



# Chronic contact with imidacloprid during development may decrease female solitary bee foraging ability and increase male competitive ability for mates

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## ARTICLE INFO

Handling editor: Michael Bank

**Keywords:**  
Solitary bee  
Imidacloprid  
Movement  
Mushroom body  
Chronic contact  
Nesting resources

## ABSTRACT

Environmentally persistent xenobiotics, such as neonicotinoid insecticides, are thought to contribute to insect declines. Much of what is known about the non-target effects on bees comes from oral exposure in eusocial species. However, most bee species are solitary and nest below ground. For them, contaminated nesting resources may represent an important, yet understudied, route of exposure. We examined the effect of chronic contact exposure with realistic soil concentrations of the neonicotinoid imidacloprid (0, 7.5, 15, or 100 ppb) during immature development on adult locomotion (movement speed and distance) and brain development of the solitary bees *Osmia lignaria* and *Megachile rotundata*. Adult locomotion and mushroom body morphology were characterized 2 (females) or 4 (males) and 14 (both sexes) days after emergence. Unlike the 0 and 7.5 ppb groups, female *O. lignaria* treated with 15 and 100 ppb did not move faster with age. If movement speed is associated with foraging or nest-building ability, this could result in a 25% reduction in nest provisioning efficiency over the first 14 days. Young male *M. rotundata* moved more quickly (7.5 and 100 ppb) and farther (100 ppb) when treated with imidacloprid, potentially increasing their ability to compete for more receptive female bees. We did not detect an effect of imidacloprid on the relative volumes of the neuropil and Kenyon cell sub-regions. We discuss how an environmentally persistent xenobiotic has the potential to alter population dynamics through changes in adult locomotion, even in the absence of a detectable effect on gross brain morphology.

## 1. Introduction

Human use of xenobiotics is thought to be one of the driving forces behind insect declines (Forister et al., 2019; Frampton and Dorne, 2007; Sánchez-Bayo and Wyckhuys, 2019). Bees have received special attention due to the economic and ecological services they provide (Goulson et al., 2015; Potts et al., 2010). However, much of what we know about xenobiotics' effects on bees comes from research on a few managed eusocial species (Pisa et al., 2017). Although such research is vital for measuring impacts on apiculture and in crop systems that currently depend on managed bees for pollination, these eusocial species differ in important ways from most bee species and likely do not adequately capture non-target effects experienced by other taxa (Scott-Dupree et al., 2009; Sgolastra et al., 2019). Over 80% of bee species in North America nest underground (Harmon-Threatt, 2020) and, unlike *Apis mellifera*, may be at risk of chronic contact with xenobiotics during immature development and adult nesting via contaminated soils. Additionally,

many of these species are solitary or exhibit less complex social behaviors, meaning the effects of xenobiotics on reproductive individuals are not buffered by workers and could have more substantial impacts on fitness and population growth (Stow et al., 2007; Straub et al., 2015). Evaluating the non-target effects of xenobiotic use on bee populations and communities requires an understanding of exposure risks and consequences over an individual's lifespan, including the role of contaminated nesting resources.

Neonicotinoids are a class of widely used neurotoxic insecticides that persist in the environment (reviewed by Goulson, 2013) and have been implicated in population declines of native bees (Woodcock et al., 2016). Neonicotinoids are commonly applied as seed coatings before sowing to provide systemic protection as the plant grows (Bromilow and Chamberlain, 1995; Simon-Delso et al., 2015). While the active ingredients are incorporated into target plant tissues (i.e., leaves, stems, and roots) and non-target plant parts that bees use (i.e., pollen, nectar, and guttation drops (Bonmatin et al., 2015, 2005a; Girolami et al.,

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2009)), treated plants only absorb 0.7–20% of the applied active ingredient (Sur and Stork, 2003). The remaining 80–99.3% accumulates in the environment, in part due to relatively long half-lives in soils (28–1250 days for imidacloprid, 7–353 days for thiamethoxam, and 148–6931 days for clothianidin; reviewed in Goulson, 2013). As a result, soil concentrations in active and former agricultural fields and neighboring semi-natural areas can be 100x higher (commonly 12–18 ppb and up to 650 ppb) than those reported in pollen and nectar (1–11 ppb) (Bonmatin et al., 2005a, 2005b, 2015, 2005a; Donnarumma et al., 2011; Jones et al., 2014; Schmuck et al., 2001; Willis Chan et al., 2019). These concentrations affect other soil fauna including increased mortality in Collembola (van Gestel et al., 2017), changes in nematode community composition (Cerevková et al., 2017), and reduced fecundity and growth in earthworms (Baylay et al., 2012; Gomez-Eyles et al., 2009; Wang et al., 2015). For solitary bees, chronic contact with field-realistic concentrations of the common neonicotinoid imidacloprid during development resulted in sex- and species-specific effects on development speed, mass, and adult longevity (Anderson and Harmon-Threatt, 2019), suggesting contaminated soils could produce consequential sublethal effects (Willis Chan and Raine, 2021).

In addition to the lethal effects of bee exposure to neonicotinoids (defined as increased mortality within 24–48 h of exposure), multiple sublethal effects on the nervous system have been described. These effects can be broadly classified into two categories: those that impact locomotory function and those that affect brain development. Neonicotinoids lower bee locomotory function resulting in fewer foraging trips and flower visits (Karahan et al., 2015) and reduced flight distance and velocity (Tosi et al., 2017). Neonicotinoid oral exposure also reduces long-term memory (Alkassab and Kirchner, 2016; Tan et al., 2015) and olfactory learning in bees (Wright et al., 2015; Yang et al., 2012). These effects may be related to abnormal connectivity and nervous system development in the mushroom bodies (Peng and Yang, 2016) – the region of the insect brain associated with olfactory, visual, and mechanosensory processing and learning (Davis, 1993; de Belle and Heisenberg, 1994; Dubnau et al., 2001; Hammer and Menzel, 1998; Liu et al., 1999; Mizunami et al., 1993, 1998, 1993; Mobbs, 1982, 1984, 1982; Strausfeld et al., 1998; Vowles, 1964). When examining movement and brain development responses simultaneously, Tomé et al. (2012) found these effects can co-occur; stingless bees (*Melipona quadrifasciata anthidioides*) fed neonicotinoids as larvae moved slower and shorter distances, and also showed reduced brain development as adults. Despite widespread reporting of the effects on bee nervous systems due to oral exposure, little is known about the chronic contact risks associated with contaminated soils.

We determined if chronic contact of immature life stages with imidacloprid, both one of the most well-studied and widely used neonicotinoid pesticide (Charpentier et al., 2014; Jeschke et al., 2011; Pisa et al., 2017), alters adult locomotory characteristics and impairs brain development of solitary bees. We quantified adult locomotion - mainly walking but also some restricted attempts at flight - as movement speed and distance traveled in timed trials in young and old adult bees. Female bees that move slower and shorter distances likely have access to fewer resources and provision fewer brood cells, whereas such males may be less competitive for mates (Greenleaf et al., 2007; Paxton, 2005; Stefan-Dewenter and Schiele, 2008). In terms of adult brain development, we measured the relative volumes of the neuropil and Kenyon cell subregions of the mushroom bodies at the same two adult ages. The volumes of these structures have been previously reported to change with adult experience and learning (Heisenberg et al., 1995; Withers et al., 1993, 2008). This study provides the first insights into the effects of chronic contact with realistic soil concentrations of imidacloprid on solitary bee neurological function and the role this exposure may play in bee declines in agricultural landscapes.

## 2. Methods

### 2.1. Study species

We used *Osmia lignaria* Say, 1837 and *Megachile rotundata* (Fabricius, 1787) (Hymenoptera: Megachilidae) to assess the effects of chronic contact exposure to imidacloprid on movement characteristics and brain development of ground-nesting bees. While often characterized as nesting above ground due to their willingness to adopt human-provided nesting materials, multiple reports indicate *O. lignaria* nests in soils (Levin, 1966; Linsley and MacSwain, 1941; Rau, 1937) and a recent review found that 92% of *Megachile* species with available nesting data were described as nesting belowground, including several observed excavating their own nests (Harmon-Threatt, 2020). As such, these species may be better categorized as "opportunistic nesters" that use available cavities both above and below ground. Physiological and behavioral differences between the opportunistic nesters observed here and ground-nesting bees are unknown or poorly documented, so we had no a priori reason to expect results from this experiment to not apply to either group and, thus, *O. lignaria* and *M. rotundata* may represent reasonable proxies for the responses of ground-nesting species to nest stressors (e.g., Cane and Neff, 2011). Additionally, these species are readily available for purchase, making it easier to obtain eggs and early instar larvae than other potential study species and *O. lignaria* has been used to describe experience-dependent changes in brain mushroom body morphology in solitary bees (Withers et al., 2008). Further, *O. lignaria* use mud partitions to separate their brood cells which may expose larvae to soil contaminants even in aboveground nests (Fortuin et al., 2021). We acknowledge that many ground-nesting bees line their nest cells and that linings may protect immature life stages from xenobiotics in the soil. However, lining use and properties vary between and within bee species making generalizations difficult (reviewed in Harmon-Threatt, 2020). The current study represents the scenario of exposure to soil contaminants in unlined nests.

### 2.2. Imidacloprid application

We purchased wild-caught, newly laid eggs and early instar larvae from Crown Bees (Seattle, WA) during the spring (*O. lignaria*) and summer (*M. rotundata*) of 2015 (Table 1). Individual bees from the same nest were stratified across treatments to reduce the potential for genetic biases when evaluating bee responses to imidacloprid (Rinkevich et al., 2015; Schmuck et al., 2001).

Starting with the second larval instar life stage, we applied 0.5 µL of 0, 7.5, 15, or 100 ppb imidacloprid (Sigma-Aldrich, PN 37894) in saline solution (Equate Sterile Multipurpose Solution, PN 68113173188) topically to the abdominal segments every 48 h using a micropipette. Once cocoons were spun, treatments were applied to the cocoon nearest to the abdominal segments until the first individual eclosed. Cocoons appeared to absorb the applied imidacloprid solution. The concentrations used reflect commonly recovered (7.5 and 15 ppb) and higher, less common (100 ppb) levels reported from soils within two years of the most recent imidacloprid application (Bonmatin et al., 2005b; Donnarumma et al., 2011; Jones et al., 2014; Schmuck et al., 2001). Imidacloprid solutions were replaced every 96 h and kept in the dark at room temperature. Individuals were treated until they were placed in overwintering conditions (4 °C) in the fall to simulate dry conditions when imidacloprid may be less mobile within soils (Gupta et al., 2002) and resumed for *M. rotundata* in the spring of 2016 as they finished their immature development. We did not treat *O. lignaria* in the spring of 2016 as they overwintered as adults and emerged shortly after being removed from overwintering conditions. To keep the number of applications consistent among individuals, we stopped applying imidacloprid to a species when the first individual of that species eclosed. Individual *O. lignaria* and *M. rotundata* that survived to adulthood received 62 (all before overwintering) and 35 (26 before overwintering and 9 after) applications,

**Table 1**

Detailed sample sizes.

Species	Imidacloprid concentrations (ppb)	Eggs and early instar larvae	Sex	2 day brains	14 day brains	2 or 4 day movement trials <sup>a</sup>	14 day movement trials
<i>Osmia lignaria</i>	0	30	Female	4	6	26	14
	7.5	33	Female	4	5	20	12
	15	31	Female	4	5	19	13
	100	32	Female	5	5	22 <sup>d</sup>	6
	0	59	Female	4	5	17	5
<i>Megachile rotundata</i>	0	59	Male	–	–	21	5
	7.5	58	Female	0 <sup>b</sup>	3 <sup>b</sup>	9	3
	15	58	Male	–	–	17 <sup>d</sup>	8
	100	58	Female	3 <sup>b</sup>	2 <sup>b c</sup>	12	3
			Male	–	–	28	12
			Female	3	3	12	3
			Male	–	–	21	8

<sup>a</sup> 2 for female bees and 4 for male bees. See Methods section.<sup>b</sup> *Megachile rotundata* females treated with 7.5 and 15 ppb were pooled for analysis.<sup>c</sup> One brain was damaged during sectioning and unavailable for measurement and analysis.<sup>d</sup> One bee did not move during the 10-min trial. It was included as 0 cm for distance traveled and excluded from the analysis of movement speed. See Methods section.

respectively. This led to cumulative doses of 0, 0.233, 0.465, and 3.1 ng a.i. bee<sup>-1</sup> for *O. lignaria*, and 0, 0.131, 0.263, and 1.75 ng a.i. bee<sup>-1</sup> for *M. rotundata* dependent upon treatment concentration of 0, 7.5, 15, and 100 ppb. Additional details about immature rearing conditions and the effect of chronic contact to imidacloprid on mortality, development speed, and mass are presented in [Anderson and Harmon-Threatt \(2019\)](#).

### 2.3. Adult holding conditions

We placed adult bees in 85 L containers (Sterilite PN 1666) grouped by imidacloprid concentration (0, 7.5, 15, and 100 ppb) and species ([Supplemental Figure 1](#)). Containers were placed in a temperature-controlled room (held at 24 °C for *O. lignaria* and 28 °C for *M. rotundata* to mimic temperatures corresponding to their natural active periods) under fluorescent lights (Philips 32 Watt Alto II PN F32T8/ADV835) set to a 14:10 light:dark cycle. Containers were rotated daily to reduce any effects of location within the temperature-controlled room on adult bees. Within each container, an artificial flower array that contained four flowers offering *Typha* sp. pollen ([YellowPollen.net](#), Kirkland, WA) and four flowers offering sucrose water provided nutritional resources. Similar resources have been used effectively in laboratory holding of adult bees ([Greenberg, 1982](#); [Roulston and Cane, 2002](#); Emily Dobbs, personal communication). To simulate variation in resource quality and spatial arrangement, every four days, we randomized flower reward (*Typha* sp. pollen or 1.0 or 2.0 M sucrose solution), color (blue, yellow, orange, pink, and purple plastic flowers), and scent (*Eugenia caryophyllata*, *Mentha spicata*, *Gaultheria procumbens*, and *Cymbopogon flexuosus* essential oils). The arrangement of the arrays was consistent across containers. We provided nesting resources in the form of uncontaminated prairie soil mixed with clay (Spring Mason Bee Mud Mix, Crown Bees, Seattle, WA) for *O. lignaria* or assorted leafy vegetation for *M. rotundata*, and an assortment of cardboard nesting tubes (6 and 8 mm diameters, Crown Bees, Seattle, WA). Despite access to nesting substrates and an abundance of pollen and sucrose resources, we only observed a single attempt to provision a nest cell by an *O. lignaria* female in the 15 ppb treatment group. However, she did not complete the pollen provision or lay an egg.

### 2.4. Movement assessment

Female bees underwent movement assays 2 and 14 days after adult emergence. As resource acquisition by female bees limits the rate of population growth ([Steffan-Dewenter and Schiele, 2008](#)), we initially focused on female bee movement. During the *M. rotundata* trials (and after all *O. lignaria* trials were complete), [Straub et al. \(2016\)](#) published a study demonstrating the adverse effects of neonicotinoids on male bees.

We decided to add male movement assays to our study, but many males were already older than 2 days. Accordingly, we assessed male movement 4 and 14 days after adult eclosion. Because male bees tend to emerge before female bees ([Eickwort and Ginsberg, 1980](#); [Szentgyörgyi and Woyciechowski, 2013](#)), the differences in the first time point between sexes likely aligns with male-female co-activity under natural conditions. Adult foraging containers were briefly (<5 min) cooled in a 4 °C cold room to capture bees. Bees were kept in 50 mL centrifuge tubes until the movement assay began. Up to four bees were assayed concurrently, and the order in which bees underwent the assay was randomized each day. Adult bees were individually placed in 9 cm diameter glass Petri dishes, allowed to acclimate for 5 min, and then filmed from above for 10 min using a web camera (Logitech c920). We included a 5 cm reference bar in each video. Bee tracks were digitized using the motion tracking software Kinovea version 0.8.15 ([Charmant, 2016](#)), and we calculated the within-trial distance traveled (i.e., the sum of each movement step) and movement speed (i.e., speed averaged across all movement steps >0 cm for an individual). To further reduce the correlation between the response variables, individuals who did not move during the trial (distance traveled = 0 cm) were excluded from the movement speed measurement as there was no movement to quantify ([Table 1](#)). Similar assays have been conducted to assess the effects of xenobiotics and other stressors on adult bee locomotor function ([Lopes et al., 2018](#); [Maze et al., 2006](#); [Tomé et al., 2012](#)).

### 2.5. Measuring mushroom bodies

Morphometric analysis of female neuropil and Kenyon cells was conducted using the methodology described in [Tomé et al. \(2012\)](#). These regions comprise the bulk of bee mushroom bodies ([Strausfeld, 2002](#)) and, as bees age and gain experience, the volume of the neuropil increases while that of the Kenyon cells decreases ([Withers et al., 1993, 2008](#)). We randomly selected bees 2 and 14 days after emergence and removed their brains in 4 °C 0.1 M phosphate-buffered saline (PBS, pH 7.4; Corning PN 21-040). Brains were then fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich PN P6148) in 0.1 M PBS for at least 24 h at 4 °C. Following fixation, brains were pre-embedded in HistoGel (Richard-Allan Scientific, San Diego, USA; PN HG-4000) to help preserve morphological features and 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). Samples were dehydrated using mixtures of 100% ethanol and infiltration solution - JB-4 monomer and benzoyl peroxide (catalyst). In the order applied, 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 them in 100% infiltration solution for three rinses for at least 30 min each. Finally, samples were embedded in JB-4 historesin (infiltration solution with an accelerator added) under a light vacuum at 4 °C for 24 h.

After being embedded in plastic, brains were cut into 7 µm-thick serial sections on an automatic microtome (Histo Range Microtome RN LKB 2218) with a glass knife. 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 compound microscope equipped with a digital camera (Zeiss Axio Imager.A2 PN 490022). We used the Cavalieri method to measure structure volume, allowing us to assess a subset of all serial sections while maintaining overall measurement accuracy (Fahrbach and Robinson, 1996; Gundersen and Jensen, 1987). We randomly selected one of the first four sections in which the mushroom bodies appeared and measured every fourth section after that unless the section was damaged, then the 3rd section was used (Fig. 1). Measurements were made using Image-J (Schneider et al., 2012).

## 2.6. Statistical analysis

Movement speed and distance traveled were modeled with generalized additive mixed effects models (GAMMs). Imidacloprid concentration, bee age, and their interaction were included as fixed effects and bee emergence day (emergence day = 0 for the first bee of a species to emerge) and intertegular distance (IT span) were included as smooth terms. Imidacloprid concentration was modeled as a factor to allow for non-linear relationships, as seen elsewhere (Anderson and Harmon-Threatt, 2019; Potts et al., 2018; Tosi et al., 2016). We used IT span as a measure of bee size because, while related to mass (Cane, 1987), it is unaffected by adult nutritional status - which could vary based on the length of adult lifespan - and it was quantifiable for female bees that were sacrificed for brain dissections. Bee identity was treated as a random effect to account for the repeated measures (individuals surviving to day 14 were also sampled during the earlier time point). We

fit our models with Gaussian distributions and evaluated model fit and adherence to the appropriate assumptions by assessing the residuals of the models graphically. Distance traveled was square-root transformed for all three models while movement speed was cube-root transformed to meet model assumptions for *M. rotundata* females and males. No transformation was necessary for *O. lignaria* female movement speed. For models with a significant fixed effect, we applied Tukey's HSD post-hoc tests.

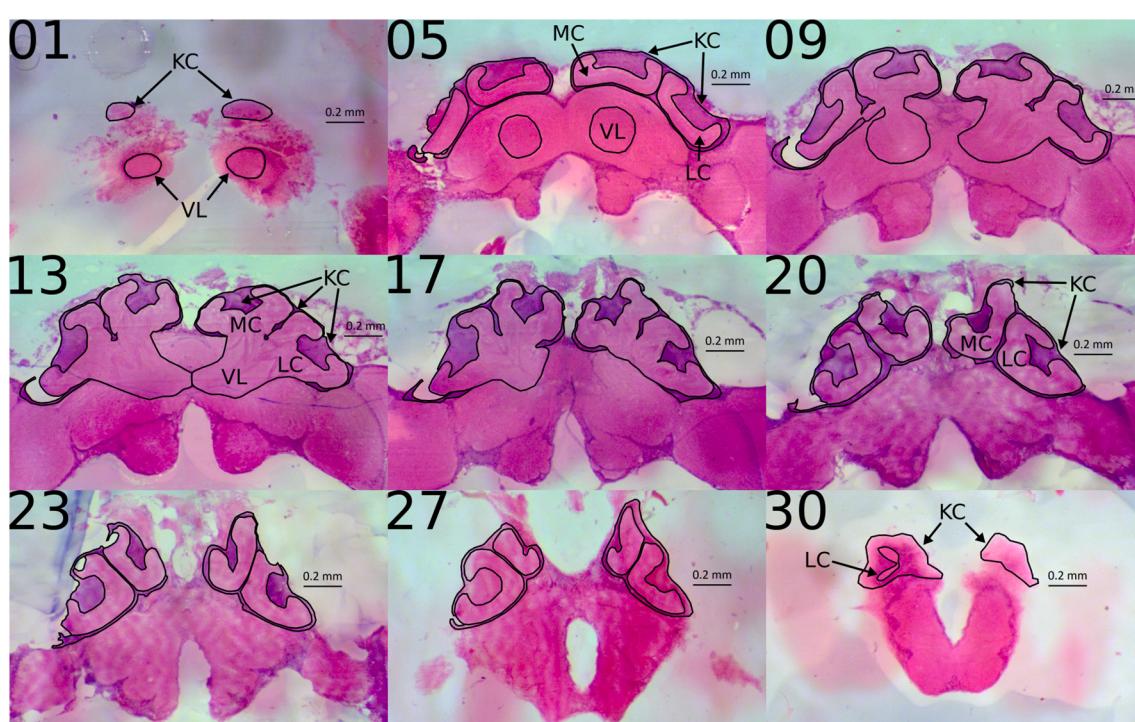
We analyzed changes in mushroom body neuropil:Kenyon cell volume (N:K) with separate generalized additive models (GAMs) for female *O. lignaria* and female *M. rotundata*. Due to low sample sizes, we grouped the 7.5 and 15 ppb treatments for *M. rotundata*. The models' fixed effects were imidacloprid concentration, bee age, and their interaction. IT span was included as a smooth term. We fit the models with Gaussian distributions and evaluated model fit and adherence to the appropriate assumptions by assessing the residuals of the models graphically. No transformations were deemed necessary.

All analyses were conducted in R 4.0.2 (R Core Team, 2020). GAMs and GAMMs were created using the package mgcv version 1.8.33 (Wood, 2004, 2011) and the significance of the modeled terms were assessed using approximate hypothesis tests and the anova.gam function (similar to type III analysis of variance). We used the package emmeans version 1.5.1 (Lenth, 2020) for post-hoc tests and estimated marginal means and standard errors. We used the packages ggplot2 version 3.3.2 (Wickham, 2016) and patchwork version 1.0.1 (Pedersen, 2020) to create the data figures. We used an  $\alpha$  of 0.1 for all analyses due to our reduced final sample sizes caused by bee mortality throughout the study (Table 1). This also allowed us to maintain a high level of power (0.9) to detect moderate and large effect sizes (Cohen, 1988), as estimated with the package pwr version 1.3.0 (Champely, 2020).

## 3. Results

### 3.1. Movement analysis

We detected an interaction between imidacloprid concentration and



**Fig. 1.** Example of brain sections from anterior (01) to posterior (30). KC = Kenyon cells; MC = medial calyx, LC = lateral calyx, VL = vertical lobe (MC + LC + VL = neuropil).

bee age for the movement speed of female *O. lignaria* (Table 2; Fig. 2A). Individuals moved approximately 1.7x faster on day 14 than day 2 in the 0 ( $t_{121} = -3.991$ ,  $p < 0.001$ ) and 7.5 ( $t_{121} = -3.795$ ,  $p < 0.001$ ) ppb imidacloprid treatments, whereas there were no detected differences at 15 ( $t_{121} = -0.863$ ,  $p = 0.390$ ) or 100 ( $t_{121} = -0.318$ ,  $p = 0.751$ ) ppb. Within trials taken at the same bee age, bees in the 0 ppb treatment were 1.6x faster than those in the 100 ppb treatment on day 14 ( $t_{121} = 2.377$ ,  $p = 0.087$ ). No other comparisons between imidacloprid levels within the same age group showed statistically significant differences ( $|t_{121}| \leq 2.278$ ,  $p \geq 0.109$ ). Overall, female *O. lignaria* traveled farther on day 14 than day 2 (Table 2, Fig. 2B). In terms of the smooth terms in the GAMM models for *O. lignaria* movement, bees that emerged later moved slower and traveled shorter distances, and larger bees (IT span) moved farther (Table 2). These relationships were linear ( $\text{edf} = 1$ ).

There was a main effect of age on the distance female *M. rotundata* traveled (Table 2; Fig. 2D). Bees moved half as far on day 14 compared to day 2. Later emerging adult female *M. rotundata* traveled farther than earlier emerging individuals. This relationship was linear ( $\text{edf} = 1$ ). We did not detect any other significant effects in our statistical models for female *M. rotundata* locomotory characteristics (Table 2; Fig. 2C).

We detected an interaction effect between imidacloprid concentration and bee age on the movement speed of and distance traveled by male *M. rotundata* (Table 2; Fig. 2E & F). When compared on day 4, bees treated with 100 ppb moved 1.3x faster than bees in the 0 ppb control ( $t_{107} = -3.111$ ,  $p = 0.013$ ) and 1.2x faster than 15 ppb treated bees ( $t_{107} = -2.361$ ,  $p = 0.091$ ). Bees treated with 7.5 ppb were 1.3x faster than 0 ppb treated bees ( $t_{107} = -2.329$ ,  $p = 0.098$ ). On day 14, *M. rotundata* male bees treated with 15 ppb imidacloprid during development moving 0.7x as fast as control bees ( $t_{107} = 2.427$ ,  $p = 0.078$ ). All other comparisons between imidacloprid concentrations within the same age group were not different ( $|t_{107}| \leq 1.935$ ,  $p \geq 0.220$ ). Within imidacloprid concentrations, male *M. rotundata* in the 0 ppb group moved 1.4x faster on day 14 than day 4 ( $t_{107} = -3.085$ ,  $p = 0.003$ ), whereas those in the 7.5 ppb group moved 0.8x as fast on day 14 compared to day 4 ( $t_{107} = 2.031$ ,  $p = 0.045$ ). We did not detect a change in movement speed

between time points for males in the 15 or 100 ppb treatment levels ( $t_{107} = 1.224$ ,  $p = 0.224$ ;  $t_{107} = 1.453$ ,  $p = 0.159$ ; respectively). For distance traveled, male *M. rotundata* treated with 100 ppb moved 2.3x farther than 0 ppb bees on day 4 ( $t_{109} = -2.364$ ,  $p = 0.090$ ). Within the 0 ppb imidacloprid level, individuals moved 3.3x farther on day 14 compared to day 4 ( $t_{109} = -2.570$ ,  $p = 0.012$ ). We did not detect any other differences between imidacloprid levels for the same value of bee age ( $|t_{109}| \leq 2.115$ ,  $p \geq 0.155$ ) or between bee ages within the imidacloprid treatment groups ( $|t_{109}| \leq 0.978$ ,  $p \geq 0.330$ ). The GAMM model smoother for IT span revealed that larger *M. rotundata* males moved faster and traveled greater distances (Table 2). There was no relationship between emergence day and either of the locomotory response variables.

### 3.2. Brain morphology

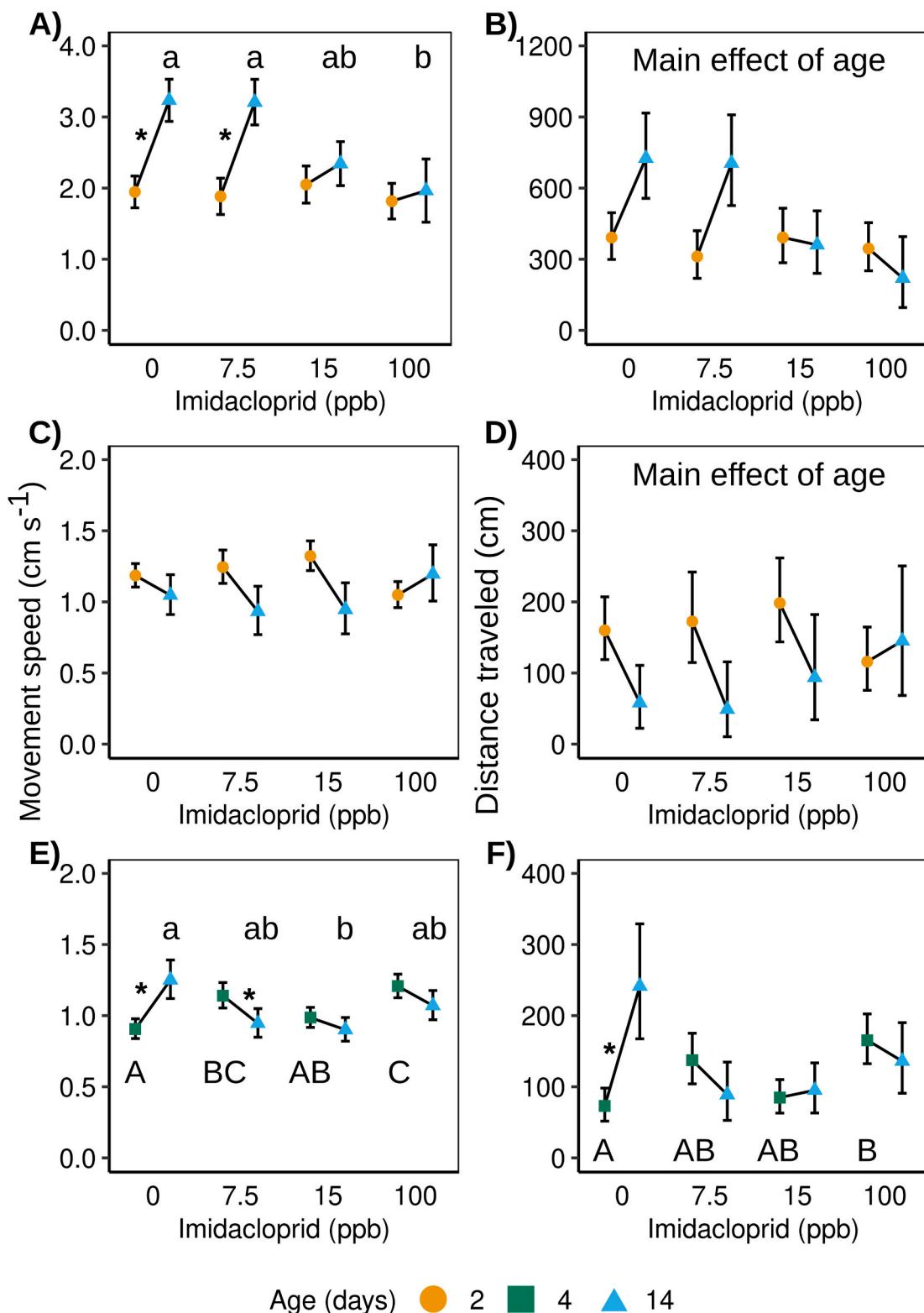
Chronic contact with imidacloprid during immature development did not affect adult female *O. lignaria* N:K ( $F_{3, 29} = 1.748$ ,  $p = 0.179$ ; Fig. 3A). There was also no interaction between imidacloprid concentration and adult bee age ( $F_{3, 29} = 1.486$ ,  $p = 0.239$ ). However, N:K values were 7% lower for female *O. lignaria* 14 days post eclosion compared to 2 days ( $F_{1, 29} = 3.431$ ,  $p = 0.074$ ). There was no relationship between IT span and N:K ( $F_{1, 29} = 1.974$ ,  $p = 0.171$ ).

Similarly, we did not detect an effect of imidacloprid on adult female *M. rotundata* N:K ( $F_{2, 11.488} = 0.346$ ,  $p = 0.715$ ; Fig. 3B). There was no significant interaction between imidacloprid concentration and adult bee age ( $F_{2, 11.488} = 2.252$ ,  $p = 0.150$ ). Adult *M. rotundata* females measured at 14 days after eclosion had N:K values 29% higher than those measured at day 2 ( $F_{1, 11.488} = 19.777$ ,  $p = 0.001$ ). The model-estimated relationship between *M. rotundata* IT span and N:K was non-linear ( $F_{5.512, 11.488} = 2.649$ ,  $p = 0.074$ ) with an absolute minimum value near an IT span of 2.24 mm, a local minimum value near 2.45 mm, and a local maximum value near 2.34 mm (overall range: 1.931–2.582 mm).

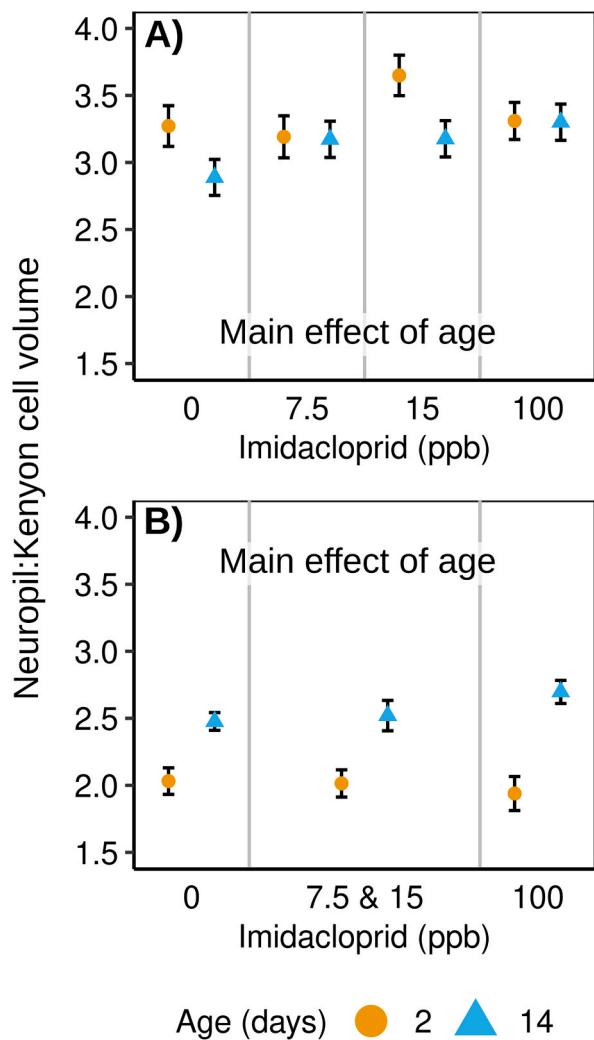
**Table 2**  
Generalized additive mixed effects model (GAMM) output for bee movement.

Species	Sex	Response	Explanatory variable	df (num., den.)	F	p		
<i>Osmia lignaria</i>	Female	Movement speed	Imidacloprid	3, 121	0.152	0.928		
			Age	1, 121	<b>15.929</b>	<b>&lt;0.001</b>	***	
			Imidacloprid x Age	3, 121	<b>2.871</b>	<b>0.039</b>	**	
			Emergence day	1, 121	<b>8.393</b>	<b>0.004</b>	**	
			IT span	1, 121	0.776	0.380		
	Distance traveled		Imidacloprid	3, 122	0.139	0.936		
			Age	1, 122	<b>3.644</b>	<b>0.059</b>	*	
			Imidacloprid x Age	3, 122	1.889	0.135		
			Emergence day	1, 122	<b>12.218</b>	<b>0.001</b>	***	
			IT span	1, 122	<b>2.956</b>	<b>0.088</b>	*	
<i>Megachile rotundata</i>	Female	Movement speed	Imidacloprid	3, 54	1.350	0.268		
			Age	1, 54	0.738	0.394		
			Imidacloprid x Age	3, 54	1.276	0.292		
			Emergence day	1, 54	2.766	0.102		
			IT span	1, 54	1.984	0.165		
	Distance traveled		Imidacloprid	3, 54	0.438	0.727		
			Age	1, 54	<b>2.941</b>	<b>0.092</b>	*	
			Imidacloprid x Age	3, 54	0.805	0.497		
			Emergence day	1, 54	<b>5.291</b>	<b>0.025</b>	**	
			IT span	1, 54	0.686	0.411		
Male	Movement speed	Imidacloprid	3, 107.048	4.103	<b>0.008</b>	**		
			Age	1, 107.048	<b>9.518</b>	<b>0.003</b>	**	
			Imidacloprid x Age	3, 107.048	<b>5.280</b>	<b>0.002</b>	**	
			Emergence day	2.952, 107.048	1.997	0.141		
			IT span	1, 107.048	<b>7.701</b>	<b>0.007</b>	**	
	Distance traveled		Imidacloprid	3, 108.815	<b>2.563</b>	<b>0.059</b>	*	
			Age	1, 108.815	<b>6.603</b>	<b>0.012</b>	**	
			Imidacloprid x Age	3, 108.815	<b>2.551</b>	<b>0.059</b>	*	
			Emergence day	2.185, 108.815	2.356	0.152		
			IT span	1, 108.815	<b>3.919</b>	<b>0.050</b>	**	

\* significant at the  $p < 0.1$  level; \*\* significant at the  $p < 0.05$  level; \*\*\* significant at the  $p < 0.001$  level.



**Fig. 2.** Effect of imidacloprid on adult movement speed and distance traveled. Panels represent female *O. lignaria* (A) movement speed and (B) distance traveled, female *M. rotundata* (C) movement speed and (D) distance traveled, and male *M. rotundata* (E) movement speed and (F) distance traveled. Points depict the estimated marginal means and the error bars the standard errors. Statistically different values ( $p \leq 0.1$ ) are depicted in different ways based on the investigated comparison. Uppercase letters were used to signal a significant difference in locomotory characteristics between imidacloprid concentrations at 2 or 4 days and lowercase letters were used to indicate a significant difference in locomotory characteristics at day 14. In both cases, points that do not share at least one letter differ according to our GAMMs and post hoc tests. An asterisk (\*) was used to signify a significant difference between the day 2 or 4 day and 14 day measurements within an imidacloprid treatment. Significant main effects in the absence of an interaction are described within the appropriate panels. Comparisons without letters or asterisks were not interpreted as differing based on the GAMMs (Table 2).



**Fig. 3.** Effect of imidacloprid on adult female (A) *O. lignaria* and (B) *M. rotundata* brain morphology. Points depict the estimated marginal means and the error bars the standard errors. Significant effects ( $p \leq 0.1$ ) are described within each plot.

#### 4. Discussion

Chronic contact with realistic soil concentrations of imidacloprid affected locomotory characteristics of solitary bees, but the effects on female adult mushroom body development were inconclusive (*O. lignaria*) or lacking (*M. rotundata*). For female *O. lignaria*, individuals treated with 0 or 7.5 ppb imidacloprid moved more quickly as they aged compared to no change in speed for individuals exposed to 15 or 100 ppb. While we did not detect an effect of imidacloprid on female *M. rotundata* movement, young males treated with imidacloprid moved more quickly (7.5 and 100 ppb) and farther (100 ppb) than 0 ppb individuals. The effect on male movement lessened as bees aged, with those in the 0 ppb group moving more quickly and traveling farther at day 14 than day 4. Similar patterns of sex- and species-specific effects have been reported elsewhere (reviewed in Pisa et al., 2015, 2017), and may be the result of different developmental strategies, haplo-diploid sex determination of bees (Carrière, 2003), or other genetic differences that may influence the responses to xenobiotics (Rinkevich et al., 2015; Schmuck et al., 2001). Additionally, commonly observed biphasic hormetic effects in response to neonicotinoids (Anderson and Harmon-Threatt, 2019; Ayyanath et al., 2013; Fortuin et al., 2021; Haddi et al., 2016; Potts et al., 2018; Tosi et al., 2016) may help explain the seemingly inconsistent effects on male *M. rotundata* movement speed

(i.e., no detected effect for young males treated with 15 ppb imidacloprid). In this case, there may be different physiological mechanisms that manifest in a similar measured outcome. Nonetheless, the changes in bee locomotion reported here have the potential to negatively impact bee populations by reducing the ability of female bees to provision brood cells efficiently or by altering male-male competition for mates.

Population growth in solitary bees is directly linked to female bees' ability to efficiently construct and provision brood cells for larvae (Danforth et al., 2019; Steffan-Dewenter and Schiele, 2008). If chronic contact with field-realistic concentrations of imidacloprid during development reduces adult movement speed and if activity level correlates with foraging and nest-building behaviors, fewer brood cells may be completed during a bee's life. The observed effect on *O. lignaria* movement speed could lead to 25% fewer provisioned nests, reducing the average number of offspring from 22.7–28.9 (Tepedino and Torchio, 1982, 1989) to 17.0–21.7. While this route of exposure increases adult female longevity for *O. lignaria* treated with 15 ppb imidacloprid (Anderson and Harmon-Threatt, 2019), the change in locomotor ability could offset the associated fitness benefits and lead to reduced offspring output observed in field and semi-field studies (Fortuin et al., 2021; Willis Chan and Raine, 2021; Stuligross and Williams unpublished data). However, as with other responses to neonicotinoids (Anderson and Harmon-Threatt, 2019; Scott-Dupree et al., 2009), adverse effects on movement may not be consistent across species. We did not observe any effect of imidacloprid on female *M. rotundata* movement speed or distance traveled. In fact, the decrease in distance traveled in this species was related to aging, as older bees covered less distance. Similar inconsistencies in activity across adulthood have been reported for these species (Artz and Pitts-Singer, 2015). Further work is needed to determine when adult bees reach their maximum foraging and nesting abilities, how the timing differs by species, and how movement traits such as speed and distance traveled relate to adult foraging and nesting ability. This information may help us better assess the consequences of changes in bee motor ability. While we did not detect changes in female *M. rotundata* locomotion related to imidacloprid, there was an effect on male *M. rotundata*.

Changes in male bee biology can have important consequences for population growth (Paxton, 2005; Powell and Powell, 1987). Hymenopterans have haplodiploid sex-determination in which female offspring are the result of fertilized, diploid eggs and reproductively viable male offspring of unfertilized, haploid eggs (Whiting, 1933). Disruptions to or ineffective matings reduce the number of fertilized eggs and female offspring in subsequent generations. Male-male competition in solitary bees is thought to take two forms, the exclusion of competitors from established territories and non-territorial, scramble competition (Paxton, 2005). In the current study, young males previously exposed to imidacloprid moved faster and farther, which could increase access to receptive female mates under both scenarios. Additionally, in most bee species, including *M. rotundata* (Blanchetot, 1992), most females are monandrous and become less receptive to mating and less attractive to potential mates following an initially copulatory event and as they age (Ayasse et al., 1999; Eickwort and Ginsberg, 1980; Roubik, 1992; Schiestl and Ayasse, 2000). Therefore, differences in young male behavior have a disproportionate impact on access to mates. Combined with earlier emergence times and increased longevity at moderate and high imidacloprid concentrations (Anderson and Harmon-Threatt, 2019), males exposed to this pesticide during development may be more likely to mate than males from uncontaminated areas within the same landscape. If imidacloprid reduces sperm quality, as is reported for other *Osmia* males (Strobl et al., 2021) and honey bee drones (Straub et al., 2016), copulatory events involving exposed males could be functionally unsuccessful. Females that mate with these individuals may be unable to create as many fertilized female eggs, potentially leading to the upward shift in male:female sex ratios reported elsewhere (Sandrock et al., 2014). Further, repeated use of contaminated soils over generations could lead to a positive feedback

loop of fewer successful matings causing an increase in the relative abundance of physically competitive but reproductively less competent males. Such an effect could cause populations of ground-nesting bees to decline with or without detectable effects on female bees of the same species, highlighting the importance of considering male bee response to xenobiotics in risk assessments.

We did not detect adverse effects of imidacloprid on adult female mushroom body morphology for either *O. lignaria* or *M. rotundata*. Interestingly, *O. lignaria* neuropil volume was lower relative to Kenyon cell volume in older individuals. Given that brain tissue is energetically expensive to maintain (Aiello and Wheeler, 1995; Isler and van Schaik, 2006; Julian and Gronenberg, 2002), this result may be an artifact of our adult holding conditions. However, when Withers et al. (2008) reared *O. lignaria* under simpler circumstances, they reported no change, rather than a decrease, over time. *Megachile rotundata* female mushroom bodies changed with age in the manner observed in other bee species - neuropil volume increased relative to Kenyon cell volume over time (Withers et al., 1993) - under nearly identical adult holding conditions. Little is known about brain development during adulthood in solitary bees, so it is unclear if the changes we report for *M. rotundata* are genetically hardwired or if we were able to provide adequate stimulation to induce olfactory and spatial learning. Whichever is the case, we did not find evidence that chronic contact with realistic soil concentrations of imidacloprid during immature development inhibits the underlying mechanism responsible for gross morphological changes in adult female *M. rotundata* mushroom bodies.

While not the focus of our study, the observed relationships between emergence day and locomotory characteristics are interesting in the context of climate change. As warm temperatures occur earlier in the growing season in temperate latitudes and at high elevations, climate change is predicted to disrupt the synchrony of plant-pollinator mutualisms (CaraDonna et al., 2018; Lee et al., 2018; Stemkovski et al., 2020) (but see Bartomeus et al., 2011; Ovaskainen et al., 2013; Rafferty and Ives, 2011). If the resulting mismatch causes adult bees to emerge into sub-optimal foraging conditions, moving faster and farther could allow individuals to access spatially scattered resources, accelerate starvation, or both. The interaction between emergence date and climate change on bee activity deserves further exploration with additional measurements of bee and forage quality. For example, reduced emerging adult bee fat content and body weight associated with temperature projections (CaraDonna et al., 2018; Fliszkiewicz et al., 2012) may offset positive effects or compound negative effects of increased activity. The direction of the effect may also be species-specific. We detected opposite responses in bee locomotory activity with later emergence dates between *O. lignaria* and *M. rotundata*, possibly related to differences in life-history traits, such as adult activity period (spring vs. summer) or overwintering stage (adult vs. prepupa) (Stemkovski et al., 2020). Understanding the role of emergence day on bee activity may help us predict the effects of climate change on specific taxa.

Taken together with the results of Anderson and Harmon-Threatt (2019) and that bees are unable to detect neonicotinoids via their olfactory senses (Kessler et al., 2015), the effects of chronic contact with realistic soil concentrations of imidacloprid on solitary bees suggest that this understudied route of exposure has the potential to harm bee populations. The most extreme responses will likely be seen in areas actively treated with neonicotinoids that accumulate over time. However, bees in areas that experience drift from active fields, or those recently converted from agricultural use into flower-rich habitats meant to conserve bees, could still experience changes in adult female and male locomotion, leading to negative population-level consequences, such as reduced offspring production and increasingly male-skewed sex ratios. Additionally, bees likely experience a combination of xenobiotics in actual field settings (Riedo et al., 2021), and these compounds may interact synergistically to exacerbate adverse effects on bees (Tosi and Nieh, 2019). To better assess the risks associated with environmentally persistent xenobiotics and to achieve pollinator conservation goals,

assays of chronic contact exposure that reflect conditions experienced by ground-nesting bees are a critical next step. Responsible use of xenobiotics, in terms of compound identity and application timing, will maximize the effectiveness of conservation strategies for bees and other non-target arthropods.

## Author contributions

N.L.A. and A.H.T. conceived the study, designed the methods, interpreted the results, and reviewed the manuscript. N.L.A. conducted the experiment, analyzed the data, and wrote the manuscript.

## Data availability

Data are available via the Illinois Data Bank ([https://doi.org/10.13012/B2IDB-2315056\\_V1](https://doi.org/10.13012/B2IDB-2315056_V1)) and by contacting the corresponding author (N.L.A. [nldrsn2@illinois.edu](mailto:nldrsn2@illinois.edu)).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgments

We want to thank Karen Doty for her assistance with the described histological methods, Katy Heath for allowing us to use her microscope and camera, Ian Tranuello and James Nardi for their help with the brain dissections, and Kapil Thacker, Lorenzo D'Alessio, Anna Grommes, Perla Magana, Delaney Demro, and Alexander Pane for their help with movement data collection and processing. This research was funded by a Clark Research Support Grant, a Harley J. Van Cleave Research Award, and a Lebus Fund Award through the School of Integrative Biology at the University of Illinois at Urbana-Champaign, as well as by additional funds provided by the University of Illinois at Urbana-Champaign.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2021.131177>.

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