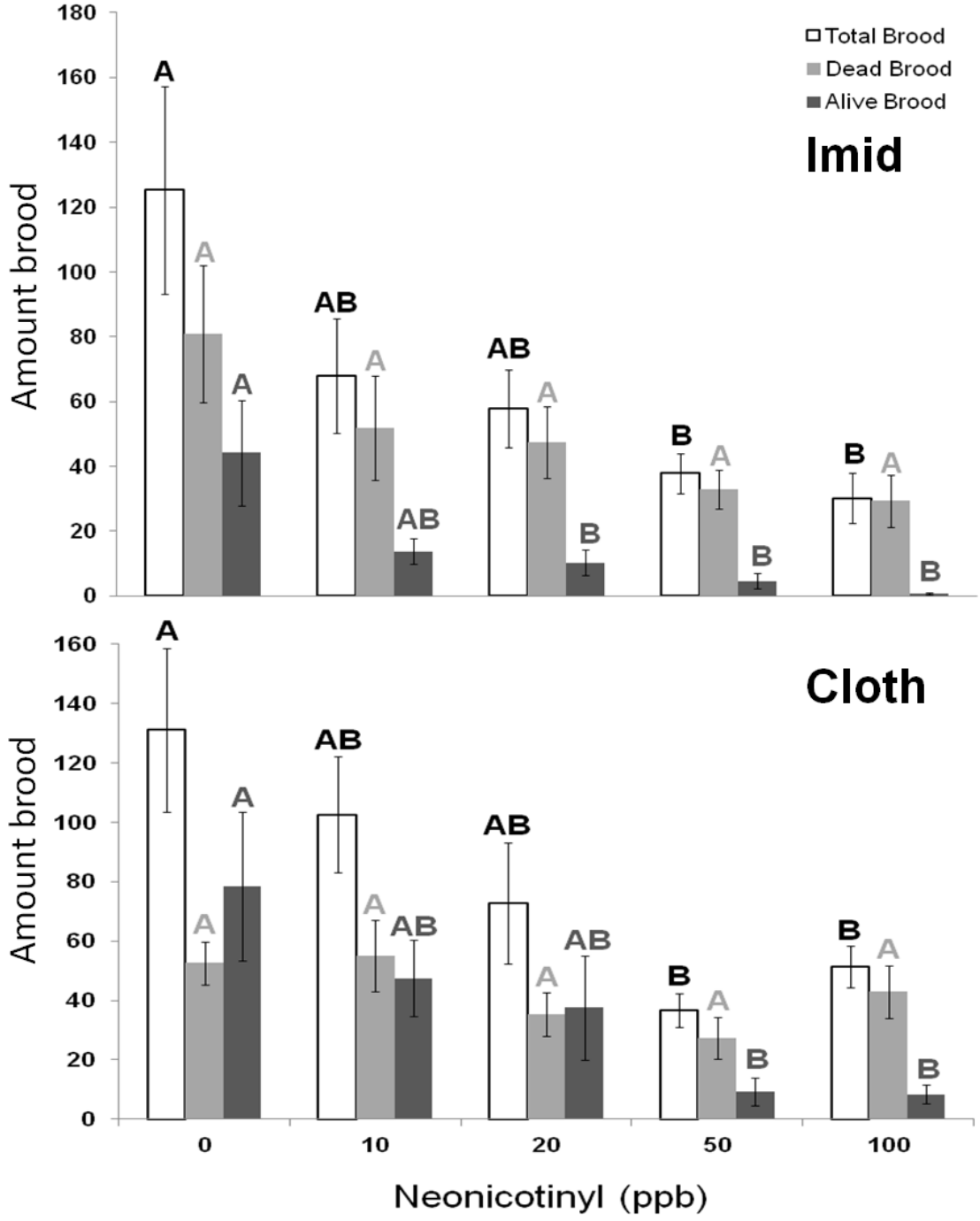

Fig. 8 Bees by caste

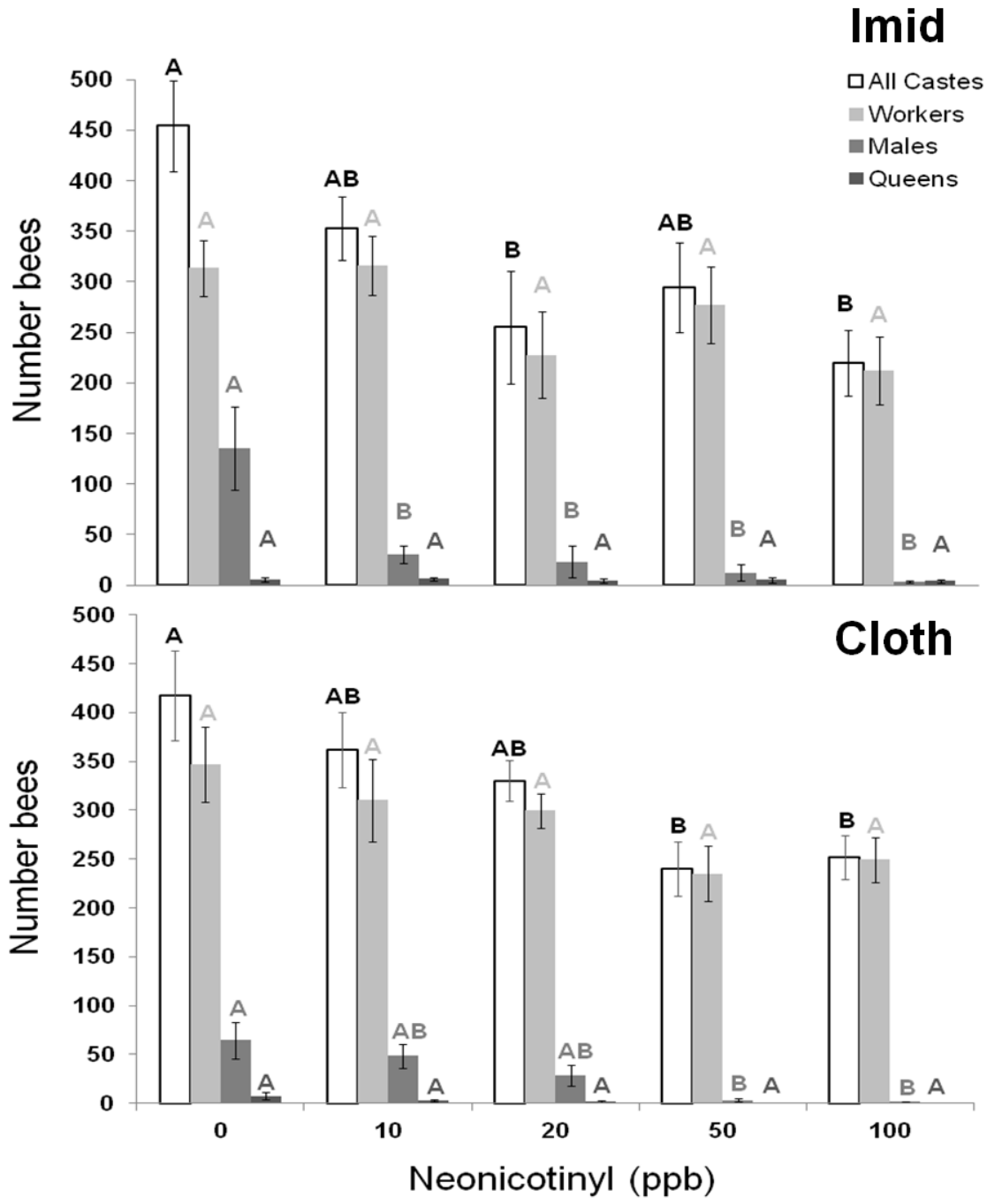

Fig. 9 Bees on nest

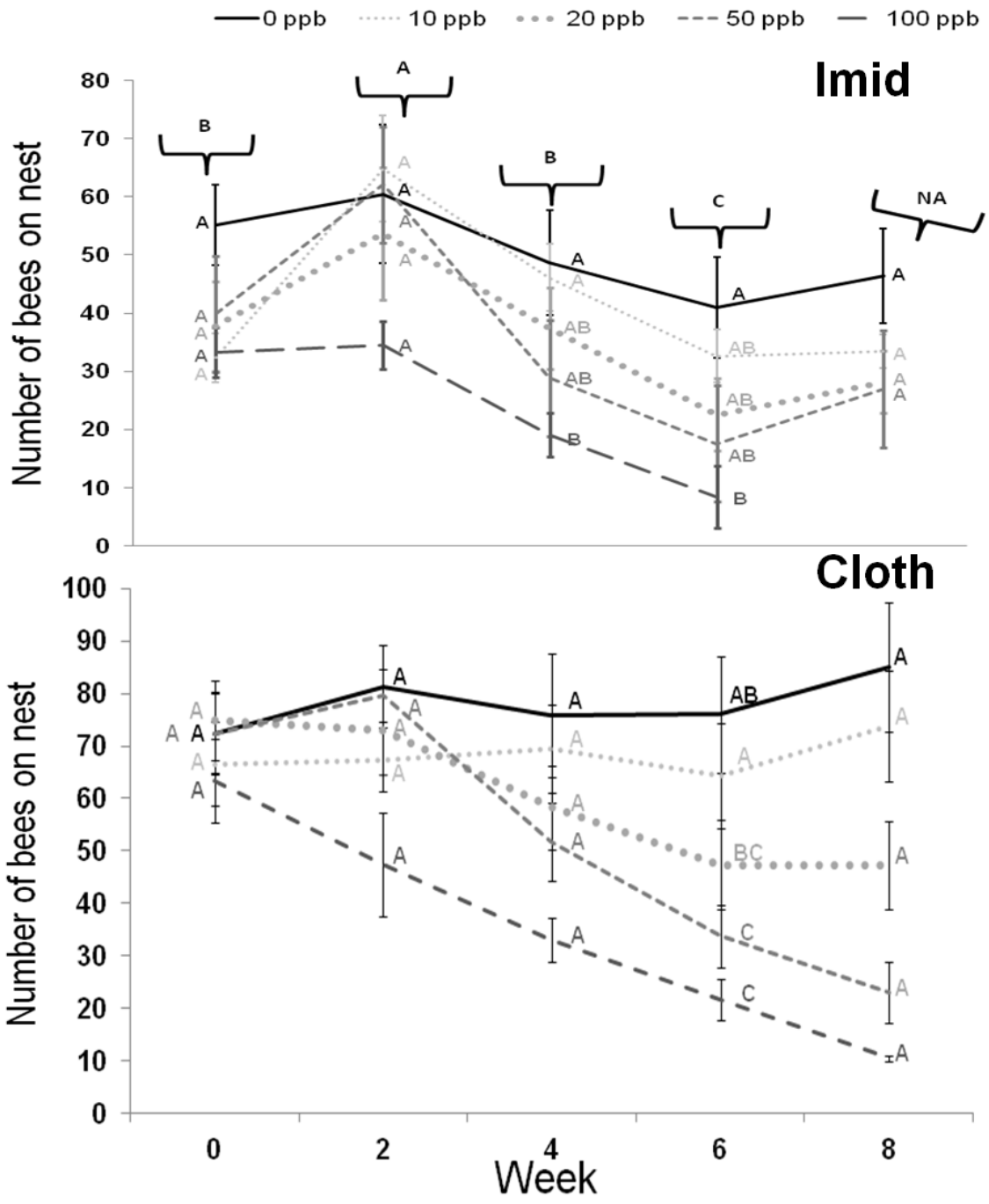

Figure legends

Figure 1. Cumulative queen mortality at weeks 1-11, Imidacloprid, Week 6: Chi-square test = 9.26, DF = 4, 235, $p<0.055$, week 11: Chi-square test = 75.49, DF = 4,435, $p<0.001$. Clothianidin queen mortality at weeks 1-11. Week 6: Chi-square test = 22.87, DF = 4, 247, $p<0.001$, week 11: Chi-square test = 102.78, DF = 4, 457, $p<0.001$, Kruskal-Wallis, Wilcoxon Test.

Figure 2. Colony consumption, Imidacloprid, Week 2: F = 52.51, DF = 4, 16, $p<0.001$, Week 4: F = 27.40, DF = 4, 14, $p<0.001$, Week 6: F = 22.61, DF = 4, 12, $p<0.001$, Week 8: F = 7.67, DF = 3,17, p = 0.002. Clothianidin, Week 2: F = 42.05, DF = 4, 17, $p<0.001$, Week 4: F = 91.96, DF = 4, 14, $p<0.001$, Week 6: F = 42.77, DF = 4, 28, $p<0.001$, Week 8: F = 48.52, DF = 4, 8, $p<0.001$, Proc Mixed, Tukey Kramer HSD due to interaction ANOVA.

Figure 3. Bee consumption, Imidacloprid, Week 2: F = 30.97, DF = 4, 16, $p<0.001$, Week 4: F = 10.31, DF = 4, 33, $p<0.001$, Week 6: F = 0.89, DF = 4, 8, p = 0.513, Week 8: F = 2.51, DF = 3, 17, p = 0.093. Clothianidin, Week 2: F = 17.68, DF = 4, 17, $p<0.001$, Week 4: F = 32.73, DF = 4, 15, $p<0.001$, Week 6: F = 9.37, DF = 4, 28, $p<0.001$, Week 8: F = 4.32, DF = 4, 8, p = 0.035, Proc Mixed, Tukey Kramer HSD and ANOVA.

Figure 4. Colony weight, Imidacloprid, Week 0: F = 1.84, DF = 4, 16, p = 0.170, Week 11: F = 16.20, DF = 4, 35, $p< 0.001$. Clothianidin, Week 0: F = 0.87, DF = 4, 37, p = 0.492, Week 11: F = 16.10, DF = 4, 37, $p<0.001$, ANOVA.

Figure 5. Stored syrup weight, Imidacloprid Week 11: F = 4.83, DF = 4,15, p = 0.011. Clothianidin, Week 11: F = 6.83, DF = 4, 16, p = 0.002, ANOVA.

Figure 6. Syrup pots added, Imidacloprid, Chi-square test = 10.23, DF = 4, p = 0.037. Clothianidin, Chi-square test = 21.54, DF = 4, $p<0.001$, Kruskal-Wallis, Wilcoxon Test.

Figure 7. Total, dead, and alive brood, Imidacloprid, Week 11: Total Brood: F = 2.99, DF = 4, 17, p = 0.049, Dead Brood: F = 1.67, DF = 4, 17, p = 0.205, Alive Brood: F = 5.74, DF = 4, 14, p = 0.006. Clothianidin, Week 11: Total Brood: F = 4.16, DF = 4,37, p = 0.007, Dead Brood: F = 1.83, DF = 4,37, p = 0.144, Alive Brood: F = 4.13, DF = 4,17, p = 0.016, ANOVA.

Figure 8. Bees produced by caste, Imidacloprid, Week 11: All Castes: F = 4.62, DF = 4, 35, p = 0.004, Workers: F = 1.92, DF = 4, 35, p = 0.129, Males: F = 4.59, DF = 4, 14, p = 0.014, Queens: F = 0.19, DF = 4, 35, p = 0.945. Clothianidin, Week 11: All Castes: F = 5.12, DF = 4, 37, p = 0.002, Workers: F = 2.15, DF = 4, 37, p = 0.094, Males: F = 7.44, DF = 4, 16, p = 0.002, Queens: F = 2.23, DF = 4, 37, p = 0.085, ANOVA.

Figure 9. Bees on nest, Imidacloprid, Week 0: F = 2.55, DF = 4, 35, p = 0.057, Week 2: F = 4.20, DF = 4, 17, p = 0.016, Week 4: F = 4.82, DF = 4, 16, p = 0.010, Week 6: F = 3.84, DF = 4, 12, p = 0.031, Week 8: F = 1.77, DF = 3, 17, p = 0.192. Clothianidin, Week 0: F = 0.39, DF = 4, 37, p = 0.813, Week 2: F = 0.21, DF = 4, 36, p = 0.928, Week 4: F = 2.16, DF = 4, 33, p = 0.095, Week 6: F = 4.52, DF = 4, 28, p = 0.006, Week 8: F = 8.29, DF = 4, 8, p = 0.005, Proc Mixed, Tukey Kramer HSD, when interaction ANOVA.

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Appendices:

Appendix 1: Neonicotinoid effects on *Bombus* spp. foraging

Species	Chemical	Trt length (days)	Treatment (ppb)		Foraging				Flower			Homing efficacy	Worker mortality
			pollen	syrup	pollen efficiency	bee #	trip #	time	# visited	specificity	handling time		
B. terrestris[1]	imidacloprid	26	Seed treated sunflower							ns	ns	ns	
B. impatiens[2]	imidacloprid	30	Irrigated Granular for grub: 0.004 mg AI/ft^2			ns							
B. impatiens[2]	imidacloprid	28	Non-irrigated WP (Spray) for grub: 0.003 mg AI/ft^2			-							
B. terrestris[3]	imidacloprid	14		2				ns					ns
B. impatiens[4]	clothianidin	77	6							ns	ns		
B. impatiens[5]	imidacloprid	84	7						ns	ns	ns		
B. terrestris[3]	imidacloprid	14		10									+
B. terrestris[6]	imidacloprid	28		10	-	+		+				-	
B. terrestris[3]	imidacloprid	14		20									+
B. impatiens[5]	imidacloprid	84	30						ns	ns	+		
B. impatiens[4]	clothianidin	77	36							ns	ns		
B. impatiens[7]	imidacloprid	1		50								-	
B. terrestris[6]	imidacloprid + λ-cyhalothrin	28	10 imidacloprid + 37,500 λ-cyhalothrin		-	+	-	+				-	+

Tasei et al. 2001[1], Gels et al. 2002[2], Mommaerts et al. 2010[3], Franklin et al. 2004[4], Morandin and Winston 2003[5], Gill et al. 2012[6], Averill et al. 2011[7]
Significant decrease (-), Significant increase (+), Not significantly different from control (ns)

Appendix 2: Neonicotinoid effects on *Bombus* spp. queen-right colony health

Species	Trt length (days)	P	S	Colony	Bee	S	Empty	S	P	Q	W	B	M	W	Q	Eggs layed	Larval ejection	Defensive behavior
		treatment (ppb)		weight		cells		consumed		mortality		produced						
B.terrestris[1]	26	Seed trt sunflower*												ns	ns			
B.terrestris[2]	14	6*	0.7*	-			ns					ns	ns	ns	-			
B.terrestris[2]	14	12*	1.4*	-			ns					ns	ns	ns	-			
B.terrestris[3]	14		20*								+					-		
B.terrestris[3]	14		10*								+					-		
B.terrestris[3]	14		2*	ns		ns		ns			ns	ns		ns		ns	ns	
B.impatiens[4]	84	7*										ns		ns				
B.impatiens[4]	84	30*										ns		ns				
B.impatiens[4]	84	7*			ns				ns			ns	ns	ns	ns			
B.impatiens[5]	77	6**			ns				ns			ns	ns	ns	ns			
B.impatiens[5]	77	36**			ns				ns			ns	ns	ns	ns			
B.terrestris[6]	28		10*	ns						ns	ns	-		-				
B.terrestris[6]	28	10* + 37,500***		ns						ns	+	-		-				
B.impatiens[7]	30	Irrigated Granular 0.004 mg AI/ft^2*		ns	ns	ns						ns	ns	ns	ns			ns
B.impatiens[7]	28	Non-irrigated Spray 0.003 mg AI/ft^2*		-	-	-						-	ns	-	ns			-
B.impatiens[7]	28	Irrigated Spray 0.003 mg AI/ft^2*		ns	ns	ns						ns	ns	ns	ns			ns

Tasei et al. 2001[1], Whitehorn et al. 2012[2], Mommaerts et al. 2010[3], Morandin and Winston 2003[4], Franklin et al. 2004[5], Gill et al. 2012[6], Gels et al. 2002[7]
*imidacloprid, **clothianidin, ***imidacloprid + λ-cyhalothrin, P = pollen, S = syrup, Q = queens, W = workers, B = brood, M = males
Significant decrease (-), Significant increase (+), Not significantly different from control (ns)

Appendix 3: Imidacloprid effects on *Bombus* spp. queen-less micro-colony health

Species	Foraging	Trt length (days)	Pollen treatment (ppb)	Syrup treatment (ppb)	Pollen consumed	Syrup consumed	Males produced	Brood produced	Worker mortality	Larval ejection	Workers w/ mature oocytes	Oocyte size
B. terrestris[1]	No	85	6	10	ns	ns	ns	-	+	-		
B. terrestris[1]	No	85	16	25	ns	ns	ns	ns	+	-		
B. terrestris[2]	No	77		200			-		+			
B. terrestris[2]	Yes	77		200			-		+			
B. terrestris[2]	No	77		20			ns		ns			
B. terrestris[2]	Yes	77		20			-		+			
B. terrestris[2]	No	77		10			ns		ns			
B. terrestris[2]	Yes	77		10			-		ns			
B. terrestris[3]	No	13		159	-	-		-	ns		-	-
B. terrestris[3]	No	13		4,10,26,64	-	-		-	ns		ns	ns
B. terrestris[3]	No	13		1.27	-	-		-	ns		ns	ns

Tasei et al. 2000[1], Mommaerts et al. 2010[2], Laycock et al. 2012[3]
Significant decrease (-), Significant increase (+), Not significantly different from control (ns)

Appendix 4: Imidacloprid and clothianidin effects This summarizes findings that are shown in the figures.

Chemical	Effect level	Effect Type	P value	F value	10 ppb	20 ppb	50 ppb	100 ppb
Imidacloprid	Colony	Cumulative queen mortality (week 6)	0.055	Chi^2 = 9.26	ns	ns	+	+
		Cumulative queen mortality (week 11)	< 0.001	Chi^2 =75.49	ns	+	+	+
		Colony weight (week 0)	0.170	1.84	ns	ns	ns	ns
		Colony weight (week 11)	< 0.001	16.20	-	-	-	-
		Colony consumption (week 2)	< 0.001	52.51	-	-	-	-
		Colony consumption (week 4)	< 0.001	27.40	ns	-	-	-
		Colony consumption (week 6)	< 0.001	22.61	-	-	-	-
		Colony consumption (week 8)	0.002	7.67	-	-	-	NA
		Stored syrup weight	0.011	4.83	ns	ns	-	-
		Bees on nest (week 0)	0.057	2.55	ns	ns	ns	ns
		Bees on nest (week 2)	0.016	4.20	ns	ns	ns	ns
		Bees on nest (week 4)	0.010	4.82	ns	ns	ns	-
		Bees on nest (week 6)	0.031	3.84	ns	ns	ns	-
		Bees on nest (week 8)	0.192	1.77	ns	ns	ns	NA
		Cumulative number of bees	0.004	4.62	ns	-	ns	-
		Cumulative number of workers	0.129	1.92	ns	ns	ns	ns
		Cumulative number of males	0.014	4.59	-	-	-	-
		Cumulative number of queens	0.945	0.19	ns	ns	ns	ns
		Total brood	0.049	2.99	ns	ns	-	-
		Total alive brood	0.006	5.74	ns	-	-	-
		Total dead brood	0.205	1.67	ns	ns	ns	ns
		Syrup pots added (Week 11 - 0)	0.037	Chi^2 = 10.23	ns	ns	-	-
		Worker movement	0.003	6.27	ns	-	-	ns
		Queen movement	0.188	1.70	ns	ns	ns	ns

Chemical	Effect level	Effect Type	P value	F value	10 ppb	20 ppb	50 ppb	100 ppb
Imidacloprid	Individual	Bee weight (week 4)	0.372	1.11	ns	ns	ns	ns
		Bee weight (week 6)	0.929	0.21	ns	ns	ns	ns
		Bee weight (week 8)	0.088	2.31	ns	ns	ns	-
		Bee consumption (week 2)	< 0.001	30.97	-	-	-	-
		Bee consumption (week 4)	< 0.001	10.31	ns	-	-	-
		Bee consumption (week 6)	0.513	0.89	ns	ns	ns	ns
		Bee consumption (week 8)	0.093	2.51	ns	ns	ns	NA
Clothianidin	Colony	Cumulative queen mortality (week 6)	< 0.001	$Chi^2 = 22.87$	ns	ns	+	+
		Cumulative queen mortality (week 11)	< 0.001	$Chi^2 = 102.78$	ns	+	+	+
		Colony weight (week 0)	0.492	0.87	ns	ns	ns	ns
		Colony weight (week 11)	< 0.001	16.10	ns	-	-	-
		Colony consumption (week 2)	< 0.001	42.05	-	-	-	-
		Colony consumption (week 4)	< 0.001	91.96	-	-	-	-
		Colony consumption (week 6)	< 0.001	42.77	-	-	-	-
		Colony consumption (week 8)	< 0.001	48.52	-	-	-	-
		Stored syrup weight	0.002	6.83	-	-	-	-
		Bees on nest (week 0)	0.813	0.39	ns	ns	ns	ns
		Bees on nest (week 2)	0.928	0.21	ns	ns	ns	ns
		Bees on nest (week 4)	0.095	2.16	ns	ns	ns	ns
		Bees on nest (week 6)	0.006	4.52	ns	ns	+	+
		Bees on nest (week 8)	0.005	8.29	ns	ns	ns	ns
		Cumulative number of bees	0.002	5.12	ns	ns	-	-
		Cumulative number of workers	0.094	2.15	ns	ns	ns	ns
		Cumulative number of males	0.002	7.44	ns	ns	-	-
		Cumulative number of queens	0.085	2.23	ns	ns	ns	ns

Chemical	Effect level	Effect Type	P value	F value	10 ppb	20 ppb	50 ppb	100 ppb
Clothianidin	Colony	Total brood	0.007	4.16	ns	ns	-	-
		Total alive brood	0.016	4.13	ns	ns	-	-
		Total dead brood	0.144	1.83	ns	ns	ns	ns
		Syrup pots added (Week 11 - 0)	<0.001	$Chi^2 = 21.54$	-	-	-	-
		Worker movement	0.006	5.60	ns	ns	-	-
		Queen movement	0.298	1.55	ns	ns	ns	ns
	Individual	Bee weight (week 4)	0.021	3.39	ns	ns	-	ns
		Bee weight (week 6)	0.004	4.90	ns	-	ns	ns
		Bee weight (week 8)	0.232	1.54	ns	ns	ns	NA
		Bee consumption (week 2)	< 0.001	17.68	ns	-	-	-
		Bee consumption (week 4)	< 0.001	32.73	ns	-	-	-
		Bee consumption (week 6)	< 0.001	9.37	ns	-	-	-
		Bee consumption (week 8)	0.035	4.32	ns	ns	ns	ns

Multiple range test compared to control, (-) = significantly less than control, (+) = significantly more than control, ns = not significantly different