CHRONIC CONTACT EXPOSURE TO REALISTIC SOIL CONCENTRATIONS OF A NEONICOTINOID INSECTICIDE REPRESENTS A POTENTIALLY IMPORTANT AND UNEXPLORED ROUTE OF EXPOSURE FOR GROUND NESTING BEES

BY

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THESIS

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Abstract

In the face of widespread declines, the non-target effects of pesticides on ecologically and economically important bees are an area of growing concern. One class of pesticides that has gained considerable attention over the past two decades is the neurotoxic neonicotinoid insecticides. Neonicotinoids are a widely used group of compounds that are often applied preemptively to protect plants from pestiferous insects. Due to their systemic nature, they are found throughout treated plants, including in pollen and nectar. This has led to an ever expanding literature concerning the effects of these chemicals when consumed by bees. However, much of the applied active ingredients are not absorbed by treated plants, return to the soil as plant material decomposes, and persist in soils due to their relatively long half-lives. Neonicotinoid contamination of soil represents a potentially important, yet under explored, route of exposure for bees, the majority of which nest in the ground.

The aims of this thesis are to examine this route of exposure and to determine potential sublethal effects. In chapter 1, I explore the effects of chronic contact exposure during to realistic soil concentrations of imidacloprid - the most widely used neonicotinoid - on pre- and post-overwintering development speed, mass, and immature and adult longevity. In chapter 2, I focus on the effects of this type of exposure on adult bee mushroom body growth.

The results presented here suggest that neonicotinoid contamination of soils represents a potentially important route of exposure for ground nesting bees. A number of sublethal effects were detected in response to treatment with chronic contract exposure to imidacloprid including: reduced development speed at low and intermediate concentrations, increased conservation of starting nest cell (food provision and egg) biomass, and changes to immature and adult longevity.

Population-level and ecological consequences of these effects are discussed, as well as implications for habitat restoration and bee conservation.

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Chapter 1: Chronic contact exposure to realistic soil concentrations of imidacloprid during immature development affects the development speed, individual mass, and longevity of solitary bees

Abstract

The non-target effects of pesticides are an area of growing concern, particularly for ecologically and economically important organisms such as bees. Systemic neonicotinoid insecticides are one such class of pesticides. Previous work on the effects of neonicotinoids on bees focused predominantly on the consumption of contaminated pollen and nectar by a limited number of eusocial species. However, neonicotinoids are known to accumulate and persist in soils at levels higher than in food resources which may represent an important and underexplored route of exposure for bees - most of which nest in the ground. The aim of this chapter was to assess the effect of chronic contact exposure to realistic soil concentrations of imidacloprid - the most widely used neonicotinoid - on bee development speed, body mass, and longevity. Cohorts of *Osmia lignaria* and *Megachile rotundata* were used as proxies for ground nesting species. A number of sublethal effects were detected in response to treatment with imidacloprid including: reduced development speed at low and intermediate concentrations, increased conservation of pollen provision mass, and changes to immature and adult longevity. These results suggest that chronic exposure to nesting substrates contaminated with neonicotinoids represents an important route of exposure that could have significant physiological and ecological consequences for bees and plant-pollinator interactions.

Introduction

Neonicotinoid insecticides are a widely used class of pesticides that have received a lot of attention for their potential impacts on non-target organisms (*reviewed in* Botías et al. 2016;

Gibbons et al. 2015; Pisa et al. 2015). These neurotoxins work by binding to nicotinic acetylcholine receptors (nAChR) in the central nervous system, which overstimulates nerve cells and results in paralysis and death (Bai et al. 1991; Elbert et al. 1991; Leicht 1993; Methfessel 1992). Neonicotinoids are most often applied as a seed-coating that is then absorbed by the growing plant and incorporated systemically throughout its tissues (Bonmatin et al. 2015; Bromilow & Chamberlain 1995). This provides preemptive protection for crops from major homopteran, coleopteran, dipteran, and lepidopteran pests (Elbert et al. 1991; Elbert et al. 1998). However, the active ingredients of these insecticides are also found in plant resources, such as pollen, nectar, and guttation drops, used by non-target organisms (Bonmatin, Marchand, et al. 2005; Bonmatin et al. 2015; Girolami et al. 2009). This has led to a growing concern about the potential non-target effects of neonicotinoids, particularly for bees.

For much of the past two decades, research on the lethal (e.g. increase in mortality over 24 - 48 hours) and sublethal (e.g. reduced performance) effects of neonicotinoid exposure in pollinators has primarily focused on oral exposure in honey bees and, more recently, bumble bees (*reviewed in* Pisa et al. 2015). In a meta-analysis of 14 laboratory and semi-field studies, Cresswell (2011) found that imidacloprid - the most widely used neonicotinoid - had no consistent effect on adult mortality rates in honey bees at dosages commonly recovered from pollen and nectar. However, the same meta-analysis detected significant sublethal effects of acute and chronic exposure. Observed sublethal effects include: delayed larval development (Abbott et al. 2008), impaired mushroom body growth and neurological function (Decourtye et al. 2003; Tomé et al. 2012; Yang et al. 2012), and disruptions to reproduction including reduced production of reproductive female offspring (Sandrock et al. 2014; Straub et al. 2016; Whitehorn et al. 2012; Wu-Smart & Spivak 2016). Such negative effects could have significant implications

for plant pollination and ultimately for agricultural and ecosystem stability (Bartomeus et al. 2013; Burkle et al. 2013; LaBar et al. 2013).

One major criticism of the previous work on neonicotinoids is that the lowest observed effect concentration (LOEC) is often higher than concentrations commonly found in field-collected pollen and nectar - 12 to 48 ppb vs 1 to 11 ppb for imidacloprid (Bonmatin, Moineau, et al. 2005; Bonmatin et al. 2015; Decourtye et al. 2003; *discussed in* Pisa et al. 2015; Whitehorn et al. 2012). This makes it difficult to interpret the ecological significance of these effects as it is unlikely that they occur with any great frequency under field conditions. Conversely, the levels of neonicotinoids found in soil samples are similar to or greater than the LOECs found in these studies. These concentrations commonly occur in the 12 to 18 ppb range, but there have been reports of levels as high as 650 ppb (Bonmatin, Moineau, et al. 2005; Donnarumma et al. 2011; Schmuck et al. 2001). Neonicotinoid concentrations reach higher and more persistent quantities because much of the applied active ingredient is not absorbed by plants and is leached into the surrounding soil (Donnarumma et al. 2011; Sur & Stork 2003), returns to the soil as treated plant material decomposes (Horwood 2007), and has a relatively long half-life in soils (Baskaran et al. 1999; Bonmatin, Moineau, et al. 2005; Cox 2001; Wagner 2016). Consequently, bees - most of which nest and develop in soil (O'Toole & Raw 1991) - are potentially exposed to large quantities of neonicotinoids during their immature stages. In addition to containing higher concentrations of neonicotinoids, contaminated nesting soils may pose a significant route of exposure for these species because the toxicity of these compounds increases with exposure time (Charpentier et al. 2014; Suchail et al. 2001) and solitary bees have so far been shown to be more sensitive to neonicotinoids than honey bees or bumble bees (Bailey et al. 2005; Scott-Dupree et al. 2009). Despite the potential impact of soil contamination on ground nesting bees, little work

to date has considered the importance of chronic contact exposure to neonicotinoids on bees, which could have major implications for current conservation and restoration practices.

The primary approach for conserving bee populations is via planting flower-rich habitats (Harmon-Threatt & Hendrix 2015; M'Gonigle et al. 2015; Morandin et al. 2014; Williams et al. 2015). However, if these areas of highly attractive floral resources provide contaminated nesting resources in or adjacent to them, they may represent ecological traps that draw bees to apparently good resources but actually serve as demographic sinks (Robertson & Hutto 2006) with the potential to cause population decline. Additionally, bees are unable to detect neonicotinoids via their olfactory senses (Kessler et al. 2015) and may be unable to assess and avoid contaminated soils. However, an assessment of the potential effects of nesting in contaminated soils has not previously been conducted and represents a major gap in our current knowledge.

The aim of the current study was to address this knowledge gap concerning an unexplored route of exposure for bees. Using imidacloprid - the archetypal member of the neonicotinoid insecticide family (Charpentier et al. 2014; Jeschke et al. 2011) - I attempted to determine the sublethal effects of chronic contact exposure during immature development on solitary bees. This encompassed larval development speed, masses at important life stages, and immature and adult longevity. I hypothesized that larvae treated with higher concentrations of imidacloprid would have delayed development, reduced masses, and shorter lives. Similarly, among the individuals that survive to adulthood, those treated with greater concentrations of imidacloprid during their development would have reduced lifespans when compared to control bees.

Methods

Study organisms - To assess the effects of chronic contact exposure to imidacloprid on ground nesting bee development and longevity, the cavity nesting bees *Osmia lignaria* Say, 1837 and *Megachile rotundata* (Fabricius, 1787) were used. These species have been previously used to approximate responses of ground nesting bees to environmental stressors because they are closely related to ground nesting species (Cane & Neff 2011), are easily collected via trap nests, and standard protocols exist for their successful rearing in a laboratory setting (Abbott et al. 2008; Huntzinger et al. 2008). Additionally, *O. lignaria* and *M. rotundata* overwinter during different life stages (Table 1A), which are representative of many bee species in temperate regions (Michener 2007) and may provide some insight into the interaction of life history and pesticide exposure. *Megachile rotundata* overwinters in its last larval stage and completes development in the spring and summer when the temperatures rise. Bees with this life history are at risk of being exposed to soil contaminants during two temporally distinct time periods of their development - specifically the late summer through fall and the following spring into early summer. *Osmia lignaria* larvae develop throughout the spring and summer and overwinter as pre-emergent adults and subsequently emerge early during the following spring. Bees with similar life histories likely face one long period of risk during development, but avoid a second round of exposure due to their early emergence the following year. However, the difference in life history, as well as size, led to some practical differences in the methodology used for each species. Differences are listed in Table 1 and will be referenced when relevant during the following description.

General methods - Reed nest tubes with newly laid eggs and early instar larvae were purchased from Crown Bees (Seattle, WA) during the spring and summer of 2015. Individual

bees and their pollen provisions were weighed together and placed into a well of a tissue culture plate (Table 1B). Individuals from the same nest were stratified across the treatments to limit the potential genetic biases that exist when exploring responses to imidacloprid (Pisa et al. 2015). Once individuals reached the second instar larval phase, they were treated every 48 hours with 0.5 μL of a solution of 0, 7.5, 15, or 100 ppb imidacloprid (Sigma-Aldrich, PN 37894) in saline solution (Equate Sterile Multipurpose Solution, PN 68113173188) applied topically to their abdominal segments. The concentrations used reflect realistic soil concentrations recovered in previous studies (Bonmatin, Moineau, et al. 2005; Donnarumma et al. 2011; Schmuck et al. 2001). Saline solution was used as the solvent because it is less detrimental for larval bees than deionized water (Craig Huntzinger, *personal communication*). Imidacloprid solutions were replaced every 96 hours and kept in the dark at room temperature. To maintain room temperature and prevent desiccation, tissue culture plates were kept inside an unheated incubator (Thermo Scientific Heratherm Incubator IMH180, PN51028068, Waltham, USA) at room temperature with a 250 mL beaker filled with water. During this time, the chamber temperature was 23.6 ± 0.6°C and the relative humidity was 84.5 ± 1.3%.

Individual development and survival was recorded daily and masses were taken at important life stages including: initial mass with pollen provision, prepupa, pupa, pre-emergent adult, and emergent adult (Mettler Toledo XS105 DualRange). Tissue culture plates were left open until individuals began spinning cocoons. At that time lids were replaced to aid in cocoon completion. Once cocoons were constructed, development was monitored by back-lighting through individual cocoons using a cell phone LED light while observing through a stereomicroscope (Zeiss Stemi 2000). In October, surviving individuals in their overwintering stages were stored at 4°C to overwinter. During this time, plates were placed in 53 L tote

containers (Sterilite 56 qt PN 1656) with a 250 mL beaker filled with water to prevent individuals from desiccating. Bees were checked twice a week to ensure humidity was appropriate and to monitor for mold growth. There were no visible signs of mold growth for either species.

In the spring of 2016, bees were removed from cold storage and allowed to emerge (*O. lignaria*) or finish their development (*M. rotundata*). To finish their development, *M. rotundata* were reared at 28.2 ± 0.1°C and 78.9 ± 1.8% relative humidity. After emergence, each adult was weighed and given a unique paint identifier on the thorax using acrylic paint (Royal Langnickel ACR12). Paint was periodically checked and reapplied as necessary (i.e. if it was damaged or partially missing). For painting, bees were temporarily anesthetized either by chilling (*O. lignaria*) or with carbon dioxide (*M. rotundata*). *Megachile rotundata* are less cold tolerant (Tim Krogh, *personal communication*) so they required a modified methodology to prevent undue stress.

Adult bees were placed in 85 L tote containers (Sterilite 90 qt PN 1666) separated by treatment and species. *Typha sp.* pollen (YellowPollen.net, Kirkland, WA) and sucrose water were provided in an artificial flower array for bees to consume *ad libitum*. Similar diets have been provided for other lab cultured bees with success (Greenberg 1982; Roulston & Cane 2002; Emily Dobbs, *personal communication*). Within the array, four flowers provided pollen, two provided a 2.0 M sucrose solution, and two provided a 1.0 M sucrose solution. Every four days the color, location within the array, sucrose concentration, and essential oil (*Eugenia caryophyllata* and *Mentha spicata*, NOW Foods, Bloomingdale, IL; *Gaultheria procumbens*, Healing Solutions, Scottsdale, AZ; *Cymbopogon flexuosus*, Aura Cacia, Norway, IA) used in the

artificial flowers was randomized and changed. Nesting tubes, nesting substrates (Table 1C), and water were also provided and replenished as needed.

Osmia lignaria adults were initially placed in a greenhouse within the totes but had to be moved to an environmental chamber to prevent overheating. A 14:10 L:D cycle was established to mimic the daylight patterns of that time of year in Illinois (Philips 32 Watt Alto II PN F32T8/ADV835) and the temperature was set to 24°C. *Megachile rotundata* adults were placed in the environmental chamber with the same conditions as for the *O. lignaria* with the exception of temperature which was set to 28°C. Adult bee mortality was assessed daily and deceased individuals were removed.

Statistics - Due to the differences in the number of treatments (Table 1D), *O. lignaria* and *M. rotundata* were analyzed separately. Additionally, except for larval longevity which was pooled across sexes, males and females were analyzed separately. Bee development speed was analyzed as the number of days it took to reach the transition points between important life stages (larva to prepupa, prepupa to pupa, pupa to pre-emergent adult, and pre-emergent adult to emergent adult). Differences in development timing was analyzed using the Prentice, Williams, and Peterson total time extension for multiple events (PWP-TT; 1981) of the Cox Proportional-Hazards Regression model because it allows for multiple ordered events such as the transitions between insect life stages (Amorim & Cai 2015; Cox 1972). The events were set as the previously listed transitions and separate models were used for the pre- and post-overwintering periods. Bees that died during the course of the experiment were censored from the development speed dataset on their last day of known activity (e.g. movement). Individual bee mass was analyzed using a linear mixed-effects model. A priori expectations that bee development speed would differ between treatments led us to use a compound symmetry covariance structure which

allowed for flexibility in the timing of repeated mass measurements. The effects of chronic contact exposure to imidacloprid on bee mass were analyzed as the proportion of the initial pollen provision and immature bee mass remaining at important life stages. When a female bee provisions a nest cell the theoretical maximum mass the offspring can achieve is the sum of the egg and food provision mass. However, in reality, bees lose much of this mass through metabolism and defecation and looking at the proportion of mass remaining helps elucidate if there are effects on these processes. Additionally, the proportion of initial mass remaining was used to limit effects of the starting food provision size as final adult size is known to be strongly correlated with this factor in solitary bees (Bosch & Vicens 2002; Klostermeyer et al. 1973). If significant differences in proportion of mass remaining were detected, post-hoc analysis was conducted using Tukey contrasts. Immature and adult longevity were analyzed using Cox Proportional-Hazards Regression (Cox 1972; Fox & Weisberg 2011). All analyses were conducted using the statistical program R (R Core Team 2014) and the packages 'survival' (Therneau 2015), 'nlme' (Pinheiro et al. 2017), and 'multcomp' (Hothorn et al. 2008).

Results

Due to equipment malfunction, some *O. lignaria* adults emerged early and were subsequently excluded from the remainder of the study. For these bees, individual sex was assigned to the immature bee data based on their starting provision mass and position within the original nest tube - both used previously to predict sex (Kemp & Bosch 2005) - and on the number of males and females that emerged early. This resulted in excluding a total of 32 female and 66 male bees from across all treatments from analysis of adult longevity (Table 2, *also see for sample sizes at different life stages*).

Development speed - Bees of both species arrived in multiple shipments and, except where noted, this date had a significant effect in the PWP-TT models for bee development ($p << 0.001$). Generally, bees that arrived later developed more quickly. This is a naturally occurring phenomena in which individuals laid later in the season develop faster than those laid earlier - the mechanism of which is yet unknown (Bosch et al. 2000). Including this factor in the models where it was a significant predictor of development speed helped reduce the variance and made it possible to discern the effects of chronic contract exposure to imidacloprid.

Prior to the overwintering period, female *O. lignaria* treated with a 0 ppb solution developed significantly faster than bees treated with 15 ppb imidacloprid ($z = -2.195$, $p = 0.0282$; Figure 1A). Time to important developmental stages did not differ significantly between any of the other comparisons of treatment levels (Table 3). Chronic contact exposure to imidacloprid during development did not affect female emergence timing (Table 3; Figure 1B). Similarly, treating male *O. lignaria* with chronic topical treatments of imidacloprid did not significantly affect the time it took to reach important developmental stages before (Figure 1C) or after (Figure 1D) the overwintering period (Table 3).

Shipment was not determined to be a significant factor in *M. rotundata* development speed before the overwintering stage for either females ($z = 0.205$, $p = 0.838$) or males ($z = 1.153$, $p = 0.249$) and thus it was removed from the models. For female *M. rotundata*, bees treated with 100 ppb developed significantly faster during the pre-overwintering phase than did those treated with 7.5 ($z = 2.058$, $p = 0.0396$) or 15 ($z = 2.387$, $p = 0.017$; Figure 2A) ppb imidacloprid solution. During the period following overwintering, where shipment timing was again a significant factor for development speed and included in the model, female *M. rotundata* treated with 15 ppb developed significantly slower than bees treated with 7.5 ($z = 2.138$, $p =$

0.033) and 100 (z = 2.253, p = 0.0243; Figure 2B) ppb imidacloprid. There were no significant differences between the other imidacloprid treatments before or after the overwintering period, regardless of sex (Table 3; Figure 2C and 2D).

Body mass - There was no significant effect of chronic contact exposure during development on the proportion of initial nest cell mass remaining at important life stages for *O. lignaria* females (t_{121}= 0.724, p = 0.47; Figure 3A) or males (t_{150}= 0.322, p = 0.748; Figure 3B). Unsurprisingly, the proportion of mass remaining decreased significantly with successive development stages in both females (t_{307}= 10.602, p << 0.001) and males (t_{311}= 7.762, p << 0.001). The interaction term between development stage and pesticide exposure was not significant for female (t_{307}= 0.332, p = 0.740) or male (t_{311}=0.114, p = 0.910) bees.

There was a significant effect of imidacloprid solution concentration on the proportion of mass remaining for female *M. rotundata* (t_{61}= 2.159, p = 0.035; Figure 3C). Bees treated with 100 ppb retained a significantly higher proportion their starting nest cell mass compared to control bees (z = 2.162, p = 0.031). There was no significant difference between control bees and bees treated with 7.5 (z = 1.687, p = 0.0916) or 15 (z = 1.781, p = 0.0749) ppb imidacloprid solution. Pairwise comparisons between bees treated with 7.5, 15, and 100 ppb revealed no significant differences in the proportion of mass remaining at important life stages (z < 0.5, p > 0.6). The proportion of mass remaining differed significantly with development stage (t_{179}= 10.601, p << 0.001), but the interaction between these factors was not significant (t_{179}= 1.320, p = 0.188). For male *M. rotundata,* mass did not differ between the different levels of pesticide exposure (t_{113}= 0.934, p = 0.3521; Figure 3D) nor was the interaction term significant (t_{339}= 0.694, p = 0.488). Again, as expected, the proportion of mass remaining for males differed significantly between the different development stages (t_{339}= 15.625, p << 0.001).

Longevity - Bees that died before reaching adulthood could not reliably be sexed. This meant that for immature longevity, bees were pooled within each species and not analyzed by sex. There were no differences in longevity between the imidacloprid treatment levels for immature *O. lignaria* (Table 4; Figure 4A). The same was true for immature *M. rotundata*, except that those treated with doses of 100 ppb imidacloprid had marginally reduced longevity compared with the saline control ($z = -1.944$, $p = 0.052$; Table 4: Figure 4B). For adult bees, female *O. lignaria* treated with 100 ppb imidacloprid during development had significantly reduced longevity compared with control bees ($z = 2.142$, $p = 0.032$) and those treated with 7.5 ppb ($z = 2.346$, $p = 0.019$; Figure 4C). Male *M. rotundata* treated with 15 ($z = 1.999$, $p = 0.046$) and 100 ($z = 2.535$, $p = 0.011$; Figure 4F) ppb had significantly increased longevity relative to control bees. No other significant differences were found between control or treated bees, regardless of species or sex (Table 4; Figure 4D and 4E).

Discussion

The effects of chronic contact exposure to realistic soil concentrations of imidacloprid during development vary based on the species and sex of the exposed individual as well as the observed life stage. Broadly, it appears that development speed, the amount of mass retained, and individual longevity are all characteristics of solitary bees that may be affected by this previously unexplored route of exposure.

The observed effects on solitary bee development speed could be explained by a hormetic response to chronic contact exposure to neonicotinoids. Hormesis is a phenomenon in which the toxicity of a compound is u-shaped as the dosage increases (Calabrese & Baldwin 2002). When bee development speed was affected by imidacloprid treatment, the general trend was that bees treated with low and intermediate dosages had delayed development relative to control and/or

high dosages of the pesticide. This may suggest that bees possess physiological mechanisms to compensate for chronic contact exposure to lower doses of neonicotinoids at the expense of development speed (Calabrese 2010). A thorough investigation of this proposed response would be required to determine if, and by what mechanism, a tradeoff is being made. However, in the cases of male bees of both species investigated here and post-overwintering female *O. lignaria*, chronic contact exposure to imidacloprid does not appear to affect development speed. A possible explanation for the lack of an effect on male bee development speed is that their haploid genome affects their gene expression levels - including genes involved in detoxification (Borges et al. 2012; Xu et al. 2013). If this is true for detoxification pathways related to neonicotinoid processing, male bees may not be able to upregulate these genes to high enough levels to divert significant energy away from development. Alternatively, if the ability of male bees to increase the production of the necessary enzymes is less than for female bees, the changes to development speed might be too small to detect with the number of replicates used here. In terms of ecological significance, the effects on bee development speed before overwintering do not appear to carry over to the post-overwintering period - possibly due to differences in gene expression across life stages (Xu et al. 2013). However, delays in the development of immature bees, especially late season bees like *M. rotundata*, could have significant fitness consequences if affected individuals do not reach their dormant stage before temperatures drop (Abbott et al. 2008). Changes to development speed in the spring could also negatively impact the synchrony of plant-pollinator mutualisms and interfere with ecosystem services and stability.

The higher proportion of mass remaining for *M. rotundata* females treated with imidacloprid suggests that differences exist between these and control bees during the larval stage. When Tomé et al. (2012) evaluated the neuromotor skills of adult *Melipona quadrifasciata*

anthidioides that were fed imidacloprid contaminated diets as larvae, they observed a reduction in movement associated with increasing pesticide dose. The same may be true of the larvae in the current study. If neonicotinoid exposure reduces activity, via inhibiting neuromotor function or otherwise, the treated bees might move less, expend less energy and, thus, retain more of the original food provision mass. The lack of a significant interaction between development stage and imidacloprid concentration also suggests that the observed differences in mass lie in some difference during the mobile larval portion of these bee's lives. More research is necessary to determine if the observed differences are due to reduced movement, changes to metabolism, or some other factor. Additionally, exploring whether these effects carry over to the adult life stage could give us a better understanding of how neonicotinoid contamination affects plant-pollinator mutualisms and the ability of female bees to provision nest cells.

The effects on longevity observed for female *O. lignaria* and male *M. rotundata* add to the growing literature of the sublethal effects of neonicotinoids on bee reproduction. If adult female bees live shorter lives and are limited in their ability to fertilize their eggs (Rosenheim & Hoy 1988; Sandrock et al. 2014; van Wilgenburg et al. 2006), then overall offspring and - more significantly - diploid female production would be expected to decline. Additionally, if exposure to imidacloprid reduces the reproductive quality of male bees for at least some species (Straub et al. 2016), but increases their lifespan, more female bees may mate with these low-quality males. Under these circumstances, a female's spermatheca may become filled with fewer viable sperm and the rate of successful fertilizations will decrease. This is particularly problematic for individuals or species that only mate a few times. The observed changes to adult longevity for *O. lignaria* females and *M. rotundata* males suggests that the effect of neonicotinoid exposure on

the reproductive output of solitary bees may differ between species, but there is the potential for significant demographic damage.

The combination of the results discussed above and the reduced longevity of larval *M. rotundata* could provide insight into the mechanisms of bee population declines described by Woodcock et al. (2016). While they hypothesized that contaminated oilseed rape floral resources were the driving force behind the observed declines, many of the affected species are not known to forage on this crop. Additionally, many of the most negatively affected species were ground nesting bees, suggesting that contaminated nesting resources may be an important factor in these declines. Future studies should collect soil concentrations of imidacloprid in conjunction with bee foraging data and attempt to determine if bees nest in contaminated soils in the field.

One limitation of the current study is that it does not take into account the role of nest cell linings and how this might influence exposure to soil contaminants. Of these, the secretions of the Dufour's gland are the most well studied and are present in a number of ground nesting bee taxa (Cane 1981; Shimron et al. 1985). These cell linings often contain a number of hydrophobic compounds (Hefetz 1987; Mitra 2013) and it is generally accepted that when such linings are present, they provide a mechanism for maintaining moisture homeostasis in the brood cell (Cane 1981). However, the use and structure of these linings varies greatly between different groups and, sometimes, even within a single species (Brooks & Cane 1984; Eickwort & Eickwort 1971; Wille & Orozco 1970). It is also hypothesized that water in the soil surrounding the brood cell crosses the cell lining, is absorbed by the pollen provision, and contributes to the mass gained by larvae (May 1972). If water is able to cross this hydrophobic barrier, then it seems reasonable to predict that molecules dissolved in the water - in particular those that are also able to cross insect cuticle (Bailey et al. 2005; Scott-Dupree et al. 2009) - may also find their way into the nest and

come into contact with developing bees. Additionally, some groups, such as the Megachilidae, do not use glandular secretions to line their nests and instead use plant-derived compounds, including resins from trees (Cane 1981; Cane 1996; Michener 1964; Michener 1974; Rozen 1967). This may represent an additional route of exposure if these resources come from neonicotinoid treated crops or trees. Future work should look to elucidate the role, if any, nest cell linings play in protecting developing bees from nest contaminants.

Despite this limitation, the results of this study suggest that chronic contact exposure to soil realistic concentrations of neonicotinoids represent a potentially important route of exposure for ground nesting bees. I also developed and described a bioassay that can be used in future toxicological studies for pesticides and other soil contaminants and demonstrated that chronic sublethal contact exposure has the potential to affect bee development speed, mass, and longevity. A better understanding of the interaction between nests conditions and bees success will help to inform more effective restoration practices and aid in the conservation of these important organisms.

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Figures

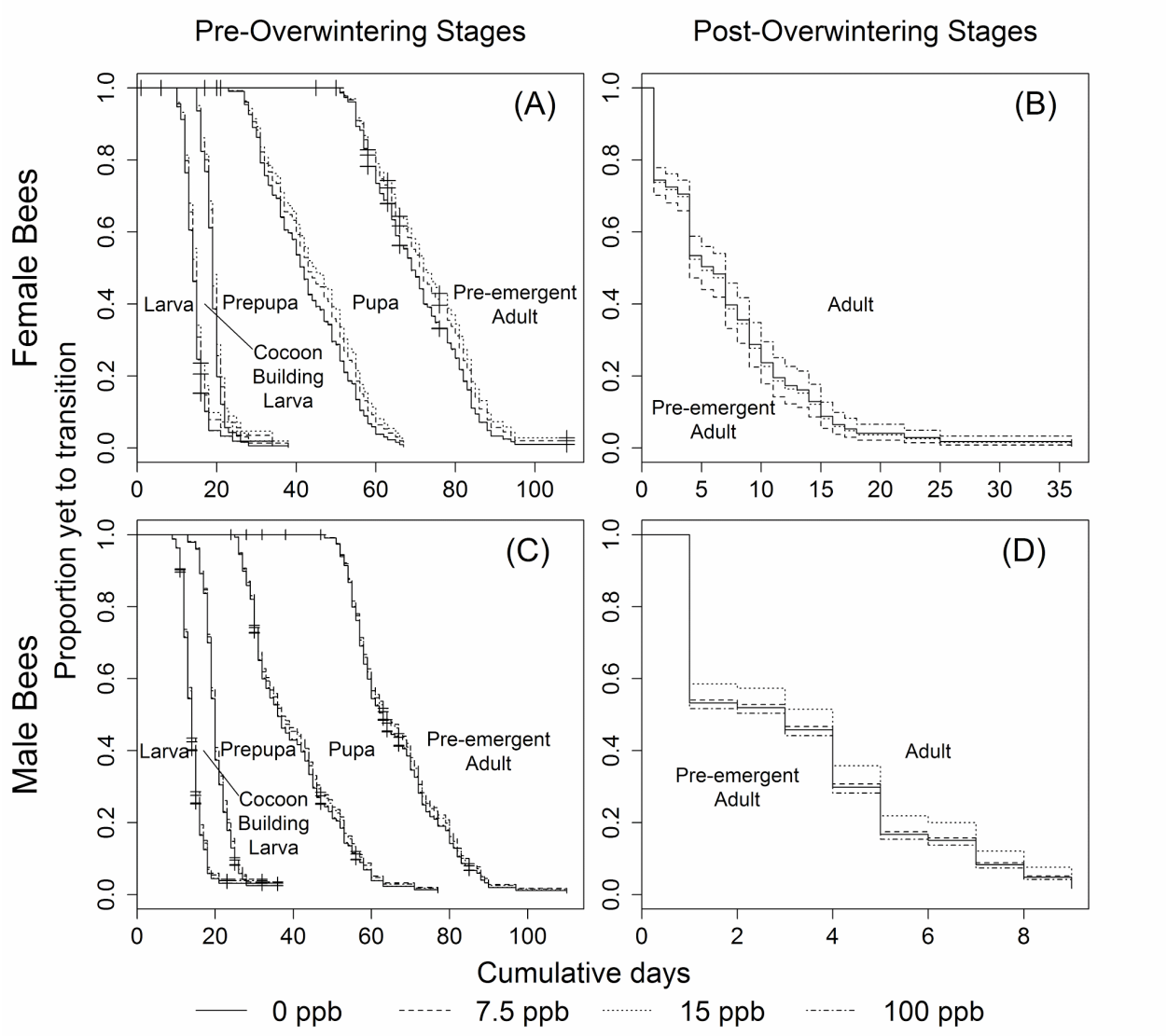

Figure 1 - *Osmia lignaria* development speed. (A) Time to development for important life stages prior to overwintering for female bees. From left to right, groups of lines represent the cumulative number of days to the beginning of cocoon construction and the prepupal, pupal, and pre-emergent adult stages. Bees treated with 15 ppb imidacloprid solution developed significantly slower than control bees. (B) Time to adult emergence for female bees after removal from overwintering conditions. There were no significant differences between treatments. (C) Cumulative time to development for important life stages before overwintering for male bees. The representation of the different important transitions are as in (A). No significant effects on development speed were detected. (D) Time to adult emergence for male *O. lignaria*. No significant difference was observed between treatments. Although not shown graphically, time when eggs were collected had a significant effect on *O. lignaria* development speed ($p << 0.001$). Crosses represent data that were censored in the model.

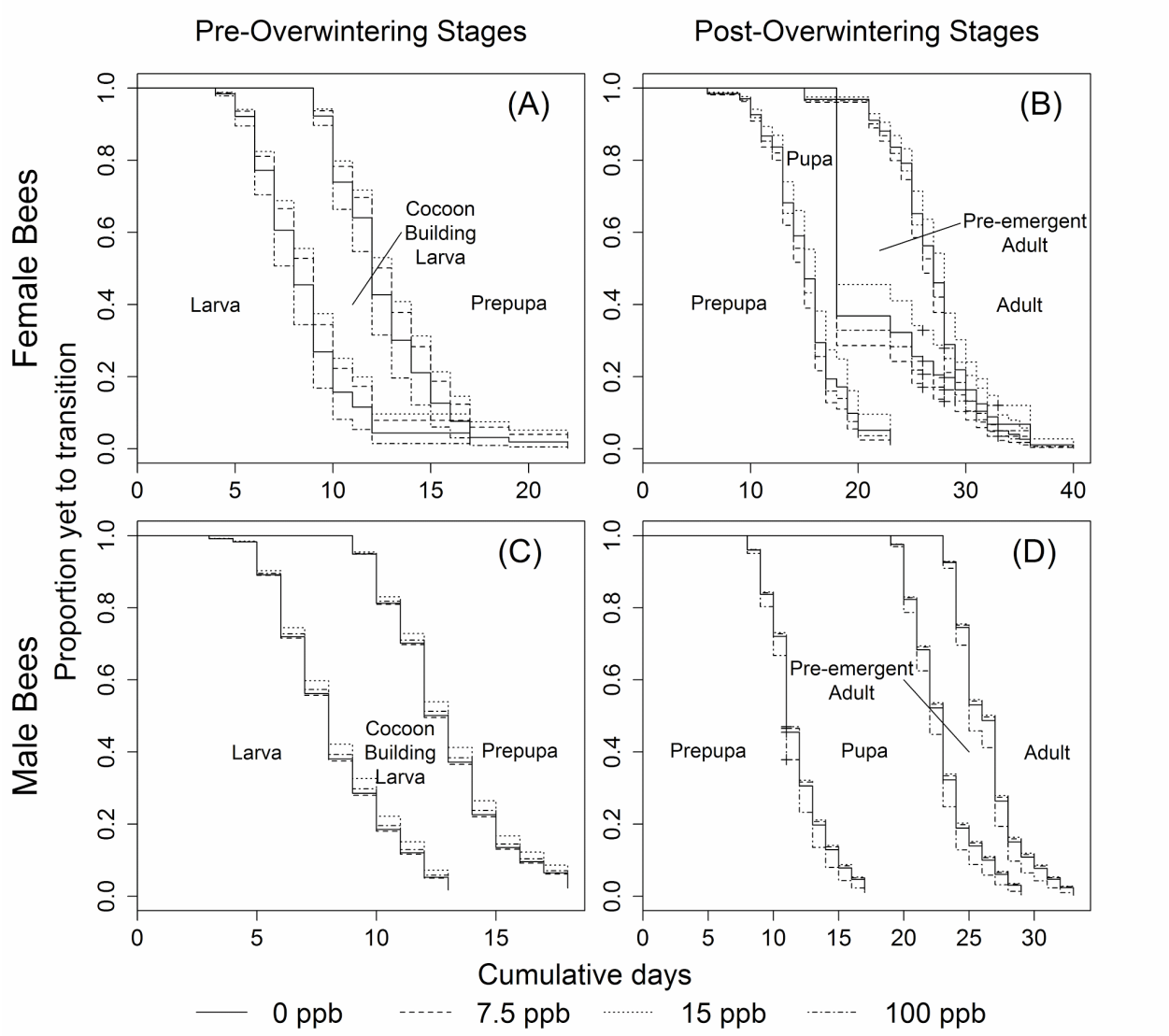

Figure 2 - ***Megachile rotundata*** **development speed.** (A) Time to the beginning of cocoon construction and the prepupal stages (events on the left and right, respectively) for female bees. Individuals treated with 100 ppb developed significantly faster than those treated with 7.5 and 15 ppb. (B) During the post-overwintering period of female bee development (pupal, pre-emergent adult, and adult stages, respectively), bees treated with 7.5 and 100 ppb developed significantly faster than those treated with 15 ppb. (C) There were no observed effects of imidacloprid treatment on male bee development before overwintering. (D) Chronic contact exposure to imidacloprid during development did not have a significant effect on post-overwintering development speed. Although not depicted graphically, time when eggs were collected had a significant effect on post-overwintering development speed (B and D). Crosses represent data that were censored in the model.

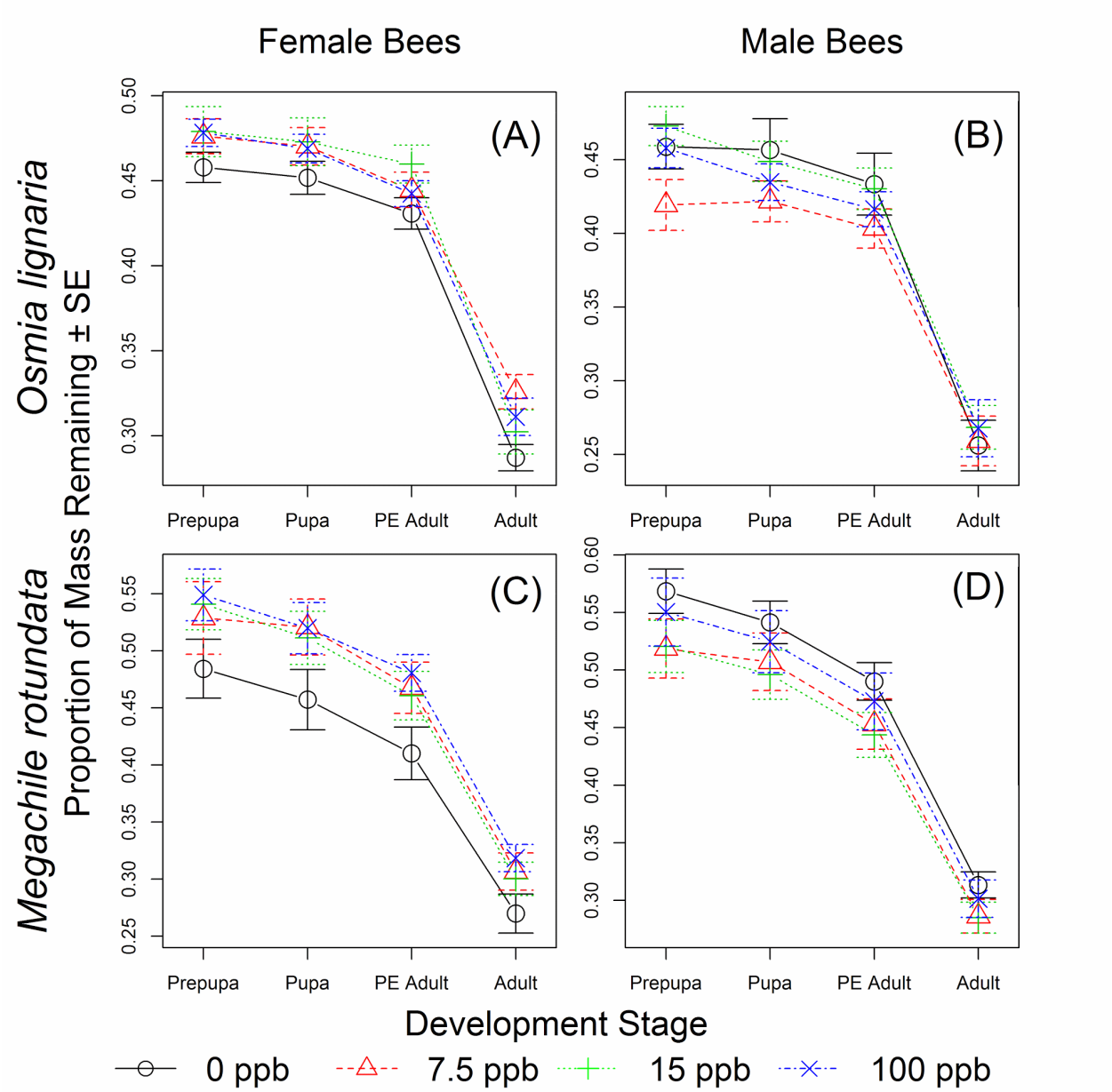

Figure 3 - The proportion of larval provision mass remaining at the start of important developmental stages. There was no significant effect of chronic contact exposure to imidacloprid on the proportion of initial nest cell mass (egg and food provision) remaining for female (A) or male (B) *O. lignaria.* (B) There was also no difference found for male *O. lignaria*. (C) Female *M. rotundata* treated with 100 ppb imidacloprid solution retain a significantly greater proportion of their initial mass than control bees. (D) There were no significant differences between imidacloprid treatment levels for *M. rotundata* males.

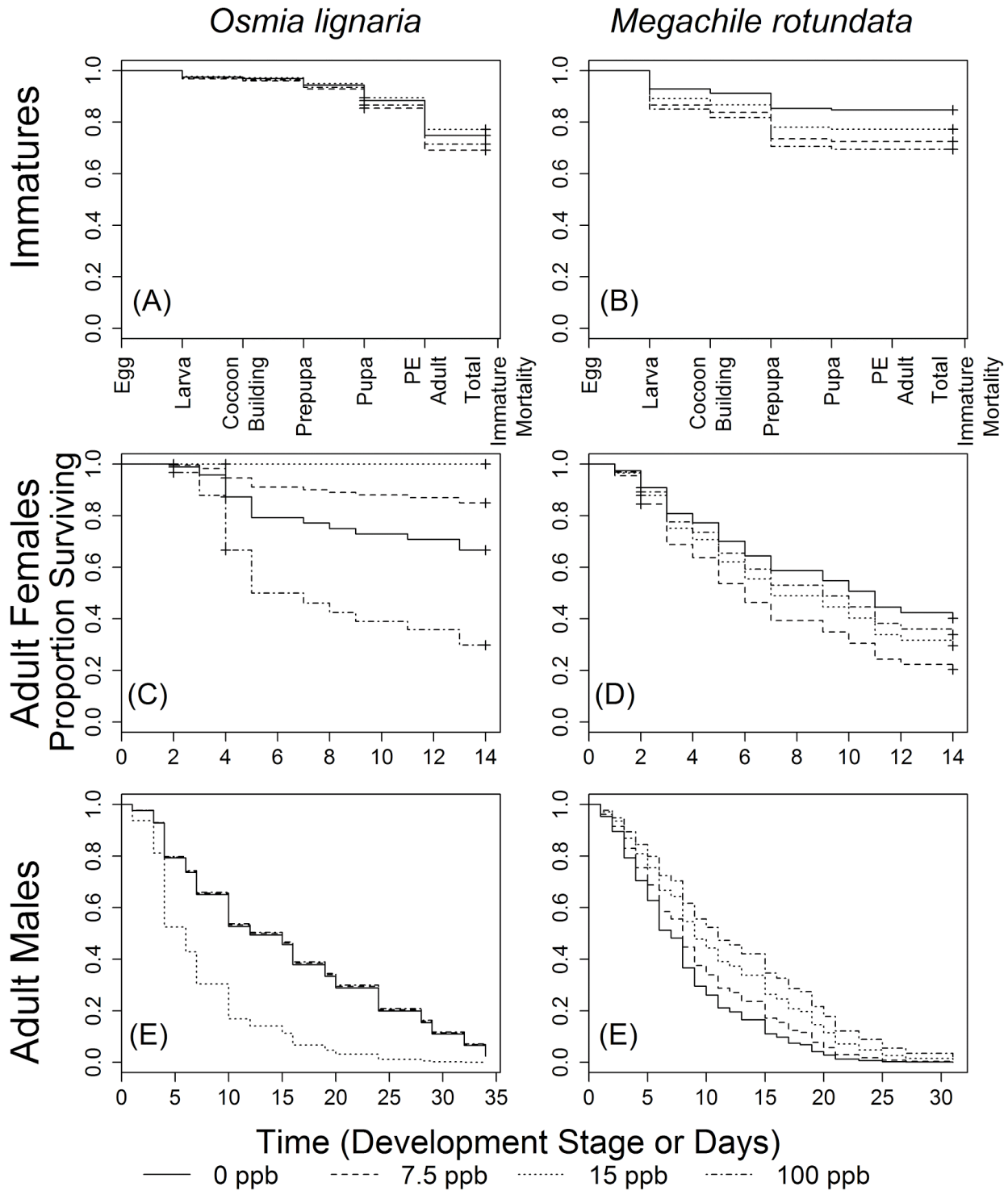

Figure 4 - Effects of chronic contact exposure to imidacloprid on longevity. Longevity of *O. lignaria* (A, C, E) and *M. rotundata* (B, D, F) during the immature (A, B) and adult female (C, D) and male (E, F) life stages. (A-B) Development stages 1-5 represent the start of the cocoon building, prepupal, pupal, pre-emergent adult, and adult life stages. Development stage 6 is only for graphical representation and was not a part of the model. (A) There was no significant effect of imidacloprid treatment on immature *O. lignaria* longevity. (B) Immature *M. rotundata* treated

Figure 4 (cont.) - with 100 ppb had a marginally significant reduction in longevity. (C) Adult female *O. lignaria* treated with 100 ppb had significantly reduced longevity compared to those treated with 0 or 7.5 ppb imidacloprid. There was no mortality in adult females treated with 15 ppb which reduces the power of this analysis when comparing this group to the others. (D) There were no significant differences between imidacloprid treatment levels in adult female *M. rotundata*. (E) No significant differences were detected for adult *O. lignaria* males in response to imidacloprid. (F) *M. rotundata* males treated with 15 and 100 ppb imidacloprid solutions had significantly increased longevity compared to control bees. Crosses represent data that were censored in the model.

Tables

Table 1 - Differences in the ecology of and the methodologies used for *Osmia lignaria* and *Megachile rotundata*. Differences in body size and life history traits of *O. lignaria* and *M. rotundata* led to practical differences in the methods used for each species.

		Osmia lignaria	***Megachile rotundata***
(A)	**Overwintering stage**	Pre-emergent Adult	Prepupa
(B)	**Tissue culture plates used**	24 well (Denville Scientific PN T1024)	Summer 2015 - Spring 2016: 96 well (Denville Scientific PN T1096) Summer 2016: 24 well
(C)	**Nesting substrates provided**	8 mm diameter paper straws (CrownBees) Clay-soil mixture (Clay: CrownBees)	6 and 8 mm diameter paper straws (CrownBees) Assorted leafy plants
(D)	**Number of imidacloprid treatments**	2015: 62 2016: 0 Total: 62 treatments	2015: 26 2016: 9 Total: 35 treatments

Table 2 - Sample sizes. The number of female and male bees, as well as those whose sex was unknown, present at the start of each life stage. Differences in numbers of bees between life stages represent mortality. *Numbers in parentheses represent bees that emerged early during the overwintering period when there was a mechanical failure. These bees were removed from analyses of adult longevity and body mass.

		Osmia lignaria				***Megachile rotundata***			
Life Stage	**Treatment (ppb)**	**Unknown**	**Female**	**Male**	**Total**	**Unknown**	**Female**	**Male**	**Total**
Larvae	**0**	2	30	43	75	8	18	33	59
	7.5	2	33	40	75	14	17	27	58
	15	0	31	41	72	12	13	33	58
	100	2	32	39	73	17	15	26	58
Cocoon Building Larvae	**0**	2	30	42	74	4	18	33	55
	7.5	2	33	39	74	5	17	27	49
	15	0	28	40	68	4	12	33	49
	100	2	32	37	71	11	15	26	52
Prepupae	**0**	1	30	42	73	3	18	33	54
	7.5	2	33	39	74	5	17	27	49
	15	0	28	40	68	3	13	33	49
	100	1	32	37	70	7	15	26	48
Pupae	**0**	1	30	40	71	0	18	32	50

Table 2 (cont.)

		Osmia lignaria				*Megachile rotundata*			
Life Stage	**Treatment (ppb)**	**Unknown**	**Female**	**Male**	**Total**	**Unknown**	**Female**	**Male**	**Total**
Pupae (cont.)	**7.5**	2	32	37	71	0	17	27	44
	15	0	28	40	68	0	12	33	45
	100	1	31	35	67	0	14	26	40
Pre-emergent Adults	**0**	-	28	38	66	-	18	32	50
	7.5	-	28	34	62	-	15	27	42
	15	-	25	37	62	-	12	33	45
	100	-	31	34	65	-	14	26	40
Emergent Adults	**0**	-	22 (6)*	7 (21)*	29 (27)*	-	18	32	50
	7.5	-	17 (10)*	9 (15)*	26 (25)*	-	15	26	41
	15	-	16 (6)*	9 (16)*	25 (22)*	-	12	32	44
	100	-	20 (10)*	5 (14)*	25 (24)*	-	14	26	40

Table 3 - Effects of chronic contract exposure to imidacloprid on bee development speed. Z-scores from the PWP-TT models for between treatment comparisons. OW = Overwintering. (***** $p < 0.05$, ****** $p < 0.01$, ******* $p < 0.001$)

	Osmia lignaria				***Megachile rotundata***			
	Females		**Males**		**Females**		**Males**	
Treatment Comparison (ppb)	**Pre-OW Stages**	**Post-OW Stages**	**Pre-OW Stages**	**Post-OW Stages**	**Pre-OW Stages**	**Post-OW Stages**	**Pre-OW Stages**	**Post-OW Stages**
0 - 7.5	-1.908	0.949	-0.204	-0.593	-0.863	0.752	0.085	-0.324
0 - 15	**-2.195 ***	0.206	0.070	-0.585	-1.170	-1.829	-0.716	-0.675
0 - 100	-0.930	0.240	1.537	-0.457	1.161	0.827	-0.213	1.758
7.5 - 15	0.557	1.119	0.557	-0.148	0.350	**2.138 ***	0.626	0.278
7.5 - 100	0.747	-0.937	1.079	0.085	**2.058 ***	0.189	-0.251	1.850
15 - 100	1.190	-0.016	1.190	0.175	**2.387 ***	**2.253 ***	0.445	1.865

Table 4 - Solitary bee longevity is affected by chronic contact exposure to imidacloprid. Z-scores for the pairwise comparisons of imidacloprid treatment derived using Cox Proportional-Hazards Regression models. (***** $p < 0.05$, ****** $p < 0.01$, ******* $p < 0.001$)

	Osmia lignaria			***Megachile rotundata***		
Treatment Comparison (ppb)	**Immature Life Stages**	**Adult Females**	**Adult Males**	**Immature Life Stages**	**Adult Females**	**Adult Males**
0 - 7.5	0.780	-1.133	-0.035	-1.599	-1.310	0.847
0 - 15	-0.330	-0.003	1.876	-1.028	-0.551	**1.999 ***
0 - 100	0.464	**2.142 ***	-0.049	-1.944	-0.402	**2.535 ***
7.5 - 15	1.083	0.002	-1.728	0.585	-0.823	1.070
7.5 - 100	-0.314	**2.346 ***	0.065	0.371	0.531	-1.644
15 - 100	0.779	0.002	-1.364	0.943	-0.213	-0.840

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Chapter 2: Evaluating the effect of chronic contact exposure during immature development on *Osmia lignaria* and *M. rotundata* adult mushroom body development

Abstract

Neonicotinoid insecticides are a widely used class of neurotoxins whose potential negative sublethal effects on bees have garnered much attention. The most dramatic of these effects are neurological in nature, including the inhibition of adult mushroom body growth - a region of the insect brain involved in processing and recalling sensory and spatial information - after oral exposure as larvae. While these studies have primarily focused on the consumption of contaminated food resources, concentrations of neonicotinoids are often higher in soils and could represent an important route of exposure for ground nesting bees. The aim of this chapter was to determine if chronic contact exposure to imidacloprid - the most widely used neonicotinoid - during immature development also inhibits adult mushroom body growth. To accomplish this, individual female *Osmia lignaria* and *Megachile rotundata* were treated with imidacloprid during their immature development and mushroom body size was assessed for 2 and 14 day old adults. Despite efforts to simulate changing resource availability and quality, control female bee mushroom bodies at 14 days were not larger than those measured at 2 days. Thus, the results of this study are inconclusive with regard to the effects of chronic contact exposure during development on mushroom body growth in adult bees. However, the mushroom bodies of untreated control *O. lignaria* were larger at 2 days than imidacloprid treated bees. While caution is necessary when interpreting this result, it may suggest differences in brain development caused by imidacloprid before adult emergence. Potential implications and suggestions for future research are discussed.

Introduction

Neonicotinoid insecticides represent a widely used class of neurotoxins that bind with high affinity to insect nicotinic acetylcholine receptors (nAChR) in the central nervous system and cause involuntary muscle contractions at low dosages and paralysis and acute death at high dosages (Bai et al. 1991; Elbert et al. 1991; Leicht 1993; Methfessel 1992). These pesticides are predominantly applied as seed coatings and as the plant germinates and grows, the active ingredient is systematically incorporated into plant tissues (Bonmatin et al. 2015; Bromilow & Chamberlain 1995). This provides constitutive protection from pestiferous insects from seedling development through crop harvest (Elbert et al. 1998). However, in addition to the active ingredients being incorporated into tissues targeted by pests, such as stem and leaf tissue, they are also found in pollen, nectar, and guttation drops and can have significant effects on non-target organisms (*see reviews by* Chagnon et al. 2015; Gibbons et al. 2015; Pisa et al. 2015).

Following reports of honey bee maladies in France after the introduction of Gaucho®, the first neonicotinoid product released to the market, interest increased in non-target effects of these pesticides on bees (Schmuck et al. 2001). While no causal link could be established in this circumstance (Schmuck 1999), additional research on this class of insecticides has uncovered a number of sublethal effects on bees. These include delayed larval development (Abbott et al. 2008), effects on reproductive success (Sandrock et al. 2014; Straub et al. 2016; Whitehorn et al. 2012; Wu-Smart & Spivak 2016), and impairments to neurological function (Decourtye et al. 2003; Tomé et al. 2012). This is still an actively growing area of research, but already one of the common themes is the reduction of neurological function.

One of the brain regions that neonicotinoids may affect are insect mushroom bodies. Mushroom bodies function in the processing, integrating, and memory of sensory information

received via the visual, mechanosensory, and olfactory systems (Davis 1993; De Belle et al. 1994; Dubnau et al. 2001; Hammer & Menzel 1998; Liu et al. 1999; Mizunami et al. 1993; Mizunami et al. 1998; Mobbs 1982; Mobbs 1984; Strausfeld et al. 1998; Vowles 1964) and are thought to play a role in movement, pattern recognition, and spatial orientation (Mizunami et al. 1993; Mizunami et al. 1998; Strausfeld et al. 1998; Vowles 1964). In at least some insects, including honey and blue orchard bees (*Apis mellifera* and *Osmia lignaria*, respectively), the size of an individual's mushroom bodies increase with experience and this is thought to directly relate to the amount of stored information (Heisenberg et al. 1995; Withers et al. 1993; Withers et al. 2008). Tomé et al. (2012) found that in the stingless bee *Melipona quadrifasciata anthidioides*, adult mushroom body growth was impeded when they were fed a neonicotinoid contaminated diet as larvae. While oral exposure via contaminated food provisions may represent one route of exposure for developing bees, the levels found in soils are often higher (Bonmatin, Marchand, et al. 2005; Bonmatin et al. 2015; Donnarumma et al. 2011; Schmuck et al. 2001) and may pose a serious risk to the vast majority of bee species which nest below ground (O'Toole & Raw 1991).

The impacts of neonicotinoid contamination of nest sites have not been investigated (Pisa et al. 2015) despite the known accumulation and persistence of neonicotinoids in soils at concentrations that are commonly higher than in pollen and nectar (Bonmatin, Moineau, et al. 2005; Bonmatin et al. 2015; Donnarumma et al. 2011; Schmuck et al. 2001). Additionally, the small body size of many ground nesting bees and their extended development time in subterranean nests increases their risk of effects as neonicotinoids are more toxic to smaller, solitary bees (Bailey et al. 2005; Scott-Dupree et al. 2009) and the toxicity of these insecticides is amplified by exposure time (Charpentier et al. 2014; Suchail et al. 2001). The combination of

these factors suggests that contaminated nesting resources may represent an important and understudied route of exposure for most bees.

The aim of this study was to determine if chronic contact exposure to realistic soil concentrations of imidacloprid - the most widely used neonicotinoid pesticide and the one that is considered the archetype of the compounds in this class (Charpentier et al. 2014; Jeschke et al. 2011) - causes inhibition of adult mushroom body growth. I hypothesized that the mushroom bodies of adult female bees treated with imidacloprid during development would exhibit reduced growth of this important brain region. Reduced growth of mushroom bodies would be indicative of deficiencies in behaviors associated with these structures - such as olfactory and spatial memory - and could have significant ecological and agricultural consequences.

Methods

Study organisms - To assess the effects of chronic contact exposure to imidacloprid on ground nesting bee brain mushroom body growth, the cavity nesting bees *Osmia lignaria* Say, 1837 and *Megachile rotundata* (Fabricius, 1787) were used. Previous studies have used these species to approximate the response of ground nesting bees to environmental stressors (Cane & Neff 2011) as they are closely related to ground nesting species and easier to obtain with trap nests. Additionally, Withers et al. (2008) demonstrated the plasticity of *O. lignaria* mushroom bodies and that these structures grew larger with increased bee experience.

Imidacloprid treatment and bee rearing - Treatment of larval bees with imidacloprid is described in detail in chapter 1 of this thesis. Briefly, starting once larvae began to consume and move about their pollen provisions, 0.5 µL of a 0 or 100 ppb imidacloprid (Sigma-Aldrich, PN 37894) and saline (Equate Sterile Multipurpose Solution, PN 68113173188) solution was applied topically to the abdominal segments every 48 hours. Saline was used instead of distilled water

because it is less detrimental to larval bees (Craig Huntzinger, *personal communication*). This was done until bees were placed in a 4°C cold room to overwinter.

During the spring and summer of 2016, bees were removed from the cold room to immediately emerge (*O. lignaria*) or continue their development (*M. rotundata*). Because *O. lignaria* emerge as adults soon after they are exposed to warm temperatures they were not treated with imidacloprid after they were removed from the cold room. Conversely, immature *M. rotundata* were treated with a second round of chronic topical imidacloprid treatment during the summer of 2016 as these bees undergo a number of development stages - pupa, pre-emergent adult, and emergent adult - after their period of dormancy. In order to keep the number of treatments consistent between individual *M. rotundata*, imidacloprid was no longer applied once the first adult bee emerged, regardless of development stage.

Following emergence, adult bees were placed in 85 L tote containers (Sterilite 90 qt PN 1666) grouped by treatment and species. Because it is known that the size of *O. lignaria* mushroom bodies is dependent on experience, I attempted to simulate a number of natural conditions within the enclosures. Nutritional resources were provided in an artificial flower array that contained four flowers offering *Typha sp.* pollen (YellowPollen.net, Kirkland, WA) and four flowers offering sucrose water rewards of differing quality and olfactory and visual cues. Similar resources have been used in the lab rearing of adult bees previously (Greenberg 1982; Roulston & Cane 2002; Emily Dobbs, *personal communication*). The association between highly rewarding 2.0 M sucrose solutions and less rewarding 1.0 M sucrose solutions and flower color (blue, orange, pink, and purple plastic flowers) and scent (*Eugenia caryophyllata*, *Mentha spicata*, *Gaultheria procumbens*, and *Cymbopogon flexuosus* essential oils) was changed every four days. Additionally, the location of each flower within the artificial array was randomized at

the same interval. This simulated changes in quality and spatial organization of nutritional resources in the natural environment. Nesting substrates (nesting tubes and nesting mud or leafy plant material; *see* Table 1C in Chapter 1) were also available in these containers. There was one observed attempt to provision a nest cell during the course of this experiment (an *O. lignaria* female treated with 15 ppb imidacloprid), but the pollen provision was not completed and no egg was laid.

Measuring mushroom bodies - Morphometric analysis of the mushroom bodies of female adult bees was achieved using the methodology described in Tomé (2012). Brains were dissected from bees 2 or 14 days after emergence (n = 5) in 4°C 0.1 M phosphate-buffer saline (PBS, pH 7.4; Corning PN 21-040) and fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich PN P6148) in 0.1 M PBS for at least 24 hours at 4°C. After fixation, brains were pre-embedded in HistoGel (Richard-Allan Scientific, San Diego, USA; PN HG-4000) to help preserve morphological features and to aid in orienting the brains during the embedding process.

Brains were embedded using a JB-4 Embedding Kit (Polysciences, Inc., Warrington, PA, USA; PN 00226) according to the manufacturer's specifications. Samples were dehydrated using mixtures of 100% ethanol and infiltration solution - JB-4 monomer and benzoyl peroxide (catalyst). These solutions were 50 : 50, 25 : 75, and 10 : 90 ethanol : infiltration solution. Samples were placed on a low-speed shaker for 30 min for each solution change. After the samples were dehydrated, they were infiltrated with JB-4 monomer by placing in three rinses of 100% infiltration solution for at least 30 minutes each. Finally, samples were embedded in JB-4 historesin (infiltration solution with an accelerator added) under a light vacuum at 4°C for 24 hrs.

After being embedded in plastic, brains were cut into 7 μm-thick serial cross sections on an automatic microtome (Histo Range Microtome RN LKB 2218) with a glass knife. Due to an

issue related to JB-4 polymerization, one of the 2 day, 0 ppb, *O. lignaria* brains was damaged during sectioning (n = 4). Sections were stained with Modified Harris Hematoxylin (Richard-Allan Scientific PN 72711) and Eosin-Y with Phloxine (Richard-Allan Scientific PN 71304) and photographed using a digital camera-equipped light microscope (Zeiss Axio Imager.A2 PN 490022). After randomly selecting one of the first four sections in which the mushroom bodies appeared, I measured the area of the medial lobe, vertical lobe, peduncle and lateral and medial calyces using the program Image-J (Rasband 1997-2016). The total volume of the mushroom bodies was determined using the Cavalieri method (Gundersen & Jensen 1987). This is a well-studied and verified method for determining the volume of morphological structures (*see* Fahrbach & Robinson 1996). Due to large variances in the measurements using this method, I also calculated the mean of the three largest sections, by cross-sectional area, as another measure of brain size.

Statistical analyses - The effects of imidacloprid treatment and time on mushroom body size - volume and cross-sectional area - were analyzed separately for *O. lignaria* and *M. rotundata* using two-way ANOVAs. If a significant interaction existed, Tukey's HSD was applied. All analysis were done using the statistical program R (R Core Team 2014).

Results

For *O. lignaria* female brains, mushroom body volume was significantly larger 2 days after emergence than at 14 days ($F_{1,15}$= 8.531, p = 0.011) and bees treated with 0 ppb imidacloprid had significantly larger mushroom bodies than those treated with 100 ppb ($F_{1,15}$= 5.146, p = 0.038; Figure 5A). The interaction term between time since adult emergence and imidacloprid treatment was also significant ($F_{1,15}$= 9.375, p = 0.008). The post-hoc analysis of this term revealed that mushroom bodies were significantly larger in control bees at 2 days after

emergence than at 14 days ($p = 0.003$) and compared to bees treated with imidacloprid at both 2 and 14 days ($p = 0.009$ and $p = 0.007$, respectively) after emergence. Control bees at 14 days did not have significantly different mushroom body volume than either 2 or 14 day old bees treated with 100 ppb imidacloprid ($p = 0.941$ and $p = 0.969$, respectively). Bees treated with chronic contact exposure to 100 ppb imidacloprid did not have significantly different mushroom body volumes between 2 and 14 days post adult emergence ($p = 0.999$).

Similar results were recorded for the mean cross-sectional area obtained from the three largest sections of *O.lignaria* female brains. While there was no significant difference in cross-sectional area between the 0 and 100 ppb imidacloprid treatments when pooled across adult bee ages ($F_{1,15}= 4.535$, $p = 0.050$), brains from 2 day old adult bees were significantly larger than those from 14 day old bees regardless of imidacloprid treatment ($F_{1,15}= 5.590$, $p = 0.032$; Figure 5B). The interaction between imidacloprid treatment and bee age was also significant ($F_{1,15}= 8.836$, $p = 0.009$). The mean cross-sectional area of the three largest sections of mushroom bodies of 2 day old bees not treated with imidacloprid was significantly greater than the same structure in 14 day old control bees ($p = 0.009$) and in 2 and 14 day bees treated with the 100 ppb imidacloprid solution ($p = 0.010$ and $p = 0.018$, respectively). There were no significant differences between 14 day old control bees and 2 or 14 day old imidacloprid treated bees ($p = 0.999$ and $p = 0.978$, respectively). Mushroom bodies from bees treated with imidacloprid were not significantly different, in terms of mean cross-sectional area, at 2 and 14 days old ($p = 0.988$).

In *M. rotundata* female brains, no significant differences were observed for mushroom body volume related to the number of days since emergence ($F_{1,11}= 0.215$, $p = 0.652$), imidacloprid treatment ($F_{1,11}= 0.536$, $p = 0.479$), or the interaction of these factors ($F_{1,11}= 0.557$,

p = 0.471; Figure 5C). However, as in the *O. lignaria* data, there was considerable variance in the volume of mushroom bodies of control bees that had their brains removed two days after adult emergence. Likewise, when the response variable was the mean cross-sectional area of the three biggest sections of female *M. rotundata* brains, there was no significant difference between the age of adult bees ($F_{1,11}$= 0.025, p = 0.878), imidacloprid treatment ($F_{1,11}$= 0.359, p = 0.561), or the interaction between these variables ($F_{1,11}$=0.626, p = 0.446; Figure 5D).

Discussion

The results of this study suggest that how imidacloprid affects adult bee mushroom body size can vary between species and depend on when the measurements are taken. While *M. rotundata* mushroom body size does not appear to change with imidacloprid treatment or adult age, those of *O. lignaria* appear to respond to both. Consistent with my original hypothesis, the mushroom bodies of bees treated with imidacloprid did not grow over time. However, the mushroom bodies of control *O. lignaria* were larger than expected at 2 days and appear to have decreased in size over time until they reached roughly the same size of those of treated bees.

While Tomé et al. (2012) did not observe differences in mushroom body volume early after adult emergence, the result for *O. lignaria* here suggest that chronic contact exposure to imidacloprid inhibits brain development before bee emergence. The discrepancy between these studies may be attributed to differences in the life histories of the study species. Unlike *O. lignaria*, which begins foraging and provisioning nest cells shortly after the onset of its adult lifestage, *M. quadrifasciata anthidioides* is eusocial and spends many of its first days as an adult inside the nest before gaining the ability to fly and subsequently foraging for pollen and nectar. Since mushroom bodies have been indicated as being important for movement, pattern recognition, and spatial orientation (Mizunami et al. 1993; Mizunami et al. 1998; Strausfeld et al.

1998; Vowles 1964), it makes intuitive sense that a larger portion of mushroom body development would occur prior to adult emergence in solitary species such as *O. lignaria* that may require immediate function of this brain region upon emergence. Others have also suggested that the brains of solitary bees are more developed at adult emergence than those of eusocial species (Withers et al. 2008). However, this is still an area that requires further exploration that could have implications for how we study the effects of xenobiotics and other factors related to bee brain development.

The smaller mushroom bodies observed in 14 day old control *O. lignaira* compared to the 2 day old bees of the same species are more difficult to explain and, to my knowledge, younger adult bees with larger mushroom bodies than older individuals has not been reported previously for bees (*although, see* Julian & Gronenberg 2002 *for an example in ant queens*). Given that brain tissue is energetically expensive to maintain (Aiello & Wheeler 1995; Isler & van Schaik 2006), it is possible that the difference in size is an artifact of the simplistic artificial conditions provided and that brain volume was lost over time. However, when Withers et al. (2008) reared adult *O. lignaria* under even simpler lab conditions, they reported that mushroom body size did not change over time. Moreover, the three large *O. lignaria* brains driving this trend have larger mushroom bodies than those measured by Withers et al. (2008) while those of 14 day old control bees and imidacloprid treated bees of both ages more closely align with the results of this previous study. It is unclear if these bees had abnormally large mushroom bodies by chance alone and thus, because of my small sample sizes, they are implying differences that are not due to imidacloprid exposure or if there is an unknown physiological mechanism underlying the observed difference. Caution must be exercised when trying to generalize these results.

Future projects could use more natural or outdoor conditions for foraging to help improve our understanding of the effects of chronic contact exposure to neonicotinoids during development on neurological growth. Previous research has demonstrated that the growth of solitary bee mushroom bodies is dependent on experience (Withers et al. 2008). Bees were kept in the laboratory due to the uncertainty about recapturing them after release and ethical concerns related to releasing the western subspecies *O. lignaria propinqua* outside of its native range. However, my attempts to replicate a natural system by altering resource availability and distribution over time did not appear to have the desired stimulatory effect on brain development. Future studies should not underestimate the immense difficulty associated with replicating natural conditions in studies designed to look at mushroom body growth.

As discussed in chapter 1, this study does not account for the role of nest cell linings and the protection they might provide for immature bees. However, the presence and structure of these linings vary both within and between species making it difficult to generalize the properties of these barriers (Brooks & Cane 1984; Eickwort & Eickwort 1971; Wille & Orozco 1970). Additionally, water from the surrounding soil is thought to cross these membranes, be absorbed by the food provisions, and account for some of the mass gained by developing bees (May 1972). It seems reasonable to hypothesize that if water is able to cross this barrier, then molecules dissolved in water may also cross it and come into contact with developing bees and their food provisions. Further research is needed to determine the level of protection nest cell linings provide and to evaluate the effects of soil contaminants on ground nesting bee communities in the field.

On a methodological note, the similar statistical results and interpretation of using the mean of the three largest cross-sectional areas versus whole structure volume suggests that

relative mushroom body size can be assessed using a more efficient method. Provided that brain orientation relative to the cutting surface is consistent, the number of sections that need to be stained, photographed, and measured is greatly reduced by focusing only on the largest sections. This would translate to fewer resources required to process each sample and may allow for a greater number of samples to be processed overall. While this methodology needs to be evaluated more rigorously for both these and other species, it has promise as a way to increase efficiency and sample sizes where time and budget are limited.

Despite the limitations described above, the results of this study suggest that chronic contact exposure to soil realistic levels of imidacloprid during immature development may have an impact on adult brain morphology. Combined with information from chapter 1, this previously unexplored route of exposure for ground nesting bees could have significant effects on bee communities and, thus, ecosystem services and stability. This may be particularly important in areas of recent restorations from agricultural fields and areas of high quality floral resources, such as field borders, located adjacent to active crop fields. If bees are unable to avoid contaminated nesting resources, areas intended to support pollinator communities may serve as ecological traps and have unintended negative consequences.

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Figures

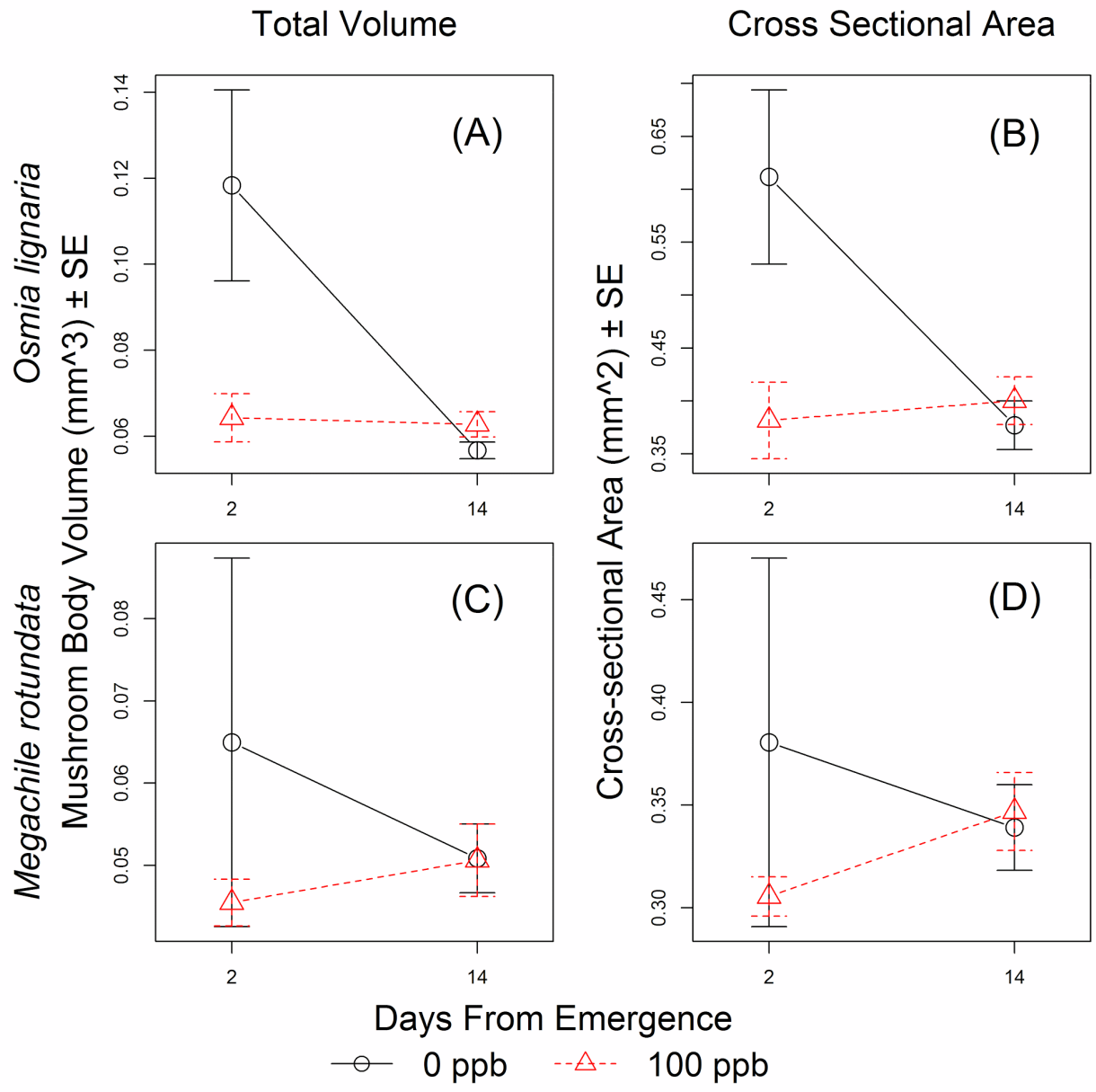

Figure 5 - Adult female bee mushroom body size. (A) The mushroom bodies of female *O. lignaria* were significantly more voluminous 2 days after emergence than for bees treated with imidacloprid after either length of time and 14 day old control bees. (B) The average cross-sectional area of the three largest sections was significantly greater for 2 day old control bees than for bees treated with 100 ppb of either age and control bees that were 14 days old. (C) No significant differences were detected in mushroom body volume for female *M. rotundata*. (D) The same was true for female *M. rotundata* when average cross-sectional area was used as the response variable.

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