Toxicity of clothianidin to common Eastern North American fireflies

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Abstract

Background. Previous research suggests that firefly larvae (Coleoptera: Lampyridae) are susceptible to commonly used insecticides. In the United States, there has been a rapid and widespread adoption of neonicotinoid insecticides, predominantly used as seed coatings on large-acreage crops like corn, soy, and cotton. Neonicotinoid insecticides are persistent in soil yet mobile in water, so they have potential to contaminate firefly habitats both in and adjacent to application sites. As a result, firefly larvae may be at high risk of exposure to neonicotinoids, possibly jeopardizing this already at-risk group of charismatic insects.

Methods. To assess the sensitivity of firefly larvae to neonicotinoids, we exposed larvae of *Photuris versicolor* complex and *Photinus pyralis* to multiple levels of clothianidin-contaminated soil.

Results. Compared to other soil invertebrates and beetle species, both *Photuris versicolor* and *Photinus pyralis* were relatively tolerant to clothianidin, only exhibiting long-term intoxication and mortality at concentrations above 1 μg g^{-1} soil. Under sub-lethal clothianidin exposure, firefly larvae fed less and spent less time in protective soil chambers, two behavioral changes which could decrease larval survival in the wild.

Discussion. Coupled with other stressors such as light pollution and habitat loss, extensive neonicotinoid contamination appears to have potential to contribute to firefly declines in the United States.

Introduction

In the United States alone, insects are estimated to provide over $50 billion in ecological services (Losey and Vaughan, 2006). Human activities, however, have put these services at risk by triggering global insect declines (Sánchez-Bayo and Wyckhuys, 2019). Some charismatic groups such as fireflies (Coleoptera: Lampyridae) are at elevated risk of at least localized extinction due to ongoing human activities such as heavy pesticide use in and around their habitats (Reed et al., 2020). Fireflies have great popular appeal and aesthetic and cultural value, but fireflies also contribute biological control of some pest species, including slugs and snails, which can be important agricultural pests (Godan, 1983; Lewis, 2016).

Despite broad agreement that pesticides are a serious extinction threat to fireflies (Lewis et al., 2020), there is a very poor understanding of the direct toxicity of insecticides on fireflies. The most commonly applied classes of insecticides (neonicotinoids, pyrethroids, or organophosphates) are broadly toxic to most insect taxa (Sparks, 2013), so fireflies are unlikely to be an exception. Indeed, full-strength organophosphate and neonicotinoid formulations are toxic to aquatic firefly larvae (Tabaru et al., 1970; Lee et al., 2008). Unfortunately, there have been no studies assessing how terrestrial firefly larvae respond to residual concentrations of these insecticides in soil, a likely route of exposure. Larvae of many common firefly species in the United States are soil-dwellers that intimately interact with soil as they forage for prey and form protective molting chambers (Buschman, 1984; Lewis, 2016). These larvae inhabit forested, suburban, and agricultural soils, where neonicotinoid insecticides are often applied directly, or via coatings on crop seeds, to protect against pests (Knoepp et al., 2012; Douglas and Tooker, 2015; Simon-Delso et al., 2015). In these habitats, neonicotinoid concentrations in soil can range from less than 5 ppb to over 4 ppm, concentrations that could plausibly influence behavior and survival of firefly larvae (Lee et al., 2008; Knoepp et al., 2012; Schaafsma et al., 2015; Pearsons et al., 2021). Some indirect evidence suggests that firefly larvae are susceptible to neonicotinoids because adult lampyrid densities have been found to be lower where neonicotinoid-coated seeds were planted (Disque et al., 2019); however, to our knowledge, there have been no direct evaluations of how terrestrial firefly larvae respond to neonicotinoid-contaminated soil.

To assess the direct sensitivity of fireflies to neonicotinoid insecticides, we measured feeding behavior, development, and survival of larvae of two common North American firefly species – *Photuris versicolor* species complex and *Photinus pyralis* (Linnaeus 1767) – exposed to clothianidin-contaminated soil. We focused on clothianidin, as it is a common seed- and soil-applied neonicotinoid and the primary metabolite of another commonly applied neonicotinoid, thiamethoxam (Douglas and Tooker, 2015). We exposed larvae to multiple levels of clothianidin-contaminated soil for 30 to 100 days with the expectation that they would be sensitive to clothianidin at concentrations that have been detected in firefly habitats.

Materials & Methods

Chemicals

We acquired clothianidin from Chem Service (West Chester, PA, USA; purity ≥ 98%), and prepared stock solutions of 0.2, 2, 20, and 200 ppm clothianidin in acetone (Sigma Aldrich, St. Louis, MO, USA, ACS reagent, purity ≥ 99.5%). Pure acetone served as a control. We stored stock solutions at 4 ˚C and allowed them to reach room temperature (20 ˚C) before applying them to soils for the assays.

Firefly Collection and Colony Care

We ran toxicity assays on three separate cohorts of fireflies: late-instar larvae from the *Photuris versicolor* species complex (hereafter referred to as *Photuris*), early-instar *Photuris versicolor* complex, and early-instar *Photinus pyralis*. Both *Photuris versicolor* and *Photinus* *pyralis* are relatively large-bodied (6-20 mm adult body length), widespread firefly species found throughout Eastern North America (Lewis, 2016). Because both species spend 1-2 years in the soil as larvae and feed on soil invertebrates (*Photuris versicolor* are thought to feed on a diversity of soil invertebrates while *Photinus pyralis* larvae are considered specialists on earthworms; McLean et al., 1972; Buschman, 1984; Lewis, 2016), they likely experience chronic contact and oral neonicotinoid exposure in contaminated habitats.

Five of the late-instar *Photuris* were reared from eggs laid by a mated female collected in late July 2019 from the Bucknell University Chillisquaque Creek Natural Area (Montour Co, PA; 41° 01′ 15″ N, 76° 44′ 53″ W), while the majority of late-instar *Photuris* were wild-collected in summer of 2019 from multiple locations throughout Pennsylvania: Bald Eagle State Park (5 August; Centre Co, 41°00'44.0"N 77°12'54.3"W), Allegheny National Forest (24-25 June; Forest Co, 41°31'29.8"N 79°17'33.9"W), and Bucknell University Forrest D. Brown Conference Center (23-24 July; Union Co, PA; 40° 57′ 28″ N, 77° 00′ 49″ W). After collection, we housed individual larvae in 16-oz clear plastic deli containers (11.5-cm diameter × 8-cm tall) lined with moist filter paper. Every 1-2 weeks, we provided each larva with one piece of cat food (Grain-Free Real Chicken Recipe Dry Cat Food, Whole Earth Farm™, Merrick Pet Care Inc., Amarillo, TX, USA), which had been softened in DI-water for 1 h. After 24 h, we removed cat food and replaced the filter paper. Occasionally there was extensive fungal growth on the cat food, which could be fatal to *Photuris* larvae; in these instances, we gently wiped larvae with DI water and a delicate task wipe then transferred them to clean containers.

Early-instar *Photuris* and *Photinus* cohorts were reared from eggs laid in July 2020. On the evening of 10 July 2020, we collected 3 male and 2 female *Photinus* adults and 3 mated *Photuris* females. Flying *Photinus* males were collected and identified based on their characteristic “J” flash pattern (Lewis, 2016) while female *Photinus* were collected from nearby patches of short grass and were identified based on their flash pattern and similar morphology to the *Photinus* males (Lewis, 2016). *Photuris* females were collected near *Photinus* females and identified based on their green-shifted flash color and morphology (Lewis, 2016). Additional *Photinus* males were collected to provision the mated *Photuris* females. We collected *Photuris* and *Photinus* in a residential area (State College, Centre Co, PA; 40° 47′ 03″ N, 77° 52′ 25″ W) into two separate 16-oz deli container "nurseries"; each nursery contained a handful of moist sphagnum moss on top of moist soil (2-in deep; silt loam, collected from certified organic fields at the Russell E. Larson Agricultural Research Center at Rock Springs, PA, U.S.A.; 40° 42′ 52″ N, 77° 56′ 46″ W). Both *Photinus* females mated within a few minutes of collection.

Female *Photuris* and *Photinus* laid eggs within the following 3 days (50+ *Photuris* eggs and 100+ *Photinus* eggs; we did not attempt more accurate counts to avoid damaging eggs). Under ambient temperatures, first-instar larvae of both species began to emerge three weeks after eggs were laid (5 August 2020). We kept *Photuris* larvae in the nursery chambers for two weeks, and then, after we observed significant cannibalism among larvae, moved them into individual soil-lined 1-oz polypropylene portion containers. As with larvae collected and reared from 2019, developing *Photuris* were fed moistened cat food (Grain-Free Real Chicken Recipe Dry Cat Food, Whole Earth Farm™, Merrick Pet Care Inc., Amarillo, TX, USA) in addition to pieces of freeze-killed *Lumbricus terrestris* (Josh's Frogs, Owosso, MI). As evidence of the hypothesis that *Photinus pyralis* larvae are specialist on earthworms, *Photinus* larvae did not feed on cat food but did feed gregariously on freeze-killed *L. terrestris*. Unlike *Photuris*, *Photinus* failed to thrive in isolation, so they were kept in the nursery chamber until starting toxicity assays.

Toxicity assays with late-instar *Photuris versicolor*

We started toxicity assays with late-instar *Photuris versicolor* on 22 June 2020. We used 1-oz polypropylene portion containers containing 8 g of soil (same soil source as nursery chambers) for our assay containers. To the soil in each assay container, we added 0.5 mL of the appropriate clothianidin stock solution, allowed the acetone to completely evaporate, then added 2-mL of DI water to moisten the soil.

After setting up assay containers, we weighed the late-instar *Photuris* and randomly assigned each to a particular clothianidin concentration (ensuring all larvae in each dose-set were sourced from the same location). For each concentration (0, 10 ng g^{-1} soil, 100 ng g^{-1} soil, 1 µg g-1 soil, 10 µg g^{-1} soil), we ran six parallel assays with late-instar *Photuris* (30 larvae in total, each in separate assay containers). We recorded firefly status at 1, 4, and 24 h, and every day for an additional 99 d. Fireflies were categorized as dead (D), exhibiting a toxic response (T), or apparently healthy (A). A larva was assumed dead if it did not respond to gentle prodding with forceps. If a larva was flipped on its back and/or demonstrating repetitive twitching of its legs or head, it was recorded as exhibiting a toxic response (T). Fireflies were recorded as apparently healthy (A) if they exhibited a usual response to prodding from blunt forceps (Fig 1A; quickly curled up on its side, often glowing). At each status check, we noted if a firefly had constructed a protective soil chamber, then carefully dismantled the chamber to check larval status. During the toxicity assays, we fed larvae once a week by carefully transferring individuals out of the assay containers into clean containers lined with moisten filter and containing a piece of moistened cat food. After 24 h, we returned fireflies to the assay containers and noted if the cat food had obvious signs of feeding (Fig 1B). Assay containers were kept in a dark drawer except when doing daily checks, and we misted containers with DI water as needed to maintain soil moisture.

Toxicity assay with early-instar *Photuris versicolor*

Toxicity assays with early-instar *Photuris versicolor* were similar to assays with late-instar larvae, except we added half the amount of soil (4 g) and half the volume of clothianidin stock solutions (0.25 mL) to each assay container. On 17 Sept 2020, we started three assays with early-instar *Photuris* (15 larvae in total), feeding them cat food once a week and recording their status at 1, 4, and 24 h, and every day for 10 d, then twice a week for an additional 90 d. Unlike for late-instar *Photuris*, we fed early-instars by directly placing moistened cat food in the assay containers (we removed the food 24 h later).

Toxicity assay with early-instar *Photinus pyralis*

As with early-instar *Photuris*, all assays with *Photinus pyralis* were run in 1-oz polypropylene portion containers containing 4 g of soil with 0.25 mL doses of clothianidin stock solutions. On 17 Sept 2020, we started fifteen assays with early-instar *Photinus* (three sets of five larvae per container, 75 larvae in total), recorded their status at 1, 4, and 24 h, and every day for 10 d, then at least twice a week for an additional 20 d. We terminated *Photinus* assays earlier than *Photuris* assays due to an acarid mite infestation, which rapidly increased larval mortality across all doses. During the assays, we fed *Photinus* pieces of earthworm (*L. terrestris*) in the same manner that early-instar *Photuris* were fed cat food.

Statistical Analysis

We performed all statistical analyses in R (v4.0.4) (R Core Team, 2021). For each firefly cohort, we calculated median toxic concentrations (TC_{50}) and median lethal concentrations

(LC_{50}) at 24 h, 7 d, and 30 d of exposure using probit analysis (LC_PROBIT from the "ecotox" package; Robertson et al., 2017; Hlina et al., 2019); for TC_{50} estimates, we included both sub-lethal and lethal responses, while LC_{50} estimates were based on mortality alone. To assess long-term survivorship across clothianidin levels, we used the Kaplan-Meier method ("survival" functions SURVDIFF and PAIRWISE_SURVDIFF; Therneau, 2021; Therneau and Grambsch, 2000). To determine how clothianidin exposure affected firefly behavior, we used non-parametric Mann-Whitney U tests (WILCOX.TEST) to compare feeding frequency and soil-chamber construction across clothianidin doses; we made pairwise comparisons using Wilcoxon rank sum tests with continuity corrections (PAIRWISE.WILCOX.TEST).

Results

24 h, 7 d, and 30 d TC_{50} and LC_{50} estimates

Dose-response curves and estimated TC_{50} and LC_{50} indicate that *Photuris versicolor* and *Photinus pyralis* were surprisingly tolerant of exposure to clothianidin (Table 1 and Fig 2-4). Reliable TC_{50} and LC_{50} estimates were limited by our small sample sizes and low acute mortality within the tested concentration range. Overall, [illegible]$_0$ values ranged from 0.5 ppm to 2 ppm while LC_{50} values exceeded our test range.

Firefly Survival

Clothianidin exposure significantly reduced long-term firefly survival at high concentrations (Fig 5). All late-instar *Photuris* exposed to the highest clothianidin concentrations (1000 and 10,000 ng g^{-1}) began to exhibit toxic responses within 24 h (Fig 2A), never recovered, and died by day 84. *Photuris* ~~was~~ somewhat tolerant to lower clothianidin concentrations (10 ng g^{-1} or 100 ng g^{-1}) and neither late- or early-instar larvae exposed to low concentrations had significantly lower 100 d survival probability compared to controls (Fig 5A-B). All *Photuris* in the control treatment either pupated (2 out of 6 late-instar larvae) or survived through day 100 (4 out of 6 late-instar larvae). For *Photinus,* exposure to 1 µg g^{-1} and 10 µg g^{-1} clothianidin led to marginally significant ($P = 0.07$) and significantly ($P < 0.0001$) lower survivorship within 30 d of exposure (Fig. 5C).

Feeding Behavior

Clothianidin exposure significantly affected the feeding behavior of firefly larvae (Fig 6). La[illegible] exposed to the highest clothianidin concentration (10 µg g^{-1} soil) never fed during the toxicity assay. Late-instar *Photuris* exposed to 1 ppm (1 µg g^{-1} soil) fed significantly less than control larvae ($\chi^2_4 = 16.3$, $P = 0.003$), and early-instar *Photinus* larvae fed significantly less at higher doses (1 µg g^{-1} and 10 µg g^{-1}) compared to the control or lower doses ($\chi^2_1 = 12.4$, $P = 0.0004$).

Soil-Chambers, Molting, and Pupation of Late-instar *Photuris versicolor*

Late-instar *Photuris* that survived through day 100 went through 1 to 5 periods where they regularly formed protective soil chambers (median = 2) and spent anywhere from 1 to 20 total days in soil chambers (median = 9). Larvae exposed to 10 ppm clothianidin (10 µg g^{-1} soil) never constructed soil chambers while larvae exposed to 1 ppm clothianidin spent significantly fewer days in soil chambers than larvae exposed to 10 ppb ($P = 0.01$; Fig 7).

Formation of protective soil chambers did not correspond with molting or pupation, and all recorded molting and pupation events occurred outside soil chambers, on the soil surface.

Late-instar *Photuris* larvae only molted once or twice, irrespective of how frequently or for how long they built soil chambers (larvae that survived through 100 days; frequency: $R^2_{adj} = -0.09$, $F_{1,10} = 0.10$, $P = 0.76$; duration: $R^2_{adj} = -0.02$, $F_{1,10} = 0.81$, $P = 0.39$). Six of the thirty late-instar *Photuris* larvae pupated; five of which successfully eclosed within 35 d of starting the assays (two controls, one at 10 ppb, two at 100 ppb) and one which was unsuccessful (1000 ppb). At 35 d, three of the larvae exposed to the highest clothianidin concentration (10,000 ppb) were still alive, but none of these larvae ever entered a pupal stage. Of individuals that successfully eclosed, three were lab-reared from eggs laid in 2019 (3 out of 5) while only two were wild-collected (2 out of 25).

Discussion

Photuris versicolor complex and *Photinus pyralis* larvae did not significantly respond to clothianidin concentrations at or below 100 ng g^{-1} soils (100 ppb), but both firefly species exhibited significant toxic responses to higher concentrations. Compared to other soil invertebrates, larvae of these two firefly species were relatively tolerant to clothianidin-contaminated soil, with over 2× and 30× the TC_{50} values for the earthworm *Eisenia andrei* and the collembolan *Folsomia candida*, respectively (de Lima e Silva et al., 2020), and higher tolerance compared to other soil-dwelling beetles (*Agriotes* spp. [Elateridae] and *Atheta coriaria* [Staphylinidae]; van Herk et al., 2007; Cloyd et al., 2009). Although we did not explicitly explore any mechanisms for why firefly larvae may be tolerant to clothianidin exposure, there are multiple behavioral, morphological, and biochemical processes could be limiting their sensitivity to clothianidin (Alyokhin et al., 2008).

Behavioral avoidance of neonicotinoids has been observed across insect orders and beetle families (Easton and Goulson, 2013; Fernandes et al., 2016; Pisa et al., 2021; Korenko et al., 2019), and the results of this current study provide some support for behavioral avoidance of neonicotinoids by Lampyridae. Although firefly larvae could not completely avoid the contaminated soil in our arenas, they could decrease oral exposure by limiting construction of their soil chambers. To form soil chambers, *Photuris* larvae manipulate soil with their mouthparts (Buschman, 1984), providing a potentially more toxic pathway for neonicotinoid exposure (Decourtye and Devillers, 2010). As neonicotinoids are repellant to other beetle species (Easton and Goulson, 2013), neonicotinoid-contaminated soil could have repulsed firefly larvae, possibly explaining reduced chamber formation above 1000 ng clothianidin g^{-1} soil. Alternatively, sub-lethal neonicotinoid exposure may simply decrease the ability of fireflies to construct soil chambers. Choice-based avoidance studies could be used to test if avoidance or toxicity at high clothianidin concentrations drove the decreased time late-instar *Photuris* spent constructing and inhabiting soil chambers.

In addition to behavioral avoidance, specific morphological and metabolic characteristics of fireflies may protect *Photuris* and *Photinus* larvae from toxic clothianidin exposure. Unlike many other soil invertebrates (e.g., earthworms and mollusks), firefly larvae have a comparably protective waxy cuticle that may act as an effective barrier against neonicotinoid uptake (Decourtye and Devillers, 2010; Wang et al., 2012). And even when clothianidin is absorbed, insects can resist target-site exposure by quickly detoxify and/or excrete neonicotinoids (Olson et al., 2000; Alyokhin et al., 2008). Although there has been no work on neonicotinoid metabolism by fireflies, *Photuris* and *Photinus* may upregulate detoxification enzymes after clothianidin exposure, similar to an aquatic firefly species after exposure to benzo[a]pyrene (Zhang et al., 2021). Additionally, *Photuris* and *Photinus* may be tolerant to clothianidin if neonicotinoids have a low binding affinity to nicotinic acetylcholine receptors of fireflies; however, this mechanism seems unlikely due to the broad affinity of neonicotinoids for nicotinic acetylcholine receptors across insect orders (Matsuda et al., 2020).

There is also the unlikely possibility that extensive neonicotinoid use has exerted selection pressure on the firefly populations in central Pennsylvania to evolve resistance to clothianidin. The way neonicotinoids are currently used is a perfect storm for developing insecticide resistance (Tooker et al., 2017), and while most concern has focused on resistance-development in herbivorous pest species, biocontrol agents and other predatory arthropods (Bielza, 2016; Mota-Sanchez and Wise, 2021) can develop insecticide tolerance and resistance in response to heavy insecticide use. Although insecticide-resistance is thought to be rare among biocontrol agents, lady beetles (Coleoptera: Coccinellidae) in particular, have been found to develop resistance to a variety of broad-spectrum insecticides, including neonicotinoids (Tang et al., 2015). Insecticide resistance has not been studied in many non-pest species (including lampyrids), but if the selection pressure is high enough, firefly populations could evolve increased tolerance or even resistance to neonicotinoid insecticides.

Differences among any of these potential mechanisms are likely driving differences in tolerance between the two firefly species, namely, the dramatically reduced feeding response of *Photinus pyralis* to clothianidin exposure. Although this difference could have been exacerbated by mite pressure and the smaller body size of early-instar *Photinus pyralis*, it is possible that *Photinus pyralis* has higher uptake, higher active-site affinity, or lower metabolism of clothianidin as compared to *Photuris.*

Despite their relative tolerance to clothianidin exposure, field-realistic neonicotinoid contamination may still pose a threat to *Photuris* and *Photinus.* Although residual neonicotinoid concentrations in soil are usually below 100 ppb (Schaafsma et al., 2016; Radolinski et al., 2019; Pearsons et al., 2021), concentrations can regularly exceed these levels after agricultural applications (as high as 594 ppb 23 days after planting neonicotinoid-coated seeds; Radolinski et al., 2019), after turf applications (3 × higher than in agronomic settings; Armbrust and Peeler, 2002) and after soil drenches to manage hemlock wooly adelgid (over 4000 ng AI g^{-1} soil;