Toxicity of clothianidin to common Eastern North American fireflies (#65786)

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Toxicity of clothianidin to common Eastern North American fireflies

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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.

1 **Toxicity of clothianidin to common Eastern North**
2 **American fireflies**

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Abstract

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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

36   by triggering global insect declines (Sánchez-Bayo and Wyckhuys, 2019). Some charismatic

37   groups such as fireflies (Coleoptera: Lampyridae) are at elevated risk of at least localized

38   extinction due to ongoing human activities such as heavy pesticide use in and around their

39   habitats (Reed et al., 2020). Fireflies have great popular appeal and aesthetic and cultural value,

40   but fireflies also contribute biological control of some pest species, including slugs and snails,

41   which can be important agricultural pests (Godan, 1983; Lewis, 2016).

42   Despite broad agreement that pesticides are a serious extinction threat to fireflies (Lewis

43   et al., 2020), there is a very poor understanding of the direct toxicity of insecticides on fireflies.

44   The most commonly applied classes of insecticides (neonicotinoids, pyrethroids, or

45   organophosphates) are broadly toxic to most insect taxa (Sparks, 2013), so fireflies are unlikely

46   to be an exception. Indeed, full-strength organophosphate and neonicotinoid formulations are

47   toxic to aquatic firefly larvae (Tabaru et al., 1970; Lee et al., 2008). Unfortunately, there have

48   been no studies assessing how terrestrial firefly larvae respond to residual concentrations of these

49   insecticides in soil, a likely route of exposure. Larvae of many common firefly species in the

50   United States are soil-dwellers that intimately interact with soil as they forage for prey and form

51   protective molting chambers (Buschman, 1984; Lewis, 2016). These larvae inhabit forested,

52   suburban, and agricultural soils, where neonicotinoid insecticides are often applied directly, or

53   via coatings on crop seeds, to protect against pests (Knoepp et al., 2012; Douglas and Tooker,

54   2015; Simon-Delso et al., 2015). In these habitats, neonicotinoid concentrations in soil can range

55   from less than 5 ppb to over 4 ppm, concentrations that could plausibly influence behavior and

56   survival of firefly larvae (Lee et al., 2008; Knoepp et al., 2012; Schaafsma et al., 2015; Pearsons

57   et al., 2021). Some indirect evidence suggests that firefly larvae are susceptible to neonicotinoids

58   because adult lampyrid densities have been found to be lower where neonicotinoid-coated seeds

59   were planted (Disque et al., 2019); however, to our knowledge, there have been no direct

60   evaluations of how terrestrial firefly larvae respond to neonicotinoid-contaminated soil.

61   To assess the direct sensitivity of fireflies to neonicotinoid insecticides, we measured

62   feeding behavior, development, and survival of larvae of two common North American firefly

63   species – *Photuris versicolor* species complex and *Photinus pyralis* (Linnaeus 1767) – exposed

64   to clothianidin-contaminated soil. We focused on clothianidin, as it is a common seed- and soil-

65   applied neonicotinoid and the primary metabolite of another commonly applied neonicotinoid,

66   thiamethoxam (Douglas and Tooker, 2015). We exposed larvae to multiple levels of

67   clothianidin-contaminated soil for 30 to 100 days with the expectation that they would be

68   sensitive to clothianidin at concentrations that have been detected in firefly habitats.

69

70   **Materials & Methods**

71   **Chemicals**

72   We acquired clothianidin from Chem Service (West Chester, PA, USA; purity ≥ 98%),

73   and prepared stock solutions of 0.2, 2, 20, and 200 ppm clothianidin in acetone (Sigma Aldrich,

74   St. Louis, MO, USA, ACS reagent, purity ≥ 99.5%). Pure acetone served as a control. We stored

75   stock solutions at 4 °C and allowed them to reach room temperature (20 °C) before applying

76   them to soils for the assays.

77

78   **Firefly Collection and Colony Care**

79   We ran toxicity assays on three separate cohorts of fireflies: late-instar larvae from the

80   *Photuris versicolor* species complex (hereafter referred to as *Photuris*), early-instar *Photuris*

81   *versicolor* complex, and early-instar *Photinus pyralis.* Both *Photuris versicolor* and *Photinus*

82   *pyralis* are relatively large-bodied (6-20 mm adult body length), widespread firefly species found

83   throughout Eastern North America (Lewis, 2016). Because both species spend 1-2 years in the

84   soil as larvae and feed on soil invertebrates (*Photuris versicolor* are thought to feed on a

85   diversity of soil invertebrates while *Photinus pyralis* larvae are considered specialists on

86   earthworms; McLean et al., 1972; Buschman, 1984; Lewis, 2016), they likely experience chronic

87   contact and oral neonicotinoid exposure in contaminated habitats.

88   Five of the late-instar *Photuris* were reared from eggs laid by a mated female collected in

89   late July 2019 from the Bucknell University Chillisquaque Creek Natural Area (Montour Co,

90   PA; 41° 01′ 15″ N, 76° 44′ 53″ W), while ==the majority of late-instar *Photuris* were wild-collected==

91   in summer of 2019 from multiple locations throughout Pennsylvania: Bald Eagle State Park (5

92   August; Centre Co, 41°00'44.0"N 77°12'54.3"W), Allegheny National Forest (24-25 June; Forest

93   Co, 41°31'29.8"N 79°17'33.9"W), and Bucknell University Forrest D. Brown Conference Center

94   (23-24 July; Union Co, PA; 40° 57′ 28″ N, 77° 00′ 49″ W). After collection, we housed

95   individual larvae in 16-oz clear plastic deli containers (11.5-cm diameter × 8-cm tall) lined with

96   moist filter paper. Every 1-2 weeks, we provided each larva with one piece of cat food (Grain-

97   Free Real Chicken Recipe Dry Cat Food, Whole Earth Farm™, Merrick Pet Care Inc., Amarillo,

98   TX, USA), which had been softened in DI-water for 1 h. After 24 h, we removed cat food and

99   replaced the filter paper. Occasionally there was extensive fungal growth on the cat food, which

100   could be fatal to *Photuris* larvae; in these instances, we gently wiped larvae with DI water and a

101   delicate task wipe then transferred them to clean containers.

102   Early-instar *Photuris* and *Photinus* cohorts were reared from eggs laid in July 2020. On the

103   evening of 10 July 2020, we collected 3 male and 2 female *Photinus* adults and 3 mated *Photuris*

104   females. Flying *Photinus* males were collected and identified based on their characteristic "J"

105   flash pattern (Lewis, 2016) while female *Photinus* were collected from nearby patches of short

106   grass and were identified based on their flash pattern and similar morphology to the *Photinus*

107   males (Lewis, 2016). *Photuris* females were collected near *Photinus* females and identified based

108   on their green-shifted flash color and morphology (Lewis, 2016). Additional *Photinus* males

109   were collected to provision the mated *Photuris* females. We collected *Photuris* and *Photinus* in a

110   residential area (State College, Centre Co, PA; 40° 47′ 03″ N, 77° 52′ 25″ W) into two separate

111   16-oz deli container "nurseries"; each nursery contained a handful of moist sphagnum moss on

112   top of moist soil (2-in deep; silt loam, collected from certified organic fields at the Russell E.

113   Larson Agricultural Research Center at Rock Springs, PA, U.S.A.; 40° 42′ 52″ N, 77° 56′ 46″

114   W). Both *Photinus* females mated within a few minutes of collection.

115   Female *Photuris* and *Photinus* laid eggs within the following 3 days (50+ *Photuris* eggs

116   and 100+ *Photinus* eggs; we did not attempt more accurate counts to avoid damaging eggs).

117   Under ambient temperatures, first-instar larvae of both species began to emerge three weeks after

118   eggs were laid (5 August 2020). We kept *Photuris* larvae in the nursery chambers for two weeks,

119   and then, after we observed significant cannibalism among larvae, moved them into individual

120   soil-lined 1-oz polypropylene portion containers. As with larvae collected and reared from 2019,

121   developing *Photuris* were fed moistened cat food (Grain-Free Real Chicken Recipe Dry Cat

122   Food, Whole Earth Farm™, Merrick Pet Care Inc., Amarillo, TX, USA) in addition to pieces of

123   freeze-killed *Lumbricus terrestris* (Josh's Frogs, Owosso, MI). As evidence of the hypothesis

124   that *Photinus pyralis* larvae are specialist on earthworms, *Photinus* larvae did not feed on cat

125   food but did feed gregariously on freeze-killed *L. terrestris*. Unlike *Photuris*, *Photinus* failed to

126   thrive in isolation, so they were kept in the nursery chamber until starting toxicity assays.

127

128 **Toxicity assays with late-instar *Photuris versicolor***

129   We started toxicity assays with late-instar *Photuris versicolor* on 22 June 2020. We used

130 1-oz polypropylene portion containers containing 8 g of soil (same soil source as nursery

131 chambers) for our assay containers. To the soil in each assay container, we added 0.5 mL of the

132 appropriate clothianidin stock solution, allowed the acetone to completely evaporate, then added

133 2-mL of DI water to moisten the soil.

134   After setting up assay containers, we weighed the late-instar *Photuris* and randomly

135 assigned each to a particular clothianidin concentration (ensuring all larvae in each dose-set were

136 sourced from the same location). For each concentration (0, 10 ng g^{-1} soil, 100 ng g^{-1} soil, 1 µg

137 g-1 soil, 10 µg g^{-1} soil), we ran six parallel assays with late-instar *Photuris* (30 larvae in total,

138 each in separate assay containers). We recorded firefly status at 1, 4, and 24 h, and every day for

139 an additional 99 d. Fireflies were categorized as dead (D), exhibiting a toxic response (T), or

140 apparently healthy (A). A larva was assumed dead if it did not respond to gentle prodding with

141 forceps. If a larva was flipped on its back and/or demonstrating repetitive twitching of its legs or

142 head, it was recorded as exhibiting a toxic response (T). Fireflies were recorded as apparently

143 healthy (A) if they exhibited a usual response to prodding from blunt forceps (Fig 1A; quickly

144 curled up on its side, often glowing). At each status check, we noted if a firefly had constructed a

145 protective soil chamber, then carefully dismantled the chamber to check larval status. During the

146 toxicity assays, we fed larvae once a week by carefully transferring individuals out of the assay

147 containers into clean containers lined with moisten filter and containing a piece of moistened cat

148 food. After 24 h, we returned fireflies to the assay containers and noted if the cat food had

149 obvious signs of feeding (Fig 1B). Assay containers were kept in a dark drawer except when

150 doing daily checks, and we misted containers with DI water as needed to maintain soil moisture.

151

152 **Toxicity assay with early-instar *Photuris versicolor***

153   Toxicity assays with early-instar *Photuris versicolor* were similar to assays with late-

154 instar larvae, except we added half the amount of soil (4 g) and half the volume of clothianidin

155 stock solutions (0.25 mL) to each assay container. On 17 Sept 2020, we started three assays with

156 early-instar *Photuris* (15 larvae in total), feeding them cat food once a week and recording their

157 status at 1, 4, and 24 h, and every day for 10 d, then twice a week for an additional 90 d. Unlike

158 for late-instar *Photuris*, we fed early-instars by directly placing moistened cat food in the assay

159 containers (we removed the food 24 h later).

160

161 **Toxicity assay with early-instar *Photinus pyralis***

162   As with early-instar *Photuris*, all assays with *Photinus pyralis* were run in 1-oz

163 polypropylene portion containers containing 4 g of soil with 0.25 mL doses of clothianidin stock

164 solutions. On 17 Sept 2020, we started fifteen assays with early-instar *Photinus* (three sets of five

165 larvae per container, 75 larvae in total), recorded their status at 1, 4, and 24 h, and every day for

166 10 d, then at least twice a week for an additional 20 d. We terminated *Photinus* assays earlier

167 than *Photuris* assays due to an acarid mite infestation, which rapidly increased larval mortality

168 across all doses. During the assays, we fed *Photinus* pieces of earthworm (*L. terrestris*) in the

169 same manner that early-instar *Photuris* were fed cat food.

170

171 **Statistical Analysis**

172   We performed all statistical analyses in R (v4.0.4) (R Core Team, 2021). For each firefly

173 cohort, we calculated median toxic concentrations (TC_{50}) and median lethal concentrations

174   (LC_{50}) at 24 h, 7 d, and 30 d of exposure using probit analysis (LC_PROBIT from the "ecotox"

175   package; Robertson et al., 2017; Hlina et al., 2019); for TC_{50} estimates, we included both sub-

176   lethal and lethal responses, while LC_{50} estimates were based on mortality alone. To assess long-

177   term survivorship across clothianidin levels, we used the Kaplan-Meier method ("survival"

178   functions SURVDIFF and PAIRWISE_SURVDIFF; Therneau, 2021; Therneau and Grambsch,

179   2000). To determine how clothianidin exposure affected firefly behavior, we used non-

180   parametric Mann-Whitney U tests (WILCOX.TEST) to compare feeding frequency and soil-

181   chamber construction across clothianidin doses; we made pairwise comparisons using Wilcoxon

182   rank sum tests with continuity corrections (PAIRWISE.WILCOX.TEST).

183

184   **Results**

185   **24 h, 7 d, and 30 d TC_{50} and LC_{50} estimates**

186   Dose-response curves and estimated TC_{50} and LC_{50} indicate that *Photuris versicolor* and

187   *Photinus pyralis* were surprisingly tolerant of exposure to clothianidin (Table 1 and Fig 2-4).

188   Reliable TC_{50} and LC_{50} estimates were limited by our small sample sizes and low acute mortality

189   within the tested concentration range. Overall, TC_{50} values ranged from 0.5 ppm to 2 ppm while

190   LC_{50} values exceeded our test range.

191

192   **Firefly Survival**

193   Clothianidin exposure significantly reduced long-term firefly survival at high

194   concentrations (Fig 5). All late-instar *Photuris* exposed to the highest clothianidin concentrations

195   (1000 and 10,000 ng g^{-1}) began to exhibit toxic responses within 24 h (Fig 2A), never recovered,

196   and died by day 84. *Photuris* was somewhat tolerant to lower clothianidin concentrations (10 ng

197   g^{-1} or 100 ng g^{-1}) and neither late- or early-instar larvae exposed to low concentrations had

198   significantly lower 100 d survival probability compared to controls (Fig 5A-B). All *Photuris* in

199   the control treatment either pupated (2 out of 6 late-instar larvae) or survived through day 100 (4

200   out of 6 late-instar larvae). For *Photinus,* exposure to 1 µg g^{-1} and 10 µg g^{-1} clothianidin led to

201   marginally significant (*P* = 0.07) and significantly (*P* < 0.0001) lower survivorship within 30 d

202   of exposure (Fig. 5C).

203

204   **Feeding Behavior**

205   Clothianidin exposure significantly affected the feeding behavior of firefly larvae (Fig 6).

206   Larvae exposed to the highest clothianidin concentration (10 µg g^{-1} soil) never fed during the

207   toxicity assay. Late-instar *Photuris* exposed to 1 ppm (1 µg g^{-1} soil) fed significantly less than

208   control larvae (χ^2_4 = 16.3, *P* = 0.003), and early-instar *Photinus* larvae fed significantly less at

209   higher doses (1 µg g^{-1} and 10 µg g^{-1}) compared to the control or lower doses (χ^2_1 = 12.4, *P* =

210   0.0004).

211

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

213   Late-instar *Photuris* that survived through day 100 went through 1 to 5 periods where

214   they regularly formed protective soil chambers (median = 2) and spent anywhere from 1 to 20

215   total days in soil chambers (median = 9). Larvae exposed to 10 ppm clothianidin (10 µg g^{-1} soil)

216   never constructed soil chambers while larvae exposed to 1 ppm clothianidin spent significantly

217   fewer days in soil chambers than larvae exposed to 10 ppb (*P* = 0.01; Fig 7).

218   Formation of protective soil chambers did not correspond with molting or pupation, and

219   all recorded molting and pupation events occurred outside soil chambers, on the soil surface.

220   Late-instar *Photuris* larvae only molted once or twice, irrespective of how frequently or for how

221   long they built soil chambers (larvae that survived through 100 days; frequency: R^2_{adj} = -0.09,

222   $F_{1,10}$ = 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

223   *Photuris* larvae pupated; five of which successfully eclosed within 35 d of starting the assays

224   (two controls, one at 10 ppb, two at 100 ppb) and one which was unsuccessful (1000 ppb). At 35

225   d, three of the larvae exposed to the highest clothianidin concentration (10,000 ppb) were still

226   alive, but none of these larvae ever entered a pupal stage. Of individuals that successfully

227   eclosed, three were lab-reared from eggs laid in 2019 (3 out of 5) while only two were wild-

228   collected (2 out of 25).

229

230   **Discussion**

231   *Photuris versicolor* complex and *Photinus pyralis* larvae did not significantly respond to

232   clothianidin concentrations at or below 100 ng g^{-1} soils (100 ppb), but both firefly species

233   exhibited significant toxic responses to higher concentrations. Compared to other soil

234   invertebrates, larvae of these two firefly species were relatively tolerant to clothianidin-

235   contaminated soil, with over 2× and 30× the TC$_{50}$ values for the earthworm *Eisenia andrei* and

236   the collembolan *Folsomia candida*, respectively (de Lima e Silva et al., 2020), and higher

237   tolerance compared to other soil-dwelling beetles (*Agriotes* spp. [Elateridae] and *Atheta coriaria*

238   [Staphylinidae]; van Herk et al., 2007; Cloyd et al., 2009). Although we did not explicitly

239   explore any mechanisms for why firefly larvae may be tolerant to clothianidin exposure, there

240   are multiple behavioral, morphological, and biochemical processes could be limiting their

241   sensitivity to clothianidin (Alyokhin et al., 2008).

242   Behavioral avoidance of neonicotinoids has been observed across insect orders and beetle

243   families (Easton and Goulson, 2013; Fernandes et al., 2016; Pisa et al., 2021; Korenko et al.,

244   2019), and the results of this current study provide some support for behavioral avoidance of

245   neonicotinoids by Lampyridae. Although firefly larvae could not completely avoid the

246   contaminated soil in our arenas, they could decrease oral exposure by limiting construction of

247   their soil chambers. To form soil chambers, *Photuris* larvae manipulate soil with their

248   mouthparts (Buschman, 1984), providing a potentially more toxic pathway for neonicotinoid

249   exposure (Decourtye and Devillers, 2010). As neonicotinoids are repellant to other beetle species

250   (Easton and Goulson, 2013), neonicotinoid-contaminated soil could have repulsed firefly larvae,

251   possibly explaining reduced chamber formation above 1000 ng clothianidin g^{-1} soil.

252   Alternatively, sub-lethal neonicotinoid exposure may simply decrease the ability of fireflies to

253   construct soil chambers. Choice-based avoidance studies could be used to test if avoidance or

254   toxicity at high clothianidin concentrations drove the decreased time late-instar *Photuris* spent

255   constructing and inhabiting soil chambers.

256   In addition to behavioral avoidance, specific morphological and metabolic characteristics

257   of fireflies may protect *Photuris* and *Photinus* larvae from toxic clothianidin exposure. Unlike

258   many other soil invertebrates (e.g., earthworms and mollusks), firefly larvae have a comparably

259   protective waxy cuticle that may act as an effective barrier against neonicotinoid uptake

260   (Decourtye and Devillers, 2010; Wang et al., 2012). And even when clothianidin is absorbed,

261   insects can resist target-site exposure by quickly detoxify and/or excrete neonicotinoids (Olson et

262   al., 2000; Alyokhin et al., 2008). Although there has been no work on neonicotinoid metabolism

263   by fireflies, *Photuris* and *Photinus* may upregulate detoxification enzymes after clothianidin

264   exposure, similar to an aquatic firefly species after exposure to benzo[a]pyrene (Zhang et al.,

265   2021). Additionally, *Photuris* and *Photinus* may be tolerant to clothianidin if neonicotinoids have

266   a low binding affinity to nicotinic acetylcholine receptors of fireflies; however, this mechanism

267   seems unlikely due to the broad affinity of neonicotinoids for nicotinic acetylcholine receptors

268   across insect orders (Matsuda et al., 2020).

269   There is also the unlikely possibility that extensive neonicotinoid use has exerted

270   selection pressure on the firefly populations in central Pennsylvania to evolve resistance to

271   clothianidin. The way neonicotinoids are currently used is a perfect storm for developing

272   insecticide resistance (Tooker et al., 2017), and while most concern has focused on resistance-

273   development in herbivorous pest species, biocontrol agents and other predatory arthropods

274   (Bielza, 2016; Mota-Sanchez and Wise, 2021) can develop insecticide tolerance and resistance in

275   response to heavy insecticide use. Although insecticide-resistance is thought to be rare among

276   biocontrol agents, lady beetles (Coleoptera: Coccinellidae) in particular, have been found to

277   develop resistance to a variety of broad-spectrum insecticides, including neonicotinoids (Tang et

278   al., 2015). Insecticide resistance has not been studied in many non-pest species (including

279   lampyrids), but if the selection pressure is high enough, firefly populations could evolve

280   increased tolerance or even resistance to neonicotinoid insecticides.

281   Differences among any of these potential mechanisms are likely driving differences in

282   tolerance between the two firefly species, namely, the dramatically reduced feeding response of

283   *Photinus pyralis* to clothianidin exposure. Although this difference could have been exacerbated

284   by mite pressure and the smaller body size of early-instar *Photinus pyralis*, it is possible that

285   *Photinus pyralis* has higher uptake, higher active-site affinity, or lower metabolism of

286   clothianidin as compared to *Photuris*.

287   Despite their relative tolerance to clothianidin exposure, field-realistic neonicotinoid

288   contamination may still pose a threat to *Photuris* and *Photinus.* Although residual neonicotinoid

289   concentrations in soil are usually below 100 ppb (Schaafsma et al., 2016; Radolinski et al., 2019;

290   Pearsons et al., 2021), concentrations can regularly exceed these levels after agricultural

291   applications (as high as 594 ppb 23 days after planting neonicotinoid-coated seeds; Radolinski et

292   al., 2019), after turf applications (3 × higher than in agronomic settings; Armbrust and Peeler,

293   2002) and after soil drenches to manage hemlock wooly adelgid (over 4000 ng AI g^{-1} soil;

294   Knoepp et al., 2012). Such high concentrations are well within the acutely toxic and chronically

295   lethal range for *Photuris* and *Photinus* (Table 1). Encountering such high concentrations are

296   likely be even more lethal under field conditions, as firefly larvae that exhibited toxic responses

297   in the laboratory would be vulnerable to predation and starvation, two risks that can increase

298   mortality from insecticides (Kunkel et al., 2001).

299   As observed with other predatory beetle species (*Cycloneda sanguinea* [Coccinellidae]

300   and *Chauliognathus flavipes* [Cantharidae]; Fernandes et al., 2016), firefly larvae exhibited

301   reduced feeding activity in response to high neonicotinoid exposure. Firefly larvae that feed less

302   frequently may have less successful eclosion rates, and those that do eclose may have lower

303   reproductive success. Additionally, the prey that fireflies encounter in neonicotinoid-

304   contaminated environments likely provide an additional neonicotinoid exposure route. *Photinus*

305   larvae primarily feed on earthworms (Lewis et al., 2020), which have been found to contain

306   neonicotinoid concentrations above 200 ppb when collected from soybean fields that were

307   planted with neonicotinoid-coated seeds (Douglas et al., 2015) and 700 ppb when collected from

308   treated cereal fields (Pelosi et al., 2021). Firefly larvae of other species are known to feed on

309   slugs (Barker, 2004), which can also contain high doses of neonicotinoids (500 ppb), leading to

310   disrupted biological control provided by carabid beetles (Douglas et al. 2015). Compounded with

311   reduced prey availability in habitats where neonicotinoids are used (Ritchie et al., 2019; Tooker

312   and Pearsons, 2021), decreased feeding activity and high risks of further neonicotinoid exposure

313   through contaminated prey may explain why adult lampyrid densities are significantly lower

314   where clothianidin has been used as a seed coating (Disque et al., 2019), despite low acute

315   mortality in our laboratory assays.

316   Despite low acute mortality, the sublethal effects of clothianidin were surprising, as some

317   *Photuris* larvae survived in a severely intoxicated state (not feeding, not building protective soil

318   chambers, only occasionally moving legs and/or mandibles) for over two months. A similar

319   phenomenon has been observed in European wireworms (*Agriotes* spp. [Coleoptera: Elateridae])

320   after exposure to clothianidin, with individuals surviving and even recovering from a severely

321   intoxicated state that can last months (van Herk et al., 2007; Vernon et al., 2007). For pests like

322   *Agriotes* spp., such sub-lethal effects of clothianidin exposure could still decrease crop damage

323   but may exacerbate the risk of *Agriotes* spp. developing neonicotinoid resistance. For predators

324   like *Photuris*, this long-term intoxication may limit their potential to provide biological control

325   beyond what would be expected based on population declines.

326

327

328   **Conclusions**

329   As larvae of the two firefly species that we studied appear to be somewhat tolerant to

330   clothianidin-contaminated soil, neonicotinoids alone are unlikely to be significant direct factors

331   in firefly declines in North America. Nevertheless, firefly populations around the world appear to

332   be suffering from other stressors (e.g., habitat loss, reduced prey availability, light pollution), and

333   ecological research has demonstrated that animal populations exposed to multiple stresses can

334   suffer disproportionally more than what is suffered from a single stress (Relyea and Mills 2001).

335   Therefore, continued widespread contamination of larval firefly habitats with neonicotinoids

336   may hold potential to exacerbate the influence of other stressors on firefly-population declines

337   (Lewis et al., 2020).

338

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Figure 1

Healthy late-instar *Photuris versicolor* larvae (A) demonstrating the typical "curl and glow" response after being prodded with blunt forceps and (B) feeding on moistened cat food.
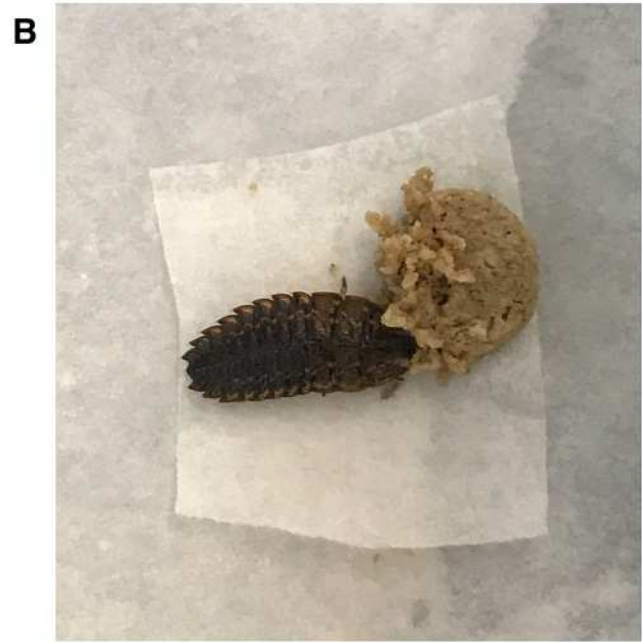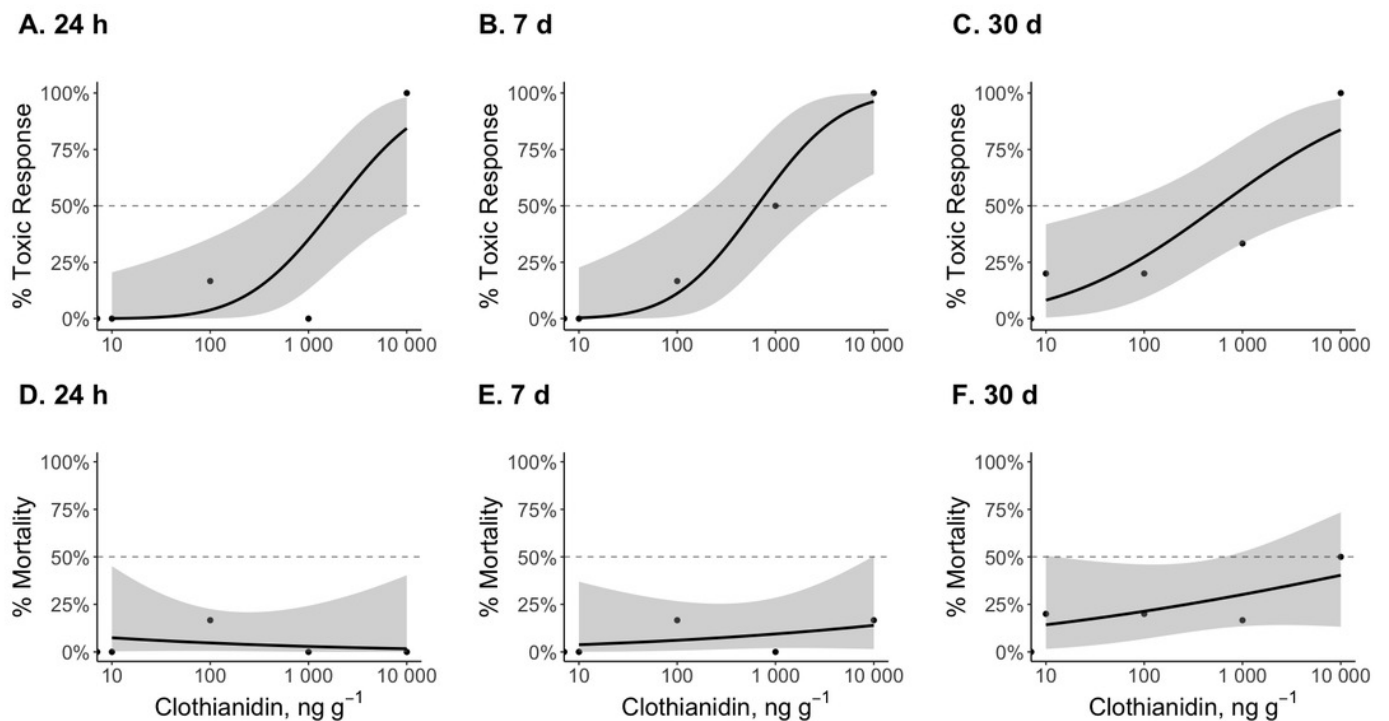
Figure 2

Dose-response curves for late-instar *Photuris versicolor* exposed to clothianidin-contaminated soil at 10, 100, 1000, and 10,000 ng clothianidin per gram of soil (n = 6 larvae for each dose).

Toxic responses after (A) 24 h, (B) 7 d, and (C) 30 d, and lethal response after (D) 24 h, (E) 7 d, and (F) 30 d. Dots in each panel represent mean responses at each insecticide concentration; the shaded area represents the 95% confidence interval for each curve. Dotted lines in each panel marks the 50% toxic response or mortality threshold.

Figure 3

Dose-response curves for early-instar *Photuris versicolor* exposed to clothianidin-contaminated soil at 10, 100, 1000, and 10,000 ng clothianidin per gram of soil (n = 3 larvae for each dose).

Toxic responses after (A) 24 h, (B) 7 d, and (C) 30 d, and lethal response after (D) 24 h, (E) 7 d, and (F) 30 d. Dots in each panel represent mean responses at each insecticide concentration; the shaded area represents the 95% confidence interval for each curve. Dotted lines in each panel marks the 50% toxic response or mortality threshold.
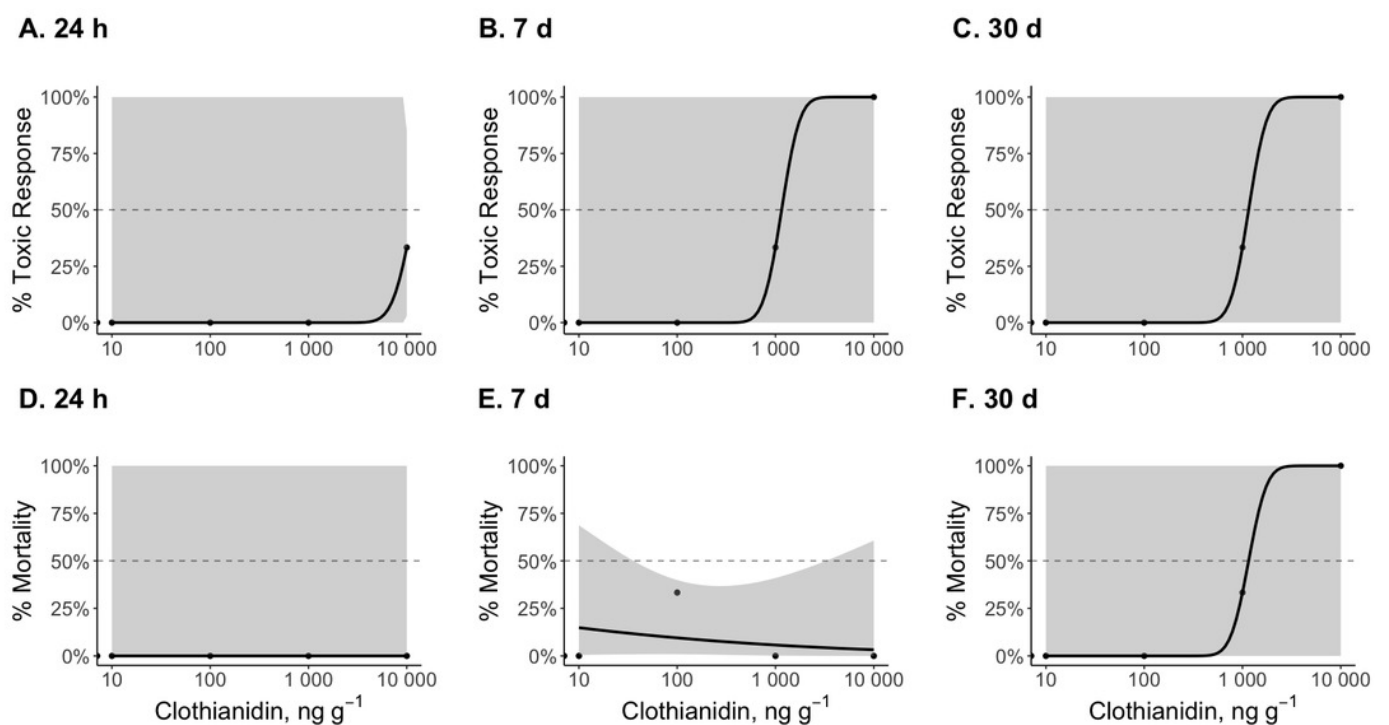
Figure 4

Dose-response curves for early-instar *Photinus pyralis* exposed to clothianidin-contaminated soil at 10, 100, 1000, and 10,000 ng clothianidin per gram of soil (n = 3 sets of 5 larvae for each dose).

Toxic responses after (A) 24 h, (B) 7 d, and (C) 30 d, and lethal response after (D) 24 h, (E) 7 d, and (F) 30 d. Dots in each panel represent mean responses at each insecticide concentration; the shaded area represents the 95% confidence interval for each curve. Dotted lines in each panel marks the 50% toxic response or mortality threshold.
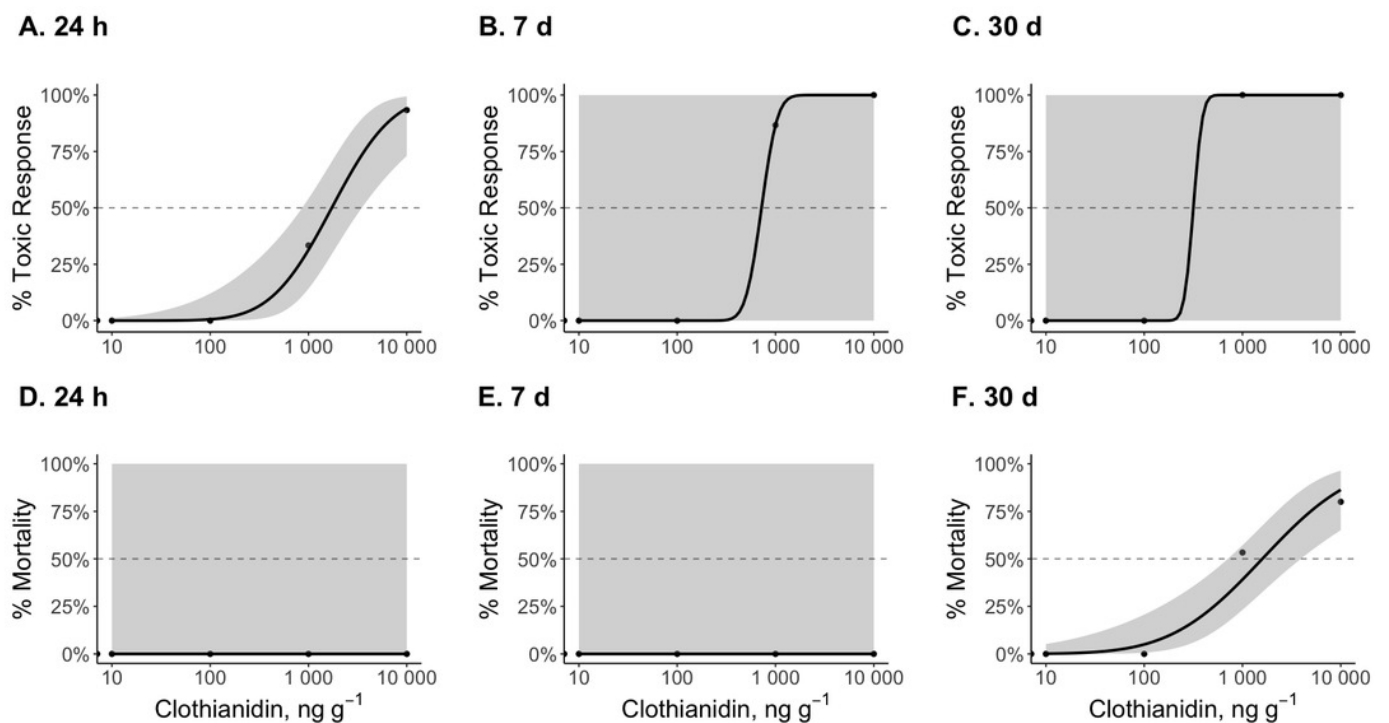
Figure 5

Survivorship curves.

(A) late-instar *Photuris* (n=6), (B) early-instar *Photuris* (n=3), and (C) early-instar *Photinus* (n=15) at different clothianidin concentrations. *P*-values next to each line indicate the significance of reduced survivorship the control (with a Benjamini-Hochberg correction for multiple comparisons). Lines and *P*-values were excluded where survivorship was 100% and perfectly overlapped with control values (100 ppb in panel B, 10 and 100 ppb in panel C).

Survival was significantly affected by clothianidin exposure (late-instar *Photuris*: $\chi^2_4 = 18$, *P* = 0.001; early-instar Photuris: $\chi^2_4 = 12.5$, *P* = 0.01; early-instar *Photinus*: $\chi^2_4 = 58.3$, *P* < 0.0001).
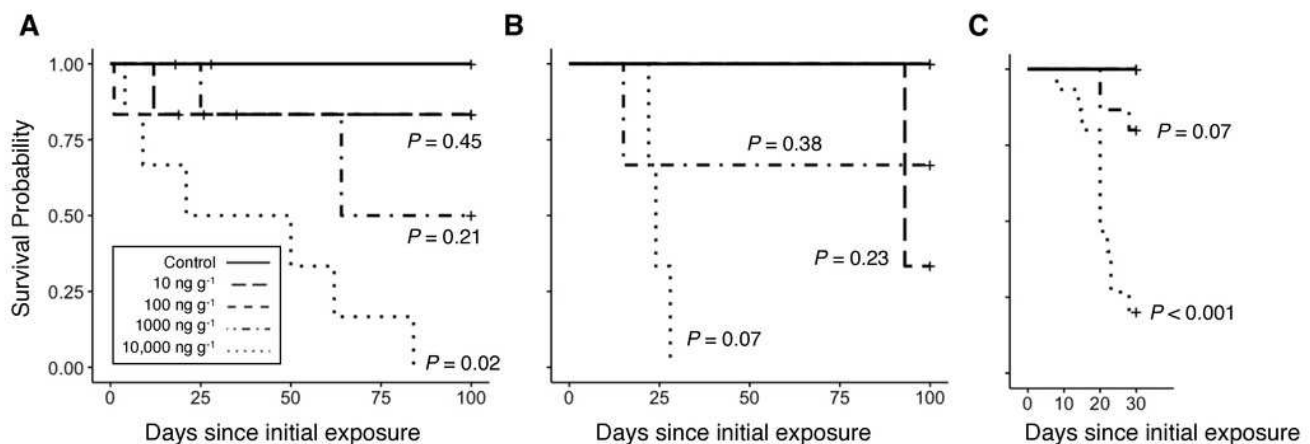
Figure 6

Percent of feeding opportunities taken by firefly larvae.

(A) late-instar *Photuris* larvae (χ^2_4 = 16.3, *P* = 0.003), (B) early-instar *Photuris* larvae (χ^2_4 = 8.2, *P* = 0.08), and (C) early-instar *Photinus* larvae (χ^2_1 = 12.4, *P* = 0.0004). Different letters indicate significant differences in late-instar *Photuris* feeding activity at *P* < 0.05 (Benjamini-Hochberg correction for multiple comparisons). The asterisk indicates significantly lower feeding activity by *Photinus* at *P* < 0.05 (Tukey HSD adjustment).
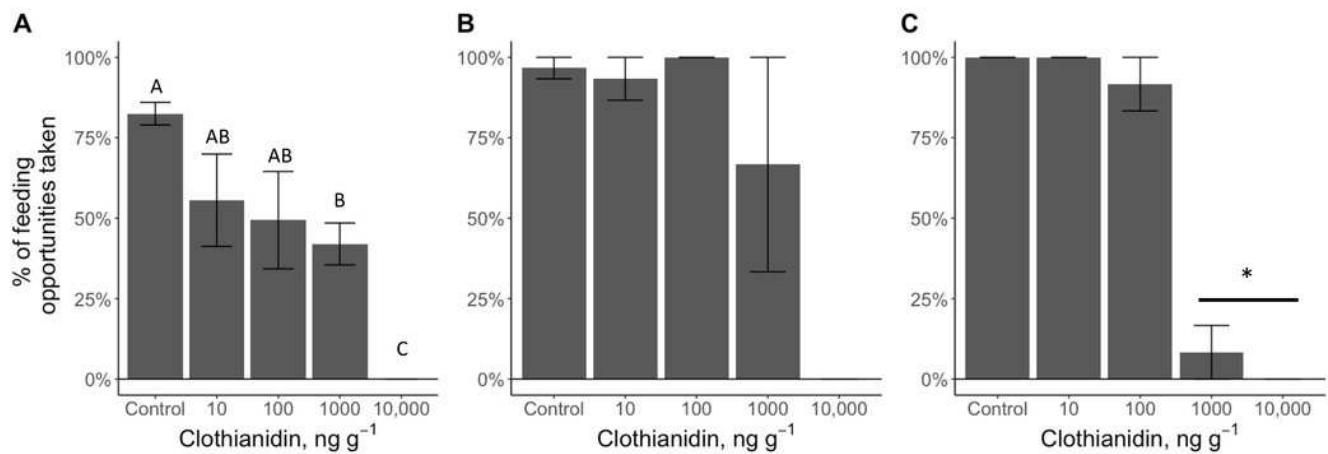
Figure 7

Amount of time that late-instar *Photuris* spent in soil chambers at different clothianidin-exposure levels ($\chi^2_4 = 18.4$, $P = 0.001$).

Different letters indicate significant differences at $P < 0.05$ (Benjamini-Hochberg correction for multiple comparisons).
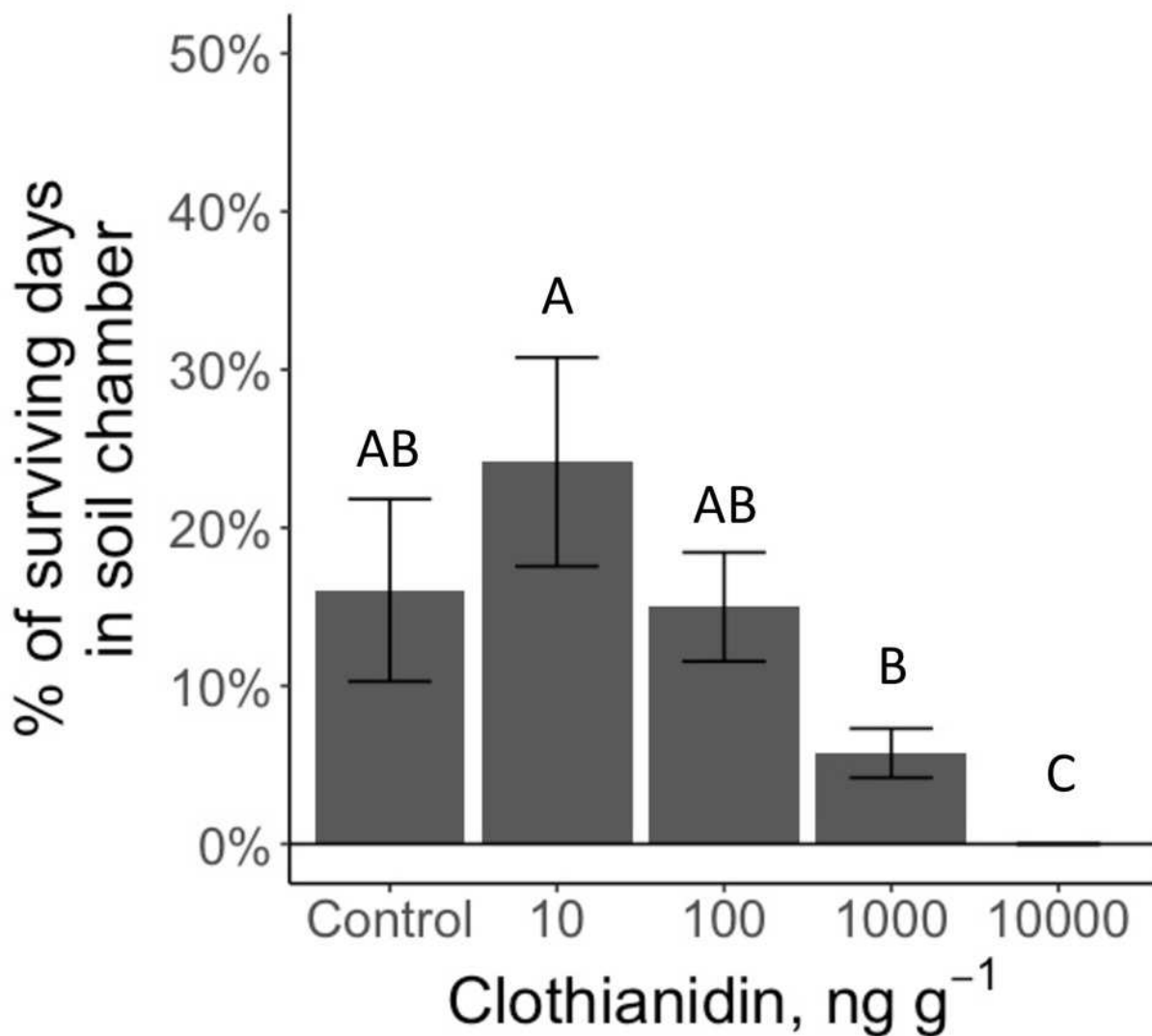
Table 1(on next page)

Estimated median toxic concentrations (TC_{50}) and lethal concentrations (LC_{50}) for *Photuris versicolor* and *Photinus pyralis* exposure to clothianidin-contaminated soil.

95% confidence intervals (CI) are based on probit analyses. CIs are not shown where data did not fit a cumulative standard normal distribution. n.r. = no response in tested range.

Species	timeframe	TC_{50} (ng g^{-1} soil)	95% CI	LC_{50} (ng g^{-1} soil)	95% CI
Photuris,	24 h	1882	136–10,000+	> 10,000	-
late-instar,	7 d	648	144–3047	> 10,000	-
6 larvae / dose	30 d	574	46–9895	> 10,000	-
Photuris,	24 h	> 10,000	-	n.r.	-
early-instar,	7 d	1169	-	> 10,000	-
3 larvae / dose	30 d	1169	-	1169	-
Photinus,	24 h	1726	836–3486	n.r.	-
early-instar,	7 d	704	-	n.r.	-
3 sets of 5 / dose	30 d	316	-	1591	246–10,000+

1