Toxicity of clothianidin to common Eastern North American fireflies

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Background. Previous research suggests that fireflies (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, fireflies 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 fireflies to neonicotinoids, we exposed larvae of *Photuris versicolor* complex and *Photinus pyralis* to multiple levels of clothianidin-treated soil and monitored feeding behavior, protective soil chamber formation, intoxication, and mortality.

Results. *Pt. versicolor* and *Pn. pyralis* larvae exhibited long-term intoxication and mortality at concentrations above 1000 ng g^{-1} soil (1 ppm). Under sub-lethal clothianidin exposure, firefly larvae fed less and spent less time in protective soil chambers, two behavioral changes that could decrease larval survival in the wild.

Discussion. Both firefly species demonstrated sub-lethal responses in the lab to clothianidin exposure at field-realistic concentrations, although *Pt. versicolor* and *Pn. pyralis* appeared to tolerate higher clothianidin exposure relative to other soil invertebrates and beetle species. While these two firefly species, which are relatively widespread in North America, appear somewhat tolerant of neonicotinoid exposure in a laboratory setting, further work is needed to extend this conclusion to wild populations, especially in rare or declining taxa.

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

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Abstract

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Introduction

36   In the United States alone, insects are estimated to provide over $50 billion in ecological

37   services (Losey and Vaughan, 2006). Fireflies have great popular appeal and aesthetic and

38   cultural value, but fireflies also contribute biological control of some pest species, including

39   slugs and snails, which can be important agricultural pests (Godan, 1983; Lewis, 2016). Human

40   activities, however, have put these services at risk by triggering global insect declines (Sánchez-

41   Bayo and Wyckhuys, 2019). Some charismatic groups such as fireflies (Coleoptera: Lampyridae)

42   may be at elevated risk of at least localized extinction due to ongoing human activities such as

43   heavy pesticide use in and around their habitats (Reed et al., 2020).

44   Despite broad agreement that pesticides can pose a serious extinction threat to fireflies

45   (Lewis et al., 2020), there is a very poor understanding of the direct toxicity of insecticides on

46   fireflies. The most commonly applied classes of insecticides (neonicotinoids, pyrethroids, and

47   organophosphates) are broadly neurotoxic to most insect taxa (Sparks, 2013), so fireflies are

48   unlikely to be an exception. Indeed, full-strength organophosphate and neonicotinoid

49   formulations are toxic to the aquatic firefly larvae *Luciola cruciata* and *Luciola lateralis*,

50   respectively (Tabaru et al., 1970; Lee et al., 2008). Unfortunately, there have been no studies

51   assessing how terrestrial firefly larvae respond to residual concentrations of these insecticides in

52   soil, a likely route of exposure. Larvae of many common firefly species in North America are

53   soil-dwellers that intimately interact with soil as they forage for prey and form protective molting

54   chambers (Buschman, 1984; Lewis, 2016). These larvae inhabit forested, suburban, and

55   agricultural soils, where neonicotinoid insecticides are often applied directly, or via coatings on

56   crop seeds, to protect against pests (Knoepp et al., 2012; Douglas and Tooker, 2015; Simon-

57   Delso et al., 2015). In these habitats, neonicotinoid concentrations in soil can range from less

58   than 5 ng g^{-1} to over 4,000 ng g^{-1} (Knoepp et al., 2012; Schaafsma et al., 2015; Pearsons et al.,

59   2021), concentrations that could plausibly influence behavior and survival of firefly larvae (Lee

60   et al., 2008). Some indirect evidence suggests that firefly larvae are susceptible to neonicotinoids

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

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

63   evaluations of how terrestrial firefly larvae respond to neonicotinoid-treated soil.

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

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

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

67   to clothianidin-treated soil. We focused on clothianidin, one of the most widely-used seed- and

68   soil-applied neonicotinoid and the primary metabolite of another commonly applied

69   neonicotinoid, thiamethoxam (Douglas and Tooker, 2015). Generally applied to combat sucking

70   and chewing insects, clothianidin disrupts insect central nervous systems, leading to paralysis

71   and death (Simon-Delso et al., 2015). We exposed larvae to multiple field-realistic levels of

72   clothianidin-treated soil for 30 to 100 days with the expectation that they would be sensitive to

73   clothianidin at concentrations that have been detected in firefly habitats.

74

Materials & Methods

Chemicals

77   We acquired clothianidin from Chem Service (West Chester, PA, USA; purity $\geq 98\%$),

78   and prepared stock solutions of 2×10^2, 2×10^3, 2×10^4, and 2×10^5 ng mL^{-1} clothianidin in

79   acetone (Sigma Aldrich, St. Louis, MO, USA, ACS reagent, purity $\geq 99.5\%$). Pure acetone

80   served as a control. We stored stock solutions at 4 ˚C and allowed them to reach room

81   temperature (20 ˚C) before applying them to soils for the assays.

82

83 **Firefly Collection and Colony Care**

84 We ran toxicity assays on three separate cohorts of fireflies (Table 1): late-instar larvae from the

85 *Photuris versicolor* species complex (*Pt. Photuris*), early-instar *Pt. versicolor,* and early-instar

86 *Photinus pyralis (Pn. pyralis)*. Both *Pt. versicolor* and *Pn. pyralis* are relatively large-bodied (6-

87 20 mm adult body length), widespread firefly species found throughout Eastern North America,

88 and their populations do not appear to be declining (Lewis, 2016). Because both species spend 1-

89 2 years in the soil as larvae and feed on soil invertebrates (*Pt. versicolor* are thought to feed on a

90 diversity of soil invertebrates whereas *Pn. pyralis* larvae are considered specialists on

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

92 contact and oral neonicotinoid exposure in contaminated habitats.

93   Five of the late-instar *Pt. versicolor* were reared from eggs laid by a mated female

94 collected in late July 2019 from the Bucknell University Chillisquaque Creek Natural Area

95 (Montour Co, PA; 41° 01′ 15″ N, 76° 44′ 53″ W), while the other 25 late-instar *Pt. versicolor*

96 were wild-collected in summer of 2019 from multiple locations throughout Pennsylvania: Bald

97 Eagle State Park (5 August; Centre Co, 41°00'44.0"N 77°12'54.3"W), Allegheny National Forest

98 (24-25 June; Forest Co, 41°31'29.8"N 79°17'33.9"W), and Bucknell University Forrest D. Brown

99 Conference Center (23-24 July; Union Co, PA; 40° 57′ 28″ N, 77° 00′ 49″ W). Larvae were

100 wild-collected at night by visually inspecting the ground for their faint glows. Larvae were

101 identified to genus by external morphology (McLean et al., 1972). After collection, we housed

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

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

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

105 TX, USA), which had been softened in DI-water for 1 h (McLean et al., 1972). After 24 h, we

106 removed cat food and replaced the filter paper. Occasionally there was extensive fungal growth

107 on the cat food, which could be fatal to *Pt. versicolor* larvae; in these instances, we gently wiped

108 larvae with DI water and a delicate task wipe then transferred them to clean containers.

109   Early-instar *Pt. versicolor* and *Pn. pyralis* cohorts were reared from eggs laid in July

110 2020. On the evening of 10 July 2020, we collected 3 male and 2 female *Pt. versicolor* adults

111 and 3 mated *Pt. versicolor* females. Flying *Pn. pyralis* males were collected and identified based

112 on their characteristic "J" flash pattern (Lewis, 2016) while female *Pn. pyralis* were collected

113 from nearby patches of short grass and were identified based on their flash pattern and similar

114 morphology to the *Pn. pyralis* males (Lewis, 2016). Female *Pt. versicolor* were collected near

115 *Pn. pyralis* females and identified based on their green-shifted flash color and morphology

116 (Lewis, 2016). Additional *Pn. pyralis* males were collected to provision the mated *Pt. versicolor*

117 females. We collected *Pt. versicolor* and *Pn. pyralis* in a residential area (State College, Centre

118 Co, PA; 40° 47′ 03″ N, 77° 52′ 25″ W) into two separate 16-oz deli container "nurseries" kept at

119 ambient room temperature (20-22 °C); each nursery contained a handful of moist sphagnum

120 moss on top of moist soil (2-in deep; silt loam, collected from certified organic fields at the

121 Russell E. Larson Agricultural Research Center at Rock Springs, PA, U.S.A.; 40° 42′ 52″ N, 77°

122 56′ 46″ W). Both *Pn. pyralis* females mated within a few minutes of collection.

123   Female *Pt. versicolor* and *Pn. pyralis* laid eggs within the following 3 days (50+ *Pt.*

124 *versicolor* eggs and 100+ *Pn. pyralis* eggs; we did not attempt more accurate counts to avoid

125 damaging eggs). Under ambient temperatures (20-22 °C), first-instar larvae of both species began

126 to emerge three weeks after eggs were laid (5 August 2020). We kept *Pt. versicolor* larvae in the

127 nursery chambers for two weeks, and then, after we observed significant cannibalism among

128   larvae, moved them into individual soil-lined 1-oz polypropylene portion containers. As with

129   larvae collected and reared from 2019, developing *Pt. versicolor* were fed moistened cat food

130   (Grain-Free Real Chicken Recipe Dry Cat Food, Whole Earth Farm™, Merrick Pet Care Inc.,

131   Amarillo, TX, USA) in addition to pieces of freeze-killed *Lumbricus terrestris* (Josh's Frogs,

132   Owosso, MI). As evidence of the hypothesis that *Pn. pyralis* larvae are specialist on earthworms,

133   *Pn. pyralis* larvae did not feed on cat food but did feed gregariously on freeze-killed *L. terrestris*.

134   Unlike *Pt. versicolor*, *Pn. pyralis* failed to thrive in isolation, so they were kept in the nursery

135   chamber until starting the toxicity assay.

136

137   **Toxicity assay on Late-instar *Photuris versicolor***

138   We started the toxicity assay with late-instar *Photuris versicolor* on 22 June 2020. We

139   used 1-oz polypropylene portion containers containing 8 g of dry soil (same soil source as

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

141   of the appropriate clothianidin stock solution, allowed the acetone to completely evaporate, then

142   added 2 mL of DI water to moisten the soil and to achieve clothianidin concentrations of 0 ng g^{-1},

143   10^1 ng g^{-1} soil, 10^2 ng g^{-1} soil, 10^3 ng g^{-1} soil , 10^4 ng g^{-1} soil. We chose this concentration

144   range (10^1 - 10^4 ng g^{-1} soil) to encompass the range of neonicotinoid concentrations in soil that

145   have been measured in potential firefly habitats (Knoepp et al., 2012; Schaafsma et al., 2015;

146   Pearsons et al., 2021).

147   After setting up assay containers, we weighed the late-instar *Pt. versicolor* and randomly

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

149   sourced from the same location). All late-instar *Pt. versicolor* were over 12 months old at the

150   start of the assay, and were over 10-mm long and >50 mg (Table 1). Each clothianidin

151   concentration (0, 10^1 ng g^{-1} soil, 10^2 ng g^{-1} soil, 10^3 ng g^{-1} soil , 10^4 ng g^{-1} soil) was replicated

152   six times (30 late-instar *Pt. versicolor* larvae in total). We recorded firefly status at 1, 4, and 24

153   h, and every day for an additional 99 d. Fireflies were categorized as dead (D), exhibiting a toxic

154   response (T), or apparently healthy (A). A larva was assumed dead if it did not respond to gentle

155   prodding with forceps. If a larva was flipped on its back and/or demonstrating repetitive

156   twitching of its legs or head, it was recorded as exhibiting a toxic response (T). Fireflies were

157   recorded as apparently healthy (A) if they exhibited a usual response to prodding from blunt

158   forceps (Fig 1A; quickly curled up on its side, often glowing). During the toxicity assay, we fed

159   larvae once a week by carefully transferring individuals out of the assay containers into clean

160   containers lined with moistened filter paper and containing a piece of moistened cat food. After

161   24 h, we returned fireflies to the assay containers and noted if the cat food had obvious signs of

162   feeding (Fig 1B). Feeding activity for each week was measured as a simple binary (0 = no

163   obvious signs of feeding, 1 = obvious signs of feeding). At each status check, we noted if a

164   firefly had constructed a protective soil chamber, then carefully dismantled the chamber to check

165   larval status. Larvae often re-built soil chambers by the next day; if a larva built soil chambers on

166   multiple consecutive days (feeding days as an exception), we noted this behavior as a "period of

167   chamber formation." Assay containers were kept in a dark drawer except when doing daily

168   checks, and we misted containers with DI water as needed to keep the soil from drying out.

169

170   **Toxicity assay on early-instar *Photuris versicolor***

171   The toxicity assay with early-instar *Photuris versicolor* was similar to the assay with late-

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

173   stock solutions (0.25 mL) to each assay container to achieve the same clothianidin concentrations

174 (0, 10^1 ng g^{-1} soil, 10^2 ng g^{-1} soil, 10^3 ng g^{-1} soil , 10^4 ng g^{-1} soil). All early-instar *Pt. versicolor*

175 were less than 3 months old and weighed between 3 and 15 mg. On 17 Sept 2020, we started

176 trials with early-instar *Pt. versicolor* (three replicates at each concentration, 15 larvae in total),

177 feeding them cat food once a week and recording their status at 1, 4, and 24 h, and every day for

178 10 d, then twice a week for an additional 90 d. Unlike for late-instar *Pt. versicolor*, we fed early-

179 instars by directly placing moistened cat food in the assay containers (we removed the food 24 h

180 later after noting if food had been damaged [1] or not [0]).

181

182 **Toxicity assay on early-instar *Photinus pyralis***

183 As with the early-instar *Pt. versicolor* assay, the *Photinus pyralis* assay was run in 1-oz

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

185 solutions (to achieve 0, 10^1 ng g^{-1} soil, 10^2 ng g^{-1} soil, 10^3 ng g^{-1} soil , 10^4 ng g^{-1} soil). All early-

186 instar *Pn. pyralis* were less than 3 months old and weighed between 0.6 and 2.4 mg. On 17 Sept

187 2020, we started the assay on early-instar *Pn. pyralis*, exposing larvae in sets of five (five larvae

188 per container, three replicates at each concentration, 75 larvae in total), recorded their status at 1,

189 4, and 24 h, and every day for 10 d, then at least twice a week for an additional 20 d. We

190 terminated the *Pn. pyralis* assay earlier than the *Pt. versicolor* assays due to an acarid mite

191 infestation, which rapidly increased larval mortality across all doses. During the assay, we fed

192 *Pn. pyralis* pieces of earthworm (*L. terrestris*) in the same manner that early-instar *Pt. versicolor*

193 were fed cat food.

194

195 **Statistical Analysis**

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

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

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

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

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

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

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

203   2000). To determine how clothianidin exposure affected firefly behavior, we used non-

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

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

206   rank sum tests with continuity corrections (PAIRWISE.WILCOX.TEST). As firefly larvae

207   reduce feeding before pupation (McLean et al. 1972), we excluded the two feeding events

208   preceding pupation for feeding assessments.

209

210   **Results**

211   **24 h, 7 d, and 30 d TC_{50} and LC_{50} estimates**

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

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

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

215   within the tested concentration range. Overall, TC_{50} values ranged from 500 ng g^{-1} to 2,000 ng g^{-1}

216   while LC_{50} values exceeded our test limit (above 10,000 ng g^{-1}).

217

218   **Firefly Survival**

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

220   concentrations (Fig 5). Between one and four hours after initial exposure, half of the late-instar

221   *Pt. versicolor* larvae and 87% of the early-instar *Pn. Pyralis* larvae exposed to the highest

222   clothianidin concentration (10,000 ng g^{-1}) began to exhibit toxic responses. By 24 h, all six late-

223   instar *Pt. versicolor* exposed to the highest clothianidin concentration (10,000 ng g^{-1}) exhibited a

224   toxic response (Fig 2A); these larvae never recovered and died by day 84. *Photuris* larvae were

225   somewhat tolerant to lower clothianidin concentrations (10 ng g^{-1} or 100 ng g^{-1}) and neither late-

226   nor early-instar larvae exposed to low concentrations had significantly lower 100 d survival

227   probability compared to controls (Fig 5A-B). All *Pt. versicolor* in the control treatment either

228   pupated (2 out of 6 late-instar larvae) or survived through day 100 (4 out of 6 late-instar larvae,

229   all three early-instar larvae). Although the experiment was terminated at 30 d due to the mite

230   infestation, early-instar *Pn. Pyralis* exposed to 1,000 ng g^{-1} clothianidin showed marginally non-

231   significant reduced survivorship ($P = 0.07$) while *Pn. pyralis* exposed to 10,000 ng g^{-1}

232   clothianidin showed significantly reduced survivorship ($P < 0.0001$) compared to controls (Fig.

233   5C).

234

235   **Feeding Behavior**

236   Clothianidin exposure significantly reduced the number of times firefly larvae fed (Fig

237   6). During the toxicity assays, no *Pn. pyralis* or *Pt. versicolor* larvae exposed to the highest

238   clothianidin concentration (10,000 ng g^{-1} soil) fed. Late-instar *Pt. versicolor* exposed to 1,000 ng

239   g^{-1} soil fed significantly less frequently than control larvae ($\chi^2_4 = 16.3$, $P = 0.003$), and early-

240   instar *Pn. pyralis* larvae fed significantly less at higher doses (1,000 ng g^{-1} and 10,000 ng g^{-1})

241   compared to the control or lower doses ($\chi^2_1 = 12.4$, $P = 0.0004$).

242

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

243

244   The 14 late-instar *Photuris* larvae that survived as larvae through day 100 went through 1

245   to 5 periods of consecutive days when they regularly formed protective soil chambers (median =

246   2 periods) and spent anywhere from 1 to 20 total days in soil chambers (median = 9 d). Larvae

247   exposed to 10,000 ng g^{-1} clothianidin never constructed soil chambers while larvae exposed to 1

248   ppm clothianidin spent significantly fewer days in soil chambers than larvae exposed to 10 ng g^{-1}

249   ($P = 0.01$; Fig 7).

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

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

252   Late-instar *Pt. versicolor* larvae only molted once or twice, irrespective of how frequently or for

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

254   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-

255   instar *Pt. versicolor* larvae pupated; five of which successfully eclosed within 35 d of starting the

256   assay (two controls, one at 10 ng g^{-1}, two at 100 ng g^{-1}) and one which was unsuccessful (1,000

257   ng g^{-1}). The unsuccessful larva failed to shed its last-instar exoskeleton and died during the pupal

258   stage. At 35 d, three of the larvae exposed to the highest clothianidin concentration (10,000 ng g^{-}

259   ^{1}) were still alive, but none of these larvae ever entered a pupal stage. Of the individuals that

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

261   were wild-collected (2 out of 25).

262

263   **Discussion**

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

265   clothianidin concentrations at or below 100 ng g^{-1} soil, but both firefly species exhibited

266   significant toxic responses to higher concentrations. Although some of the larvae exposed to

267   10,000 ng clothianidin g^{-1} soil showed a toxic response within four hours of exposure, compared

268   to other soil invertebrates, larvae of these two firefly species were relatively tolerant to

269   clothianidin-treated soil. Twenty-four hour TC_{50} values for *Pt. versicolor* and *Pn. pyralis* were

270   over 2× and 30× the TC_{50} values for the earthworm *Eisenia andrei* and the collembolan *Folsomia*

271   *candida*, respectively (de Lima e Silva et al., 2020). Twenty-four hour LC_{50} values exceeded our

272   maximum test concentration of 10^4 ng clothianidin g^{-1} soil (10 ppm), indicating higher tolerance

273   to clothianidin compared to other soil-dwelling beetles (*Agriotes* spp. [Elateridae] and *Atheta*

274   *coriaria* [Staphylinidae]; van Herk et al., 2007; Cloyd et al., 2009). The one other study which

275   tested neonicotinoid toxicity to fireflies observed 13% survival of aquatic *Luciola lateralis* larvae

276   after 24 h of exposure to 10^5 ng thiamethoxam mL^{-1} in water (Lee et al., 2008); these results

277   suggest that fireflies as a group may be somewhat tolerant to neonicotinoid exposure, although

278   this is likely a tenuous conclusion because it is based on just two studies that represent less than

279   0.2% of all described firefly species (Lewis et al., 2020). Tolerance to neonicotinoids may partly

280   explain why populations of *Pt. versicolor* and *Pn. pyralis* do not appear to be declining as fast as

281   rarer firefly species (Reed et al. 2020), which may be more sensitive to neonicotinoid exposure.

282   *Pt. versicolor* and *Pn. pyralis* may tolerate clothianidin exposure due to multiple behavioral,

283   morphological, and biochemical processes that could limit their sensitivity to clothianidin

284   (Alyokhin et al., 2008).

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

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

287   2019), and the results of this current study provide some support for behavioral avoidance of

288   neonicotinoids by Lampyridae. Although firefly larvae could not avoid dermal exposure to the

289   treated soil in our arenas, they may have decreased oral exposure by limiting construction of

290   their soil chambers. To form soil chambers, *Pt. versicolor* larvae manipulate soil with their

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

292   exposure (Decourtye and Devillers, 2010). Because neonicotinoids are repellant to other beetle

293   species (Easton and Goulson, 2013), neonicotinoid-treated soil could have repulsed firefly

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

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

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

297   direct toxicity drove the decreased time late-instar *Pt. versicolor* spent constructing and

298   inhabiting soil chambers at high-clothianidin concentrations.

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

300   of fireflies may protect *Pt. versicolor* and *Pn. pyralis* larvae from toxic clothianidin exposure.

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

302   comparably protective cuticle that may act as an effective barrier against neonicotinoid uptake

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

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

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

306   by fireflies, *Pt. versicolor* and *Pn. pyralis* may upregulate detoxification enzymes after

307   clothianidin exposure, similar to an aquatic firefly species after exposure to benzo[a]pyrene

308   (Zhang et al., 2021). Additionally, *Pt. versicolor* and *Pn. pyralis* may be tolerant to clothianidin

309   if neonicotinoids have a low binding affinity to target sites on firefly neurons. Neonicotinoids

310   primarily target nicotinic acetylcholine receptors (nAChRs), which regulate cation movement

311   and neuron firing in response to acetylcholine levels (Matsuda et al., 2020). Neonicotinoid

312   insecticides agonistically bind to these receptors, forcing ion channels open, leading to spasms

313   and eventual paralysis (Simon-Delso et al., 2015). As neonicotinoids have broad activity across

314   insect orders (Matsuda et al., 2020), it is unlikely that clothianidin has a low binding affinity for

315   nAChRs of *Pt. versicolor* and *Pn. pyralis*.

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

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

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

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

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

321   develop insecticide tolerance and resistance in response to heavy insecticide use (Bielza, 2016;

322   Mota-Sanchez and Wise, 2021). Although insecticide-resistance is thought to be rare among

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

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

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

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

327   increased tolerance or even resistance to neonicotinoid insecticides.

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

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

330   *Pn. pyralis* to clothianidin exposure. Although this difference could have been exacerbated by

331   mite pressure and the smaller body size of early-instar *Pn. pyralis*, it is possible that *Pn. pyralis*

332   has higher uptake, higher active-site affinity, or lower metabolism of clothianidin as compared to

333   *Pt. versicolor.*

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

335   concentrations may still pose a chronic threat to *Pt. versicolor* and *Pn. pyralis.* Although residual

336   neonicotinoid concentrations in soil are often below 100 ng g^{-1} (Schaafsma et al., 2016;

337   Radolinski et al., 2019; Pearsons et al., 2021), concentrations can regularly exceed these levels

338   after agricultural applications (as high as 594 ng g^{-1} 23 days after planting neonicotinoid-coated

339   seeds; Radolinski et al., 2019), after turf applications (3 × higher than in agronomic settings;

340   Armbrust and Peeler, 2002) and after soil drenches to manage hemlock wooly adelgid (over

341   4,000 ng AI g^{-1} soil; Knoepp et al., 2012). Such high concentrations are well within the acutely

342   toxic and chronically lethal range for *Pt. versicolor* and *Pn. pyralis* larvae (Table 2).

343   Encountering such high concentrations are likely to be even more lethal under field conditions,

344   as firefly larvae that exhibited toxic responses in the laboratory would be vulnerable to predation

345   and starvation, two risks that can increase mortality from insecticides (Kunkel et al., 2001).

346   Additionally, further work is needed to assess if neonicotinoid exposure can exacerbate other

347   stressors affecting firefly populations (i.e., light pollution) or if neonicotinoids pose a significant

348   risk to firefly eggs or adults.

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

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

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

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

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

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

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

356   neonicotinoid concentrations above 200 ng g^{-1} when collected from soybean fields that were

357   planted with neonicotinoid-coated seeds (Douglas et al., 2015) and 700 ng g^{-1} when collected

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

359   on slugs (Barker, 2004), which can also contain high doses of neonicotinoids (500 ng g^{-1}),

360   leading to disrupted biological control provided by carabid beetles (Douglas et al. 2015).

361   Compounded with reduced prey availability in habitats where neonicotinoids are used (Ritchie et

362   al., 2019; Tooker and Pearsons, 2021), decreased feeding activity and high risks of further

363   neonicotinoid exposure through contaminated prey may explain why adult lampyrid densities are

364   significantly lower where clothianidin has been used as a seed coating (Disque et al., 2019), even

365   if acute mortality is low. Adult fireflies may also encounter neonicotinoid residues while resting

366   on sprayed vegetation or during oviposition into soil (Pisa et al, 2021), although the risk of such

367   exposure does not appear to have been explored.

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

369   *Pt. versicolor* larvae survived in a severely intoxicated state (not feeding, not building protective

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

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

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

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

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

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

376   like *Pt. versicolor*, this long-term intoxication may limit their potential to provide biological

377   control beyond what would be expected based on population declines.

378

379

Conclusions

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

382 clothianidin-treated soil, neonicotinoids alone may not be significant direct factors in firefly

383 declines in North America, at least for common species. Nevertheless, firefly populations around

384 the world appear to be suffering from other stressors (e.g., habitat loss, reduced prey availability,

385 light pollution), and ecological research has demonstrated that animal populations exposed to

386 multiple stressors can suffer disproportionally more than what is suffered from a single stress

387 (Relyea and Mills 2001). Therefore, continued widespread contamination of larval firefly

388 habitats with neonicotinoids may hold potential to exacerbate the influence of other stressors on

389 declining firefly populations (Lewis et al., 2020). We encourage researchers with access to other

390 species of fireflies, particularly those with declining populations in areas where neonicotinoids

391 are commonly used, to explore their toxicological responses to insecticides.

392

Acknowledgements

394 We would like to thank Cheyenne McKinley and Nellie Heitzman for assistance with

395 specimen collection and rearing. Specimens were collected in Bald Eagle State Park,

396 Pennsylvania under permit SFRA-1907 to S. Lower and in Allegheny National Forest under

397 firefly monitoring permit to the Pennsylvania Firefly Festival. We would also like to thank Larry

398 Buschman for his advice on firefly collection and rearing.

399

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Summary details of the firefly larvae (three cohorts of two species) used for toxicological assays.

Species	Instar group	Age (months)	Mean weight ± SD (mg)	# used in assay	Source
Pt. versicolor	late	> 12	77 ± 17	30	wild-collected
	early	< 3	9 ± 3	15	lab-reared
Pn. pyralis	early	< 3	1.4 ± 0.5	75	lab-reared

Estimated median toxic concentrations (TC$_{50}$) and lethal concentrations (LC$_{50}$) for *Pt. versicolor* and *Pn. 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
Pt. versicolor,	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	-
Pt. versicolor,	24 h	> 10,000	-	n.r.	-
early-instar,	7 d	1169	-	> 10,000	-
3 larvae / dose	30 d	1169	-	1169	-
Pn. pyralis,	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+

Figure 1

Healthy *Pt. versicolor* larvae (A) demonstrating a typical "curl and glow" response after being prodded with blunt forceps and (B) feeding on moistened cat food. (C) An intoxicated *Pt. versicolor* larva on its back, unable to right itself.
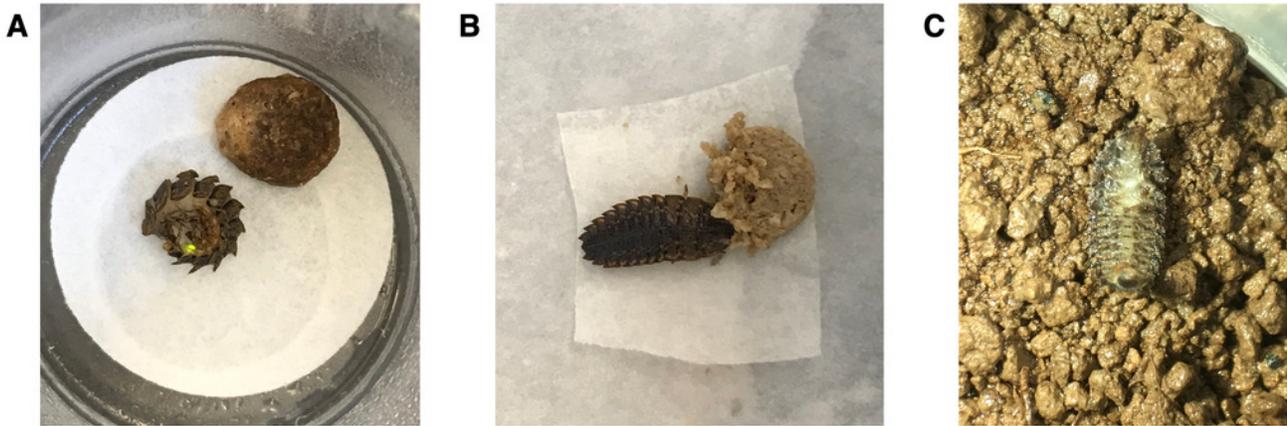
Figure 2

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

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. Black dots in each panel represent mean responses at each insecticide concentration; the shaded area represents the 95% confidence interval for each curve. Blue diamonds represent the response of the control group. Dotted lines in each panel marks the 50% toxic response or mortality threshold.
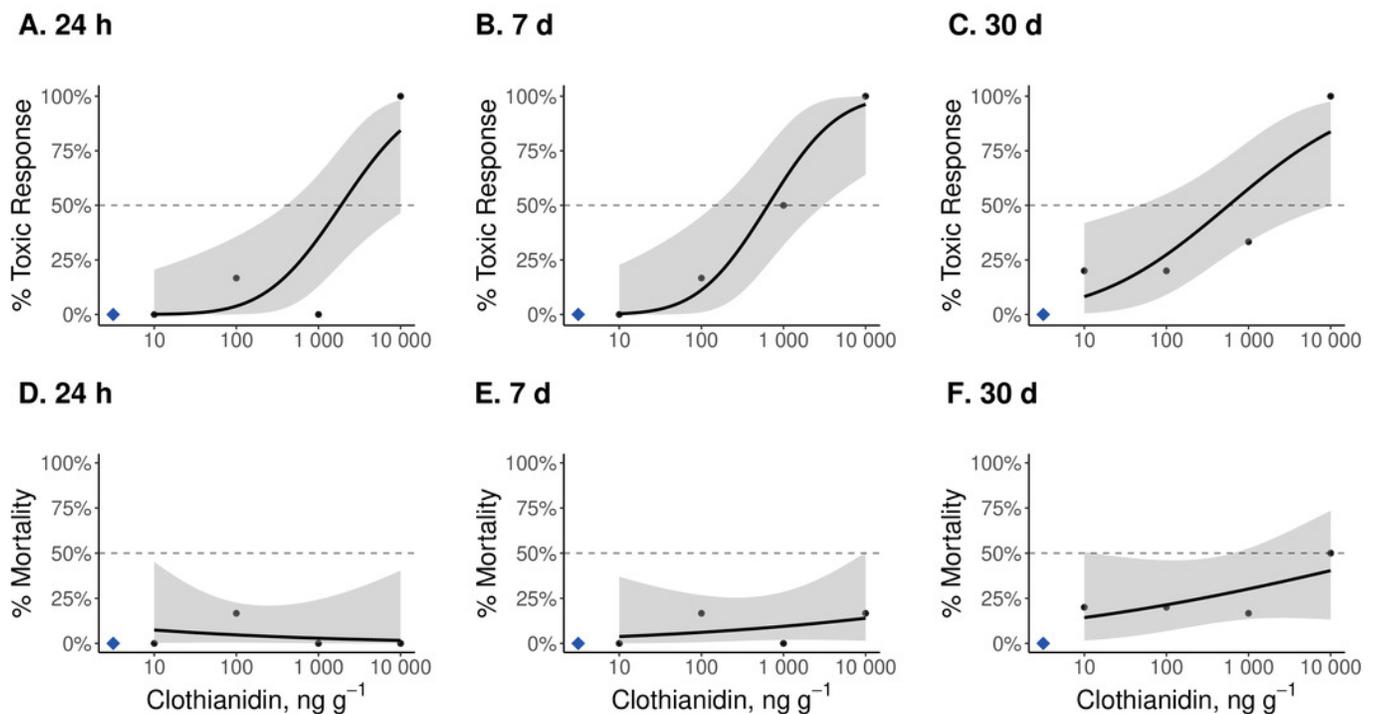
Figure 3

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

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. Black dots in each panel represent mean responses at each insecticide concentration; the shaded area represents the 95% confidence interval for each curve. Blue diamonds represent the response of the control group. Dotted lines in each panel marks the 50% toxic response or mortality threshold.
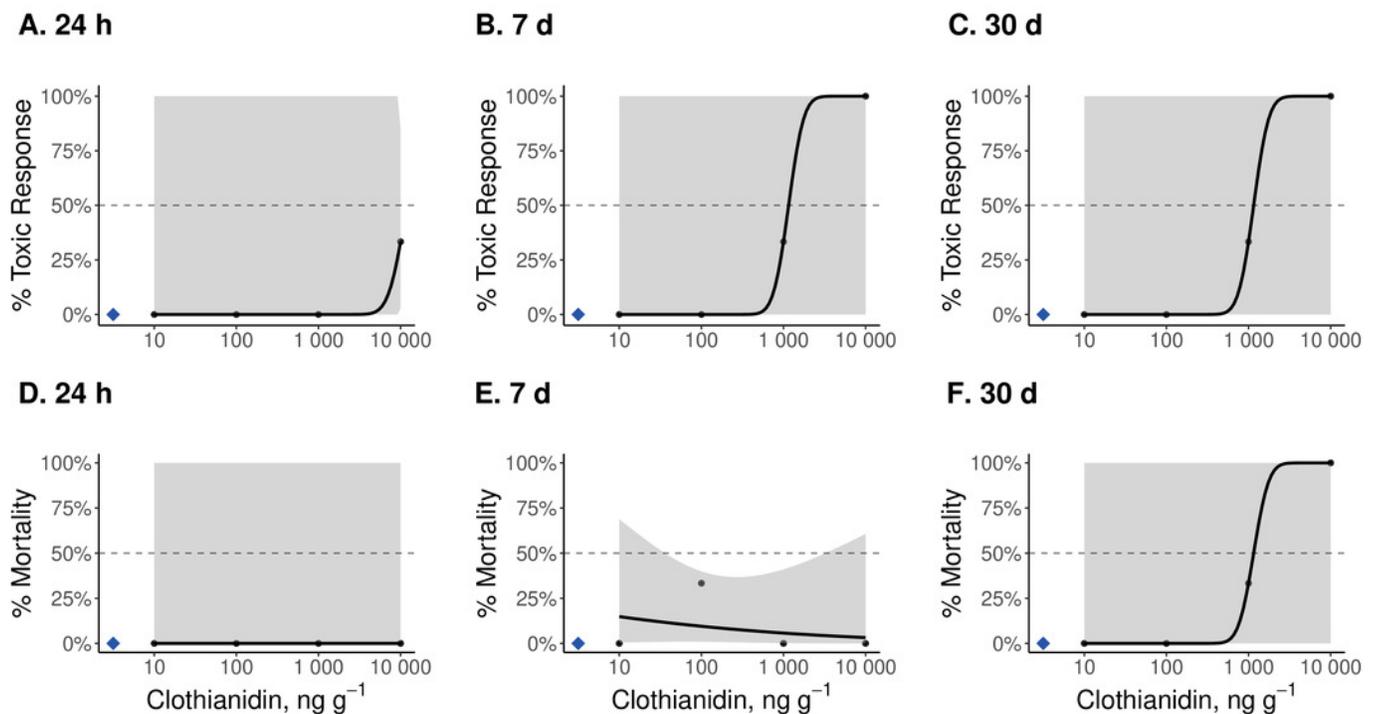
Figure 4

Dose-response curves for early-instar *Pn. 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 concentration).

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. Black dots in each panel represent mean responses at each insecticide concentration; the shaded area represents the 95% confidence interval for each curve. Blue diamonds represent the response of the control group. Dotted lines in each panel marks the 50% toxic response or mortality threshold.
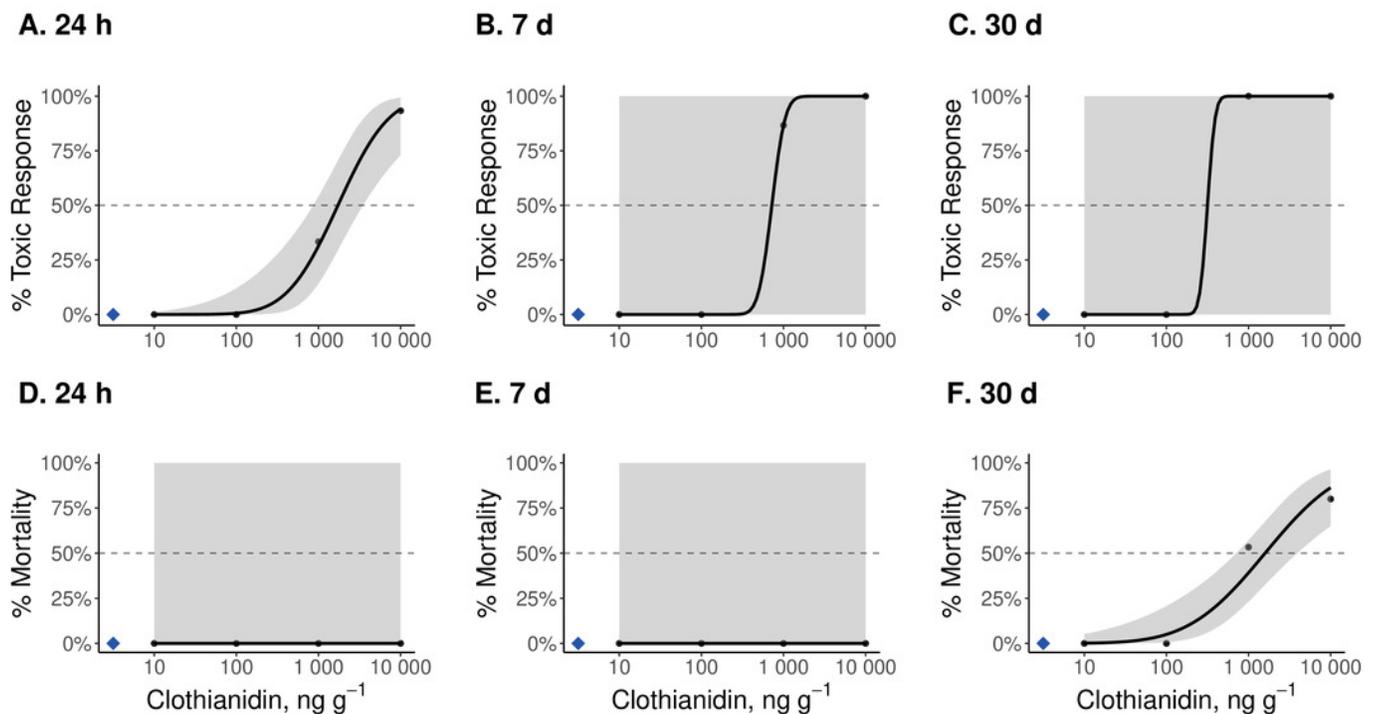
Figure 5

Survivorship curves.

A) late-instar *Pt. versicolor* (n=6 per concentration), B) early-instar *Pt. versicolor* (n=3 per concentration), and C) early-instar *Pn. pyralis* (n=15 per concentration) at different clothianidin concentrations. *P*-values next to each line indicate the significance of reduced survivorship compared to the control (with a Benjamini-Hochberg correction for multiple comparisons). *P*-values were excluded where survivorship was 100% and perfectly overlapped with control values (100 ng g^{-1} in panel B, 10 and 100 ng g^{-1} in panel C). Survival was significantly affected by clothianidin exposure (late-instar *Pt. versicolor*: χ^2_4 = 18, *P* = 0.001; early-instar *Pt. versicolor*: χ^2_4 = 12.5, *P* = 0.01; early-instar *Pn. pyralis*: χ^2_4 = 58.3, *P* < 0.0001).
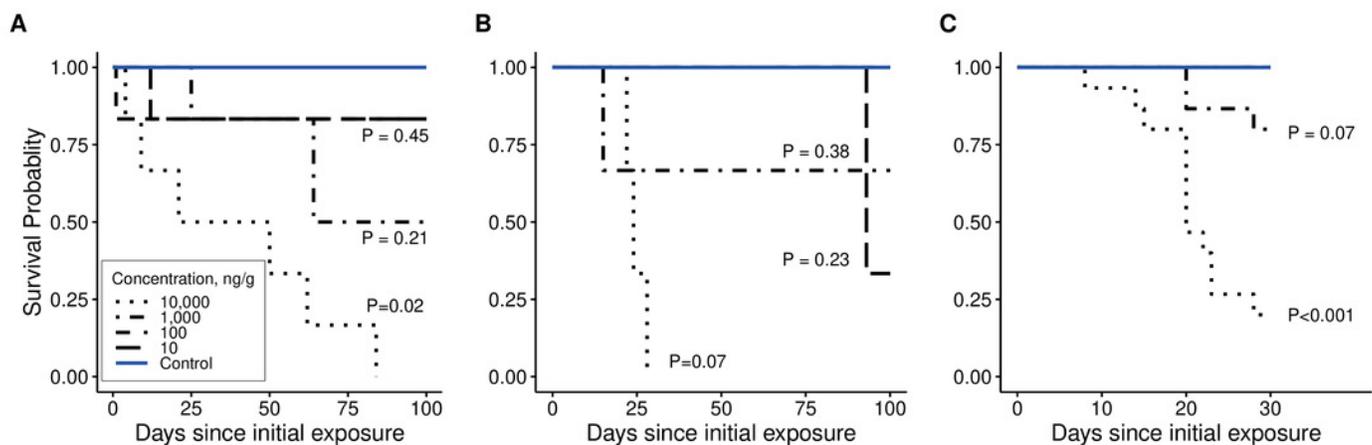
Figure 6

Percent of feeding opportunities taken by firefly larvae.

A) late-instar *Pt. versicolor* larvae (χ^2_4 = 15.8, *P* = 0.003), (B) early-instar *Pt. versicolor* larvae (χ^2_4 = 8.2, *P* = 0.08), and (C) early-instar *Pn. pyralis* larvae (χ^2_1 = 12.4, *P* = 0.0004). Different letters indicate significant differences in feeding activity within each cohort at *P* < 0.05 (Benjamini-Hochberg correction for multiple comparisons).
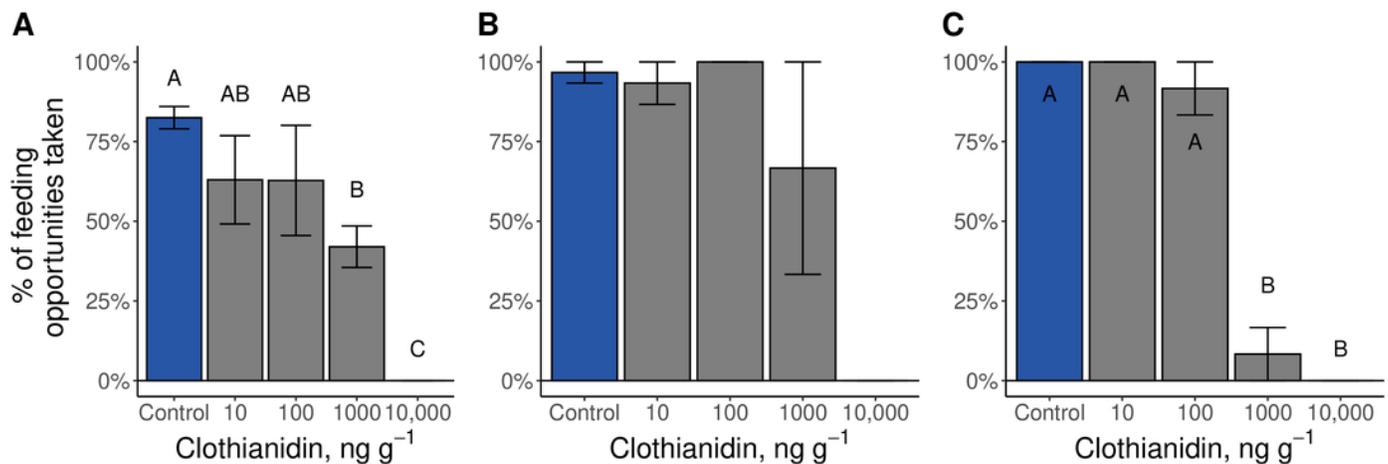
Figure 7

Amount of time that late-instar *Pt. versicolor* 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).