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Non-target effects of neonicotinoid seed treatments; mortality of coccinellid larvae related to zoophytophagy

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ABSTRACT

Third and fourth instar Harmonia axyridis Pallas larvae feed directly on corn seedlings; therefore they may be at risk if the seedlings are chemically treated. Neonicotinoids are broad-spectrum systemic insecticides that are frequently applied to corn seeds prior to planting to protect seedlings from early-season root and leaf-feeding. In this study, H. axvridis larvae were exposed for 360 min to corn seedlings that had been grown from seeds treated with either thiamethoxam or clothianidin. Neurotoxic symptoms (trembling, paralysis, and loss of coordination) were observed in 72% of the H. axvridis larvae; recovery following neurotoxic symptoms rarely occurred (7%). Starvation of larvae for 48 h prior to seedling exposure may increase tissue consumption; however, there was no difference in the number of starved or satiated larvae that displayed neurotoxic symptoms and died. Neurotoxic symptoms and larval mortality were significantly more likely to occur if larvae were exposed to seedlings grown from seeds treated with neonicotinoids compared to the control. In addition, clothianidin resulted in significantly greater larval mortality (80%) compared to thiamethoxam (53%). Symptoms and mortality were rarely observed if seedlings were not treated with a neonicotinoid seed treatment (3% and 3%, respectively). Coccinellids are abundant in agroecosystems in which neonicotinoid seed treatments are likely to be applied. Therefore, the use of neonicotinoids may have negative effects on these non-target species if early-season leaf-feeding occurs in the field.

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1. Introduction

Zoophytophagy is a type of omnivorous feeding behavior that occurs when plant material is consumed by primarily predaceous species (Coll and Guershon, 2002). Nectar-feeding commonly occurs among parasitoids and predators, and pollen-feeding has been recorded for several species of insect predators (Lundgren, 2009). For example, adults and larvae of the predaceous coccinellid, Coleomegilla maculata De Geer (Coleoptera: Coccinellidae), consume pollen (Smith, 1960; Lundgren et al., 2004) and nectar (Smith and Krischik, 1999). Ingestion of leaf tissue by insect predators is less often reported and it has frequently been explained by low prey availability (Hodek and Honěk, 1996; Eubanks and Denno, 1999; Coll and Guershon, 2002). There are relatively few studies of leaf tissue ingestion by insect predators other than hemipterans (Coll, 1998; Gillespie and McGregor, 2000; Lundgren et al., 2009), but recent findings have documented that larvae of two species of coccinellids (C. maculata and Harmonia axyridis Pallas) feed directly on leaf tissue (Moser et al., 2008). Ingestion of leaves by a predaceous

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coccinellid species had previously been reported for adult *Coccinella septempunctata* L. (Brassler, 1930), *Exochomus nigromaculatus* (Pathak and Khan, 1994), and several *Micraspis* species (Pathak and Khan, 1994).

Zoophytophagous feeding behavior increases fecundity, and reduces development time and cannibalism in some predatory species (Coll, 1998; Eubanks and Denno, 1999; Gillespie and McGregor, 2000; Patt et al., 2003; Leon-Beck and Coll, 2007; Berkvens et al., 2008). However, zoophytophagy can also be harmful if the plant material is morphologically or chemically defended (Coll, 1998) or contains plant-incorporated pest resistance traits (Lundgren, 2009). For example, nectar-feeding by beneficial insects is detrimental if neonicotinoid insecticides are applied to the soil or foliage during flowering because neonicotinoids are highly systemic; changes in behavior and/or mortality have been recorded for honey bees (see review Maus et al., 2003; Rortais et al., 2005), C. maculata (Smith and Krischik, 1999), Chrysoperla carnea (Neuroptera: Chrysopidae) (Rogers et al., 2007), Orius spp. (Sclar et al., 1998; Al-Deeb et al., 2001) and several parasitoid species (Stapel et al., 2000; Rebek and Sadof, 2003; Krischik et al., 2007).

Neonicotinoid seed treatments are becoming increasingly important in agricultural crops because they target organisms that are not controlled by Bt δ -endotoxins (Tomizawa and Casida, 2005). In corn, the use of neonicotinoid seed treatments protects





corn seedlings against a range of insect pests from the orders Coleoptera, Lepidoptera, Diptera, Hemiptera and Hymenoptera (Elbert et al., 2008). Neonicotinoid seed treatments have been used in soybeans for protection against bean leaf beetles, Cerotoma trifurcata Forster (Coleoptera: Chrysomalidae) (Bradshaw et al., 2003) and may be used to reduce soybean aphid, Aphis glycines (Homoptera: Aphididae), populations (Magalhaes et al., 2008, 2009). Seed treatments containing neonicotinoids are considered relatively safe for non-target species because the concentration of the insecticide decreases as the plant increases in biomass. Thus, when seed treatments are used, the concentration of insecticide distributed throughout the plant has been greatly reduced by the time the mature plant has entered its flowering phase, minimizing its effect on non-target nectar and pollen-feeders. However, non-target organisms that feed on seedling leaf tissue may be exposed to lethal concentrations of the insecticide.

The coccinellid, *H. axyridis*, occurs in many agricultural habitats, including soybean and corn, and feeds directly on pollen (Lundgren et al., 2004) and leaf tissue in the laboratory (Moser et al., 2008); therefore, if *H. axyridis* feeds on leaf tissue from plants grown from treated seeds, it may be at risk due to the prevalent use of these insecticides.

The objective of this study is to determine the effect of two types of neonicotinoid seed treatments, thiamethoxam and clothianidin, on *H. axyridis* larval mortality following ingestion of leaf tissue from corn seedlings grown from treated seeds. Furthermore, we quantified the amount of time 4th instars initially spent on seedlings to determine if seedlings grown from treated seeds had a repellent effect. The use of alternative food may be influenced by prey availability (Hodek and Honěk, 1996); therefore, we also examined the influence of hunger on larval behavior and mortality in response to seedlings grown from treated seeds.

2. Materials and methods

Corn seedlings, Zea mays L. (Poales: Poaceae), were used to test the effect of seed treatments on coccinellid larvae because corn seeds are treated with neonicotinoids and H. axyridis larvae feed on corn seedlings (Moser et al., 2008). Two neonicotinoid seed treatments were used: thiamethoxam (0.250 mg AI/seed) and clothianidin (0.250 mg AI/seed). Control seeds did not contain a neonicotinoid seed treatment. Seeds (10-12) were planted in 110 mm diameter pots (Kord products, Toronto, Canada) with Promix BX[®] soil (Premier[®] Horticulture Inc., Quakertown, PA) and watered daily; seedlings were grown under greenhouse conditions (25-29 °C; L:D 14:10 h). Because many seed treatments are water-soluble (Maienfisch et al., 2001; Nault et al., 2006), pots containing different seed treatments were placed in separate plastic saucers to prevent contamination due to water run-off. Seedlings used in this study were about 8 cm in height, and between the vegetative first leaf stage (V1) and vegetative second leaf stage (V2).

Adult *H. axyridis* were collected from alfalfa fields during summer 2008 at the University of Kentucky Spindletop Research Farm, Lexington, KY (Universal Trans-Mercator Grid: 4224676 N, 689850 E, Zone 16). Egg-producing *H. axyridis* females were separated into individual Petri dishes (Fisherbrand[®], polystyrene, 100×15 mm, Fisher Scientific Company LLC, Pittsburgh, PA) and were provided cotton soaked with 10% sucrose water and an *ad libitum* supply of *Acyrthosiphon pisum* Harris (Homoptera: Aphididae). Faba bean, *Vicia faba* L. (Fabales: Fabaceae) was used to rear *A. pisum*. Larvae used in this study were first-generation offspring from the field-collected females. Following egg eclosion, larvae were separated and placed individually within plastic Petri dishes (Fisherbrand[®], polystyrene, 60×15 mm) and provided with an *ad libitum* supply of *A. pisum* and cotton soaked with 10% sucrose water daily. Adults

and larvae were maintained at 23.0 \pm 1.0 °C with a photoperiod of L:D 16:8 h.

2.1. Experimental design

Within 24 h of 4th instar eclosion, larvae were provided an ad libitum supply of A. pisum or no aphid prey for 48 h prior to exposure to corn seedlings. After 48 h, larvae were individually placed in arenas containing a corn seedling growing from a seed treated with thiamethoxam, clothianidin or no insecticide (control). Arenas consisted of dishes containing a transplanted corn seedling; corn seedlings were individually transplanted into Reynolds[®] Del-Pak® plastic containers (diameter 10.5 cm, height 6 cm, Reynolds Metal Company, Richmond, VA) containing Promix BX[®] soil. To prevent larvae from having direct contact with the soil, an additional plastic container with a central opening (2.5 cm diameter) was placed over the seedling. The central opening was covered with water-soaked cotton to prevent soil exposure and larval escape. A lid was created by inverting an additional Reynolds[®] Del-Pak® plastic container and loosely sealing it over the container. A wire-mesh vent (5 cm by 5 cm) was added to the center of the lid to increase air circulation. During the plant exposure interval, containers were maintained under fluorescent lighting and a temperature of 21.0 ± 2.0 °C. Treatments were randomly assigned to larvae and a new seedling and container were used for each replication; each treatment was replicated 15 times (N = 90).

2.2. Data collection

Insecticidal seed treatments used in this study may have a repellent effect that may reduce contact by *H. axyridis* larvae with the plant surface. To determine if there is a difference in the initial time spent on seedlings, we recorded the time 4th instars initially spent on a corn seedling. Larvae were individually placed with a small paint brush on the water-soaked cotton at the base of the seedling and timing began once a larva walked onto the surface of the plant and ended when the larva left the plant, or at 5 min. The larva was then observed within the arena once every 30 min for 240 min and once every 60 min for an additional 120 min; each larva was confined to the container with the corn seedling for a total of 360 min. Mortality and the number of larvae displaying neurotoxic symptoms, including trembling, paralysis, and loss of coordination (De Cock et al., 1996), were recorded during this period. Following the 360 min plant exposure, larvae were returned to individual Petri dishes and provided A. pisum. Larvae were examined about 24 h after seedling exposure and mortality was recorded. A larva was considered dead if it did not move its legs when touched with a small paint brush (Youn et al., 2003).

2.3. Statistical analysis

Factorial ANOVA using ranks was used to examine differences in initial time spent on seedlings based on seed treatment, satiation level, and their interaction (SAS 9.1; Brunner and Puri, 2001). Factorial ANOVA using ranks was also used to compare the time until larvae displayed neurotoxic symptoms based on seed treatment, satiation level, and their interaction. In addition, *t*-tests were used to compare treatments within ranks if the interaction was significant (SAS 9.1). Larvae that did not display neurotoxic symptoms during the 360 min exposure period were assigned the highest rank (360 min). Likelihood-ratio χ^2 analyses were performed to examine observations of neurotoxic symptoms and mortality based on treatments (JMP 7.0.2). All tests were examined at the $p \leq 0.05$ level of significance.

3. Results

Seed treatments did not influence time larvae spent on corn seedlings (thiamethoxam = 168.9 ± 22.5 s; clothianidin = 190 ± 22.4 s; control = 182.4 ± 21 s; F = 0.42, df = 1.98, 78.5, p = 0.66) and there was no interaction between seed treatment and satiation (F = 2.60, df = 1.98, 78.5, p = 0.08). Well-fed 4th instar *H. axyridis* spent more time initially on seedlings (mean \pm SE; 218.4 ± 16.2 s) compared to individuals that were starved for 48 h prior (143 ± 17.7 s) (F = 10.03, df = 1, 78.5, p = 0.0015).

There was a significant interaction between satiation level and seed treatment on the onset of neurotoxic symptoms (trembling, loss of coordination, and inability to walk) (F = 4.27, df = 1.6, 53.8, p = 0.0210; Fig. 1). On the control seedlings, no well-fed larvae displayed neurotoxic symptoms, but one starved larva (1 of 45) displayed symptoms at 5 h (Fig. 2). Both starved and well-fed larvae that were exposed to seedlings grown from treated seeds displayed neurotoxic symptoms faster than larvae exposed to control seedlings (well-fed larvae: clothianidin, t = -8.75, df = 28, p < 0.0001; thiamethoxam, t = -3.81; df = 28; p = 0.0007; starved larvae: clothianidin, t = -5.57, df = 28, p < 0.0001, thiamethoxam, t = -5.57-4.57, df = 28, p < 0.0001; Fig. 1). There was no difference between seed treatments in the onset of neurotoxic symptoms in starved larvae (t = 0.37, df = 28, p = 0.71; Fig. 1a), but well-fed larvae displayed symptoms faster in the clothianidin seed treatment $(92 \pm 29 \text{ min})$ compared to the thiamethoxam seed treatment $(228 \pm 35 \text{ min}; t = -3.23, df = 28, p = 0.0032; Fig. 1b).$

Of the 44 larvae displaying neurotoxic symptoms, 93% died within 24 h of the bioassay. The remaining three larvae recovered; one well-fed larva exposed to a seedling grown from a clothianidin-treated seed and two starved larvae exposed to seedlings grown from thiamethoxam-treated seeds. The number of dead larvae and those displaying neurotoxic symptoms were significantly greater if larvae were exposed to seedlings grown from seeds treated with neonicotinoids (thiamethoxam and clothianidin combined; 67% mortality; 72% symptoms) compared to control (3% mortality; 3% symptoms) seedlings (mortality: χ^2 = 38.90, df = 1,

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p < 0.0001; symptoms: $\chi^2 = 44.42$, df = 1, p < 0.0001; Fig. 2). Exposure to seedlings grown from clothianidin-treated seeds resulted in significantly greater number of larvae displaying neurotoxic symptoms and mortality (83% symptoms; 80% mortality) compared to exposure to seedlings grown from thiamethoxam-treated seeds (60% symptoms; 53% mortality; symptoms: $\chi^2 = 4.11$, df = 1, p = 0.0425; mortality: $\chi^2 = 4.90$, df = 1, p = 0.0268; Fig. 3a and b). Larval satiation prior to the bioassay had no effect on the number of larvae displaying neurotoxic symptoms or on the number that died (symptoms: $\chi^2 = 0.18$, df = 1, p = 0.67; mortality: $\chi^2 = 0.05$, df = 1, p = 0.83; Fig. 3a and b).

4. Discussion

Insecticides can be disruptive to natural and biological control by decreasing natural enemy populations (Johnson and Tabashnik, 1999). Plant-incorporated pest resistance, such as genetic modification, can limit exposure to many non-target organisms in comparison to broad-spectrum insecticidal sprays (Lundgren, 2009). However, these management techniques cannot be assumed to influence only the target organism because large variations exist in insect behavioral and physiological adaptations. The use of neonicotinoid seed treatments is a technique that can reduce the impact on some non-target organisms (Mizell and Sconvers, 1992; Albajes et al., 2003; Elbert et al., 2008; Jeschke and Nauen, 2008), but this current study and previous studies indicate that the use of insecticidal seed treatments may have negative effects on non-target zoophytophagous species. Exposure to seedlings grown from neonicotinoid-treated seeds resulted in high mortality (67%) of H. axyridis larvae; clothianidin-treated seeds were more toxic to H. axyridis larvae than thiamethoxam-treated seeds (Fig. 3). Satiation level did not play a role in the ingestion of systemically-treated leaf tissue because starved and well-fed larvae had similar rates of mortality. However, larval satiation influenced the time larvae initially spent on seedlings; H. axyridis larvae spent less time initially on seedlings if they were starved for 48 h prior to the bioassay. Neonicotinoid seed treatments are detrimental to all



Fig. 1. Mean (±SE) time (min) before neurotoxic symptoms were observed in *Harmonia axyridis* 4th instars exposed to corn seedlings grown from seeds treated with thiamethoxam, clothianidin, or not treated (control). Larvae were (A) starved for 48 h or, (B) fed an *ad libitum* supply of *Acyrthosiphon pisum* (well-fed) prior to the study. Larvae were exposed to seedlings for 360 min (*N* = 90).



Fig. 2. Percentage of *Harmonia axyridis* 4th instars displaying neurotoxic symptoms over time (min) when exposed to corn seedlings grown from (A) clothianidin-treated, or (B) thiamethoxam-treated seeds. Larvae were fed an *ad libitum* supply of *Acyrthosiphon pisum* (well-fed) or starved for 48 h prior to the bioassay.

leaf-feeding insects during the early growing stages of plants grown from treated seeds and further studies are needed to examine the early-season presence of zoophytophagous predators in agroecosystems. Although larval feeding on seedling leaf tissue has only recently been reported for *H. axyridis* (Moser et al., 2008), observations of *C. septempunctata* adults feeding on leaves in the field were made nearly 80 years ago (Brassler, 1930).

4.1. Neonicotinoids and coccinellid mortality

Neonicotinoids can cause mortality and negatively impact coccinellids through several routes of entry, including: topical contact, residual contact, inhalation of volatiles, ingestion of toxified plant products and ingestion of toxified prey tissues (Ruberson et al., 1998; Johnson and Tabashnik, 1999; Smith and Krischik, 1999). During this study, the amount of tissue consumed was not quantified but personal observations of leaf feeding frequently occurred; larvae feed for brief periods (<3 min) prior to the display of neurotoxic symptoms (Moser, S.E., personal observation). The exposure to these toxic plant tissues resulted in relatively high mortality (53–80% mortality; Fig. 3) and recovery after exhibiting neurotoxic



Fig. 3. Percentage of *Harmonia axyridis* 4th instars displaying (A) neurotoxic symptoms and, (B) mortality when exposed to corn seedlings treated as seeds with thiamethoxam, clothianidin, or not treated (control). Larvae were fed an *ad libitum* supply of *Acyrthosiphon pisum* (well-fed) or starved for 48 h prior to the study. Likelihood χ^2 analyses were used to determine if there was difference in the number of larvae displaying neurotoxic symptoms or mortality for both factors (plant type, hunger level). There was no difference due to hunger, but larvae were more likely to display symptoms and die if they were exposed to neonicotinoid treatments (symptoms, p < 0.0001; mortality, p < 0.0001) or if they were exposed to clothianidin compared to thiamethoxam treatments (symptoms, p = 0.04; mortality, p = 0.03).

symptoms rarely occurred (7% recovery). Recovery may be less frequent in the field because larvae exhibiting neurotoxic symptoms are incapacitated and vulnerable to predation by other predators, including other coccinellids (Hodek and Honěk, 1996; Lucas, 2005; Moser and Obrycki, 2009). Therefore, debilitated larvae may provide an additional toxic source to other coccinellids. Coccinellids may also be exposed to neonicotinoids through contaminated prey (Lucas et al., 2004; Galvan et al., 2006; Papachristos and Milonas, 2008). For example, mortality of C. maculata adults and larvae was 50-100% following consumption of Leptinotarsa decemlineata (Coleoptera: Chrysomelidae) egg masses that were treated with a neonicotinoid (imidacloprid) foliar application (Lucas et al., 2004). Soil application of imidacloprid resulted in >50% mortality of Hippodamia undecimnotata (Coleoptera: Coccinellidae) larvae due to toxification of their aphid prey which were reared on treated plants and sublethal effects (reduced fecundity, reduced adult longevity) were also observed (Papachristos and Milonas, 2008).

Coccinellids may be vulnerable to systemically translocated neonicotinoids in corn seedlings because prior to ingestion, they are unable to detect the presence of these insecticides. The neonicotinoids used in this study are systemically translocated to seedlings and *H. axyridis* larvae were unlikely to contact the chemical prior to ingestion. In contrast, when insecticidal (dimethoate) residues are present on the surface of plants, *C. septempunctata* (Coleoptera: Coccinellidae) spent less time on treated plants (Singh et al., 2001). In the current study, *H. axyridis* larvae initially spent similar amounts of time on systemically-treated and untreated seedlings. Although direct observations of leaf tissue feeding were not recorded, it is assumed that neurotoxic symptoms and mortality were due to tissue ingestion because contact with imidacloprid residues do not kill *H. axyridis* larvae (Vincent et al., 2000) and *H. axyridis* 4th instars have been observed to repeatedly feed on corn seedlings in previous studies (Moser et al., 2008). Acceptance of inappropriate or potentially harmful food sources by coccinellids has been frequently reported (Hodek and Honěk, 1996).

4.2. Differences between seed treatments

Clothianidin seed treatments resulted in significantly greater larval mortality compared to thiamethoxam seed treatments. Similarly, clothianidin compared to thiamethoxam seed treatments were more effective in controlling larval onion maggots, *Delia antiqua* (Diptera: Anthomyiidae), in the field (Nault et al., 2006). Thiamethoxam and clothianidin have a common mode of action, but thiamethoxam is a partial agonist while clothianidin is a full or nearly full agonist (Tan et al., 2007) and thiamethoxam is more water-soluble than clothianidin (Maienfisch et al., 2001; Nault et al., 2006). Water-solubility may have been a factor in its reduced toxicity to *H. axyridis* larvae; watering during seed germination may have partially removed thiamethoxam from the seed treatment, reducing the thiamethoxam concentration within the seedlings.

4.3. Neonicotinoid use and zoophytophagous predators

Since the discovery of neonicotinoids in the 1990's, there have been few studies examining the systemic and translaminar action of neonicotinoid seed treatments on zoophytophagous species through leaf tissue ingestion. Many zoophytophagous species, in addition to *H. axyridis*, are likely susceptible to neonicotinoid seed treatments. For example, when exposed to corn seedlings grown from seeds treated with neonicotinoid seed treatments, nearly 100% mortality occurred in eighteen species of carabids (Mullin et al., 2005). Ingestion of thiamethoxam (through treated water) was about 200 times more toxic than residue contact for the zoophytophagous species, Podisus nigrispinus (Heteroptera: Pentatomidae) (Torres and Ruberson, 2004). Reduced P. nigrispinus survival in field cages also occurred in fields treated with thiamethoxam and imidacloprid foliar sprays (Torres and Ruberson, 2004). In addition, exposure to foliar applications of clothianidin, thiamethoxam, and imidacloprid, can cause coccinellid mortality (Mizell and Sconyers, 1992; James, 2003; Bozsik, 2006; Cloyd and Dickinson, 2006) and suppress populations of several coccinellid species (Wells et al., 2001; Grafton-Cardwell et al., 2008). Mortality due to topical exposure and ingestion of imidacloprid has also been recorded for the zoophytophagous species, Podisus maculiventris (Heteroptera: Pentatomidae) (De Cock et al., 1996).

Albajes et al. (2003) completed a comprehensive multi-year survey of predatory arthropods in corn grown from neonicotinoid-treated (imidacloprid) seeds and in untreated corn fields throughout the growing season. Based on the number of individuals sampled, most predator groups (grouped by order) did not appear to be influenced by the addition of neonicotinoid seed treatments (Albajes et al., 2003). However, zoophytophagous feeding behavior varies widely within orders and the leaf-feeding predators may have been disproportionately affected by insecticidal seed treatments. For example, Albajes et al. (2003) reported that, contingent on year, Heteroptera populations were reduced in treated fields and that *Orius* spp. were the most commonly collected species (88%) within this group. Feeding on leaf material is common among many *Orius* spp., which may explain why fewer *Orius* spp. were recorded from treated fields (Albajes et al., 2003). An important early-season predator of the soybean, *Orius insidiosus* feeds directly on the xylem and mesophyll of soybean plants in addition to soybean aphids (Armer et al., 1998; Harwood et al., 2007, 2009). When confined with corn seedlings treated with imidacloprid seed treatments, *O. insidiosus* mortality significantly increased (Al-Deeb et al., 2001). Neonicotinoid seed treatments containing thiamethoxam and imidacloprid have been proposed for soybean aphid control in soybeans (Magalhaes et al., 2008, 2009) and the use of these seed treatments in soybean may also impact other soybean aphid predators, such as *H. axyridis* (Harwood et al., 2007, 2009).

5. Conclusion

Neonicotinoids are well suited for use in seed treatments because of their systemic action (Elbert et al., 2008). Seed treatments containing neonicotinoids are used in many crops, including cotton, corn, cereals, sugar beet and oilseed rape (Elbert et al., 2008) and all Cry-protected corn seeds are treated with neonicotinoid seed treatments (Smith et al., 2004). Non-target impacts on beneficial pollen and nectar feeders are likely to decline with reduced use of foliar sprays containing neonicotinoids; however, the use of neonicotinoid seed treatments may negatively impact beneficial leaf-feeding insects that are important biological and natural control agents in integrated pest management programs. For example, feeding on new vegetative growth from seeds treated with neonicotinoids by zoophytophagous species may impact early-season colonization and may reduce survival of zoophytophagous predators in agroecosystems. Although early-season colonization by predators and parasitoids can help prevent pest outbreaks later in the season (Daane and Yokota, 1997; Campbell and Lilley, 1999; Jung et al., 2004), there are few non-target studies addressing the potential impact of neonicotinoids on zoophytophagous species. Therefore, it is vital that additional studies are conducted on earlyseason establishment of zoophytophagous species in agroecosystems in which seed treatments are utilized.

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