Neonicotinoid insecticide travels through a soil food chain, disrupting biological control of non-target pests and decreasing soya bean yield

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Summary

1. Neonicotinoids are the most widely used insecticides world-wide, but their fate in the environment remains unclear, as does their potential to influence non-target species and the roles they play in agroecosystems.

2. We investigated in laboratory and field studies the influence of the neonicotinoid thiamethoxam, applied as a coating to soya bean seeds, on interactions among soya beans, non-target molluscan herbivores and their insect predators.

3. In the laboratory, the pest slug *Deroceras reticulatum* was unaffected by thiamethoxam, but transmitted the toxin to predaceous beetles (*Chlaenius tricolor*), impairing or killing > 60%.

4. In the field, thiamethoxam-based seed treatments depressed activity–density of arthropod predators, thereby relaxing predation of slugs and reducing soya bean densities by 19% and yield by 5%.

5. Neonicotinoid residue analyses revealed that insecticide concentrations declined through the food chain, but levels in field-collected slugs (up to 500 ng g^{-1}) were still high enough to harm insect predators.

6. *Synthesis and applications*. Our findings reveal a previously unconsidered ecological pathway through which neonicotinoid use can unintentionally reduce biological control and crop yield. Trophic transfer of neonicotinoids challenges the notion that seed-applied toxins precisely target herbivorous pests and highlights the need to consider predatory arthropods and soil communities in neonicotinoid risk assessment and stewardship.

Key-words: agroecosystem, Carabidae, clothianidin, *Deroceras*, ecosystem service, thiamethoxam, tritrophic interactions, trophic cascade

Introduction

Neonicotinoid insecticides are the most widely used class of insecticides world-wide (Sparks 2013), and mounting evidence suggests that they can undermine populations of non-target animals in natural and agricultural ecosystems (van der Sluijs *et al.* 2014). Neonicotinoids are neurotoxins that can have sublethal effects on bees (Goulson 2013), and their concentrations in surface waters have been negatively correlated with abundance of aquatic invertebrates and insectivorous birds (van Dijk, van Staalduinen & van der Sluijs 2013; Hallmann *et al.* 2014).

Despite this recent scrutiny, major gaps remain in our knowledge about the fate of neonicotinoids and their consequences for animal communities, even for invertebrates in agroecosystems where these compounds are most commonly used (Goulson 2013; van der Sluijs *et al.* 2014). Neonicotinoids are routinely applied as seed coatings to large-acreage crops, such as corn and soya beans, and so are used preventatively on millions of hectares of farmland annually (U. S. Geological Survey 2014) to counter early season insect pests, many of which are sporadic in space and time. Neonicotinoid seed coatings are absorbed systemically into crop tissues and then decline over the season (Laurent & Rathahao 2003; Sur & Stork 2003). Non-target effects of these coatings might be particularly important

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for soil organisms, given their proximity to the insecticides (Goulson 2013). Populations of soil-dwelling arthropod predators have been depressed by neonicotinoid seed coatings in some field studies (e.g. Leslie *et al.* 2010), but effects have been variable and exposure pathways remain obscure. It is also virtually unknown what significance these effects may have for biological control of pests. There is, therefore, a strong need to investigate more closely the fate of neonicotinoid seed treatments in the soil environment and their influence on predatory arthropods that provide pest-control benefits to farmers.

Molluscan pests (slugs and snails) are often overlooked but in many cropping systems are among the most challenging pests farmers face (South 1992). In Great Britain alone, slugs cost wheat and rapeseed farmers upwards of £9 million annually in control costs (UK DEFRA 2010). Slugs are also a mounting problem in grain and forage production the Mid-Atlantic U.S., where their populations have increased with adoption of conservation-tillage farming techniques (Douglas & Tooker 2012). The introduced species *Deroceras reticulatum* Müller and its native congener *D. laeve* Müller are the major pest slugs in the Mid-Atlantic region, where they feed upon emerging seedlings and compromise crop establishment, sometimes requiring costly replanting (Hammond & Byers 2002).

Slugs are likely to consume neonicotinoids when they feed early in the growing season upon seedlings grown from coated seeds, but as molluscs they may not be sensitive to these insecticides. In Silent Spring, Carson noted 'For some reason, snail-like mollusks seem to be almost immune to the effects of insecticides' (1962; p. 257). This rule-of-thumb appears to hold for imidacloprid, which has low acute toxicity to *Deroceras reticulatum* (Simms, Ester & Wilson 2006; but see effects on freshwater snails, van Dijk, van Staalduinen & van der Sluijs 2013). If slugs ingest neonicotinoids without dying, they may serve as toxic prey for predators that attack slugs, potentially disrupting biological control. Such a phenomenon would bear a strong resemblance to the protection some insect herbivores derive from toxic secondary metabolites, such as nicotine, that they acquire from their host plants (Thorpe & Barbosa 1986; Kumar *et al.* 2014). Notably, neonicotinoids and nicotine share a common mode of action, but neonicotinoids are roughly 10 000 times more toxic to insects (Jeschke & Nauen 2008). Predators of slugs in temperate agroecosystems include epigeal beetles, especially certain species of ground beetles (Carabidae; Symondson 2004), which are physiologically susceptible to neonicotinoids (Mullin *et al.* 2005).

Here, we examine the influence of neonicotinoid seed treatments on slug pests and the potential for these insecticides to disrupt slug predators through dietary transfer of the toxin. We studied soya beans *Glycine max* L. Merr. coated with thiamethoxam because they are the most popular no-till crop in the U.S. (50% of acres; Horowitz, Ebel & Ueda 2010), and thiamethoxam

is one of two neonicotinoids commonly used on soya beans (U. S. Geological Survey 2014). We began our investigation with laboratory experiments to test whether: (i) slugs are susceptible to thiamethoxam applied as a seed coating and (ii) thiamethoxam and its metabolites move from slugs to their predators. After finding that slugs could transfer neonicotinoids to their predators, we tested whether thiamethoxam influences trophic relationships among soya beans, slugs and predators in the field. Our primary hypothesis was that thiamethoxam would disrupt predation of slugs, fostering larger slug populations that would in turn hinder soya bean establishment, potentially decreasing yield (Fig. 1). We complemented our experiments with neonicotinoid sampling to quantify insecticide residue transfer through the food chain under laboratory and field conditions. To our knowledge, the results we present here are the first to describe the flow of neonicotinoids through any food chain and to rigorously investigate the potential for neonicotinoid seed treatments to disrupt biological control under field conditions.
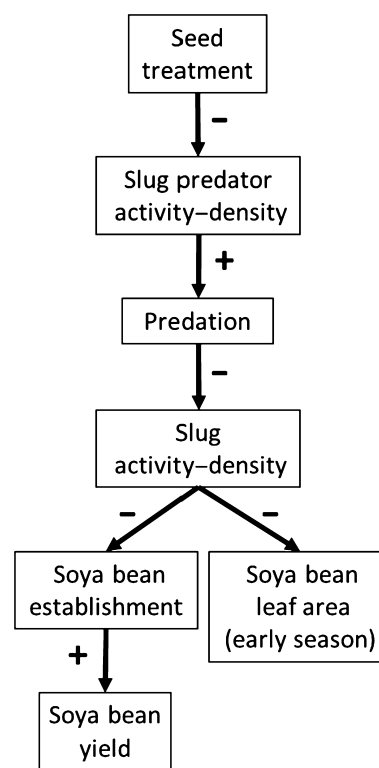

Fig. 1. A schematic representation of our hypothesis for the potential influence of seed treatments on the ecological community in no-till soya beans. The '+' and '−' signs indicate the anticipated effect (positive or negative) of the preceding factor on the following factor. We would expect this model to hold when slugs are the dominant early season soya bean herbivore. Based on previous findings that moderate early season leaf damage has little effect on soya bean yield (Hammond 2000), we expected slugs to reduce yield mainly by killing plants rather than by eating leaf tissue.

Materials and methods

LABORATORY EXPERIMENTS

Seeds, slugs and beetles

To explore the influence of soya bean seed treatments on slug–predator interactions, we used a single soya bean variety (A1016495, FS HiSOY® RR2; Growmark, Bloomington, IL, USA) treated in one of four ways to represent a range of commercially available seed treatments: (i) untreated control; (ii) fungicide-alone [ApronMaxx®, active ingredients (a.i.): mefenoxam, ~0·0068 mg ai seed^{-1}; fludioxonil, ~0·0045 mg ai seed^{-1}; Syngenta, Basel, Switzerland]; (iii) fungicides plus low rate insecticide (CruiserMaxx®, a.i.: thiamethoxam, 0·0756 mg ai seed^{-1}; Syngenta); and (iv) fungicides plus high rate insecticide (thiamethoxam, 0·152 mg ai seed^{-1}). Neonicotinoids on soya bean seed are virtually always combined with fungicides.

We collected grey garden slugs *D. reticulatum* in State College, PA (+40·78, −77·87) in areas free from insecticide use, primarily an old field and a residential backyard, from early spring to early summer. In our area, this species has a roughly annual life cycle with juveniles hatching in spring (Douglas 2012). Because slugs do not have distinct growth stages, we standardized our experiments by slug mass (see details below). We kept slugs at room temperature in covered plastic boxes lined with moist potting soil and fed them organic cabbage.

We collected adults of the ground beetle *Chlaenius tricolor* Dejean from crop fields at Russell E. Larson Agricultural Research Farm (LARF; Pennsylvania Furnace, PA; +40·71, −77·95) using dry pitfall traps and hand collection. This carabid was previously identified as an important slug predator in the eastern U.S. (Eskelson *et al.* 2011). We housed beetles individually in 16-oz plastic containers (Reynolds Del-Pak®, Lake Forest, IL, USA) with moist potting soil, in a growth chamber (21 °C, 14:10 L:D). Beetles were fed dry kitten food (Purina® ProPlan® Selects®; Nestlé Purina PetCare, St. Louis, MO, USA) that we moistened with water.

Soya bean–slug and slug–ground beetle bioassays

To determine whether seed treatments alter *D. reticulatum* feeding, in fall 2011, we conducted a factorial experiment with the four types of seed treatments crossed with the presence or absence of slugs. The no-slug treatment accounted for possible direct effects of thiamethoxam on plant growth. On day zero, we planted four soya bean seeds in 16-oz clear plastic containers, and on day one, added one juvenile slug [0·22 ± 0·09 (SD) g] per container and placed them in a growth chamber (21 °C, 14:10 L: D; *n* = 34 containers per treatment with slugs; *n* = 24 containers per treatment without slugs). For a week, we recorded daily the status of seedlings and slugs. On day eight, we recovered slugs, weighed them and held them for use in ground beetle assays. This experiment was blocked into three consecutive trials due to space limitations.

We next investigated whether *D. reticulatum* can transmit seed-applied insecticides from seedlings to ground beetle predators. On day eight, we transferred previously weighed slugs to new 16-oz plastic containers with ~1 cm of moist potting soil (one slug per container), and introduced *C. tricolor* (starved for 6 days, 47% male, one beetle per container), randomly assigning beetles to containers (*n* = 17–19 container per treatment). Six days is within the normal range of starvation for carabids (Bilde & Toft 1998). We tracked the status of slugs and beetles closely for the first 3·5 h in the evening when beetles were introduced, and then daily for 1 week when the containers were stored in a growth chamber (21 °C, 14:10 L:D). Because neonicotinoids can impair motor control at sublethal doses (Goulson 2013), we recorded beetle flip time to assess beetle coordination (Lundgren & Wiedenmann 2002). For each beetle, we flipped the beetle on its back using forceps and used a stopwatch to record the time necessary for the beetle to right itself, ending a trial after 30 s if the beetle failed to flip over (four trials per beetle per day to reduce variability). Beetle flip time was bimodal with most beetles flipping either in <1 s or not at all, so we considered a beetle 'normal' if it had an average flip time ≤1 s and 'impaired' if it had an average flip time >1 s. From day 8 to 16, the one slug we provided was the only food available to each beetle. Starting on day 16, we maintained beetles with kitten food (Purina® ProPlan® Selects®) in a growth chamber until day 24 of the experiment, when all beetles had either recovered (defined as flip time ≤ 1 s) or died. See Appendix S3 (Supporting information) for further details on bioassays.

FIELD EXPERIMENT

Study site, experimental design and crop management

To explore the effects of seed-applied insecticides on interactions among plants, slugs and predators, we conducted a field experiment in 2012 at LARF where plots were arranged in a replicated Latin square design (2 × 6 array), in a field that had been farmed using no-till practices for 7 years. Using a four-row planter with graphite as a seed lubricant, we planted a regional soya bean variety on May 18th at a rate of 444 600 seeds ha^{-1} (76-cm row spacing), either with commercially applied fungicide (mefenoxam and fludioxonil, concentrations as above) and insecticide (thiamethoxam, 0·152 mg ai seed^{-1}; *n* = 6 plots) or without a seed coating (untreated control; *n* = 6 plots). We only used two experimental treatments to improve statistical power and because our laboratory-based results demonstrated that fungicides did not influence slug–soya bean or slug–predator interactions (see Results). Plots (27 × 40 m) abutted one another but we collected all samples in a central area in each plot (15 × 22 m), leaving a buffer of at least 6 m to adjacent plots or edges. We managed weeds in all plots by spraying glyphosate on May 2nd, paraquat on May 17th and glyphosate again on June 14th. See Appendix S3 (Supporting information) for more details on crop management and experimental design.

Stand establishment, early season herbivory and yield

To assess the influence of the seed treatment on crop establishment and productivity, we measured soya bean plant populations and herbivore damage during three early soya bean growth stages (cotyledon, one-trifoliate and three-trifoliate stages). On each sampling date, we counted the number of plants in 3-m stretches of row (six stretches per plot, random locations). We also examined the first 15 seedlings in each sample for evidence of herbivory, recording damage to each cotyledon (0: none; 1: some; 2: cotyledon missing) and the approximate percentage of leaf area removed (on true leaves) using a four-point scale (0: 0%; 0·4: <10%; 1: 10–25%; 2: 25–50%; 3: 50–75%; 4: >75%).

We harvested soya beans on 9 November (at 12·9% moisture), taking yield samples 30·5-m long and four rows wide (two samples per plot).

Invertebrate activity–density

To assess the influence of the treatments on activity–density of slugs and their predators, we installed pitfall traps (4 per plot; 15 and 26 m down rows 16 and 24 of each plot) that we opened monthly for 72 h from June to September. Depending on the taxon, we identified captured specimens to genus, family or order (see Appendix S3, Supporting information). To provide an additional measure of slug activity–density, we used square-foot pieces of roofing material (Owens Corning Rolled Roofing, colour: Shasta White) as artificial slug shelters (6 per plot, random locations). We checked shelters in the morning, weekly from planting through harvest and identified slugs to species in the field (Chichester & Getz 1973; McDonnell, Paine & Gormally 2009).

Predation

In addition to measuring predator activity–density, we more directly measured the prey-consuming function of the generalist predator community by deploying waxworm caterpillars *Galleria mellonella* L. as sentinel prey. While sentinel slugs would have been more relevant to our study, their lack of exoskeleton makes restraining them difficult and impractical. In our previous work (Douglas 2012), predation on waxworm caterpillars was positively related to activity–density of large (>9 mm) ground beetles, which are thought to be among the most important predators of slugs in agroecosystems (Ayre 2001; Symondson 2004). Within several days of pitfall sample dates (June 8th, July 12th, August 15th), we deployed sentinel caterpillars (0·21 ± 0·06 g, 10 per plot, equally spaced from 10 to 30 m along the 14th and 22nd row of each plot) in two 12-h periods (day and night). We restrained each waxworm to a clay ball with an insect pin through its last abdominal segment and placed each waxworm in the field under a wire mesh cage (mesh size: 1·3 cm) to exclude vertebrate predators (after Lundgren *et al*. 2006; Appendix S3, Supporting information).

INSECTICIDE ANALYSES

To further understand the potential movement of neonicotinoid residues through the plant–slug–beetle food chain, we tested both laboratory- and field-exposed organisms for neonicotinoid insecticides and their major degradates. We deposited samples into preweighed 50-ml tubes and stored them at −80° C before shipping them on dry ice to the USDA's National Science Laboratory (Gastonia, NC, USA) for analysis with LC/MS-MS (methods adapted from Kamel 2010). In June 2012, we repeated a subset of our laboratory experiments to describe neonicotinoid concentrations in organisms in the low and high thiamethoxam treatments. Replication was minimal (*n* = 2 per treatment for soil and soya beans; *n* = 1 per treatment for slugs and beetles) because of the expense of insecticide analyses and the need to pool numerous organisms to generate the mass required for an acceptable limit of detection. In the field study previously described, we collected soil, soya bean seedlings and slugs *D. reticulatum* for insecticide analysis (*n* = 3 plots per treatment, pooling subsamples

within plots). At the cotyledon stage, we sampled the aboveground portion of soya bean seedlings and collected soil from cores centred on soya bean stems (10 cm deep, 10·8 cm diameter). In the course of our soil sampling, we also found and collected several earthworms (rinsed in water to remove soil particles). We collected slugs from plants at night at the cotyledon and one-trifoliate stages, and from under refuge traps shortly before soya bean harvest, when plants had senesced. See Appendix S3 (Supporting information) for more details on pooling our subsamples and for reasons why we did not sample predators in the field.

STATISTICAL ANALYSES

We performed all statistical analyses in R 3.1.0 (R Development Core Team 2014), using the 'lm' function for fixed-effects models and the 'lme' function for mixed-effects models (Pinheiro *et al*. 2013). For repeated measures analyses, we chose among candidate covariance structures using Akaike information criteria. We report results based on type II sum of squares for models with multiple fixed effects. Because our blocking factors (trial in the laboratory; 'row' and 'column' blocks in the field) had relatively few levels and were not sampled randomly from a larger population of blocks, we treated them as fixed effects.

Laboratory experiments

To test whether seed treatment influenced slug mass gain, we used analysis of variance (ANOVA) with seed treatment and trial as fixed effects. For slug damage to soya beans over time, we fit a similar model, but with a random effect of microcosm to account for repeated measurements, and an AR(1) covariance structure.

In the slug–ground beetle experiment, to test whether slug-feeding history influenced likelihood of attack by *C. tricolor*, we fit a Cox proportional hazards regression model on slug survival, stratified by trial (Therneau 2014). We compared numbers of impaired and normal beetles across treatments using a Fisher's exact test.

Field experiment

We expected seed treatments to influence mainly early season trophic interactions, so our primary analyses focused on response variables measured during the first 2 weeks after soya beans emerged (~21–35 days after planting). Because we sampled many different predatory taxa, we created a variable for potential slug predators by summing Carabidae, Lampyridae, Staphylinidae and Opiliones, the major arthropod groups at our site that include slug predators (Barker 2004). With the early season data set, we first conducted ANOVAs to test whether seed treatment affected each response in the hypothesized direction (Fig. 1). Then, to see if seed treatment effects could have been caused by our proposed mechanisms, we fit linear regressions between (i) predator activity–density and predation, (ii) predation and slug activity–density, (iii) slug activity–density and soya bean damage, (iv) slug activity–density and soya bean populations and (v) soya bean populations and grain yield, using a Bonferroni-corrected α = 0·01 to account for five interdependent regression analyses. For soya bean damage, we created a factor (loadings for leaf damage: 0·89; loadings for cotyledon damage: 0·89) to represent

variation in overall soya bean damage. All analyses included blocking factors as fixed effects to help account for environmental variation unrelated to treatments.

To examine whether seed treatment had lasting effects on activity–density of slugs and their potential predators, we analysed the remaining sample dates. We fit mixed-effects models with fixed effects of blocks, seed treatment, and their interactions with time and a random effect of plot to account for repeated measurements.

Insecticide analyses

To describe changes in neonicotinoid residues across trophic levels and test for differences between laboratory and field experiments, we fit a regression model to the combined residue data, treating trophic level as a numeric predictor (soya beans = 0; slugs = 1; ground beetles = 2) and setting as a categorical predictor (laboratory, field). We treated trophic level as a numeric predictor because this allowed us to test for a consistent change in neonicotinoid concentration across trophic levels. Laboratory data were from both low and high thiamethoxam treatments, and field data were from treated plots in the cotyledon stage.

Results

LABORATORY EXPERIMENTS

Soya bean–slug bioassays

Slugs readily attacked soya bean seedlings grown from each of the four seed treatments, with no significant differences in the number of seedlings damaged over time (Fig. 2a; seed treatment: $F_{3,116} = 0.26$, $P = 0.85$; Day × Seed treatment: $F_{18,708} = 0.54$, $P = 0.94$). Slug survival was similar in all treatments (85–94%; Fisher's exact test, $P = 0.46$), and there was no evidence that seed treatment influenced slug mass gain (Fig. 2b; $F_{3,116} = 0.41$, $P = 0.75$). These results suggest that fungicidal and insecticidal seed coatings did not alter slug herbivory, survival,

growth or behaviour, a finding that we also confirmed in an additional experiment with smaller slugs (Appendix S1, Supporting information).

Slug–ground beetle bioassays

Overall, 75% (54 of 72) of *C. tricolor* beetles killed the slug with which they were confined. Beetles attacked slugs at a similar rate regardless of slug-feeding history (Fig. S1, Supporting information; Likelihood ratio test, D = 2·18, d.f. = 3, P = 0·54). All beetles appeared normal after eating slugs from the untreated and fungicide-only treatments (e.g. Video S1, Supporting information), while the majority of beetles that fed upon slugs from the low and high thiamethoxam treatments were impaired (Video S2, Supporting information; Fig. 2c; Fisher's exact test, P < 0·0001). Symptoms of beetle poisoning ranged from twitching and mild motor difficulty, to partial paralysis (especially of hind legs), extensive paralysis and death. Of the sixteen beetles impaired in the two insecticide treatments, seven died (3 high, 4 low thiamethoxam); the rest eventually recovered. Beetles that recovered took several days to do so [4·3 ± 0·4 days (SEM), $n = 9$].

FIELD EXPERIMENT

Experimental conditions and community composition

Slug populations and damage were intense across our region in Spring 2012, and *D. reticulatum* was the dominant slug species at our site (see Appendix S2, Supporting information). We observed few above-ground, non-slug herbivores during the early growth stages of soya bean except for occasional caterpillars and bean leaf beetles *Cerotoma trifurcata* Forster (<1 beetle per 10 plants). Diverse natural enemies were represented in our pitfall samples (3861 individuals), about a quarter of which were
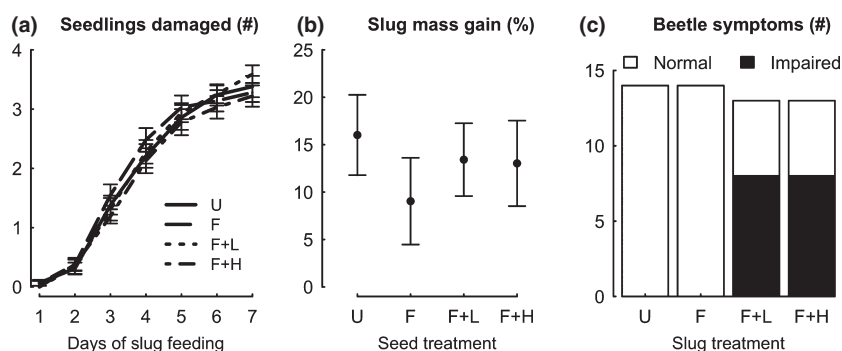

Fig. 2. Outcomes from laboratory experiments investigating the influence of neonicotinoid seed treatments on interactions between soya beans *Glycine max*, slugs *Deroceras reticulatum* and ground beetles *Chlaenius tricolor*. Soya bean seed treatments: U = untreated, F = fungicide-only, F+L = fungicide + low rate thiamethoxam, F+H = fungicide + high rate thiamethoxam. Error bars show ± one standard error. (a) Number of soya bean seedlings (out of four) damaged by slugs over 7 days ($n = 34$ microcosms treatment^{-1}; no statistical differences among treatments). (b) Slug mass gain (%) after 7 days of feeding on soya bean seedlings ($n = 34$ microcosms treatment^{-1}; no statistical differences among treatments). (c) Beetle symptoms after consuming slugs fed upon the four seed treatments; beetles exposed to insecticides via slugs suffered significantly higher frequency of impairment (Fisher's exact test, P < 0·0001).

potential slug predators (1052 individuals), represented by Carabidae (49%), Staphylinidae (36%), Opiliones (12%) and Lampyridae (4%; Table S1, Supporting information).

Effects of seed treatment on early season trophic dynamics and yield

Consistent with our predictions, seed treatment had the following significant effects during the first 35 days after planting: reduced activity–density of potential slug predators by 31%, reduced predation on sentinel prey by 33%, increased slug activity–density by 67%, decreased soya bean population by 19% ($P < 0.02$ for all; Table 1). Seed treatment also reduced grain yield by 5% (Table 1). Furthermore, regression analyses (with Bonferroni-corrected $\alpha = 0.01$) revealed that predation was positively associated with activity–density of potential slug predators, and slug activity–density was negatively related to predation (Fig. 3). In turn, slug activity–density was marginally, negatively related to soya bean plant population, which was positively related to grain yield (Fig. 3). Slug activity–density was not significantly related to soya bean leaf and cotyledon damage (partial $R^2 = 0.52$; $P = 0.11$). With the exception of damage, all of these results were consistent with our hypothesis that seed treatments would disrupt a trophic cascade, indirectly fostering slugs and reducing soya bean yield (Fig. 1).

Effects of seed treatment on slugs and predators over the season

Seed treatment had lasting effects on slug activity–density; slug captures were greater in pitfall traps in treated plots through the end of the season (Fig. S2, Supporting infor-mation; Treatment: $F_{1,4} = 9.74$, $P = 0.04$; Treatment × Date: $F_{1,4} = 0.08$, $P = 0.79$). Slug activity–density under shelter traps was also consistent with these findings (Fig. S2, Supporting information). In contrast to slugs, potential slug predators appeared to rebound quickly, with no significant differences in activity–density between treatments after the first sampling date (Fig. S2, Supporting information; Treatment: $F_{1,4} = 0.31$, $P = 0.61$; Treatment × Date: $F_{2,8} = 0.48$, $P = 0.64$). Predation on sentinel prey also seemed to recover quickly (Fig. S3, Supporting information; Treatment: $F_{1,4} = 0.0$, $P = 1.0$; Treatment × Date: $F_{1,4} = 0.07$, $P = 0.80$).

INSECTICIDE ANALYSES

From our laboratory-collected samples, we confirmed that neonicotinoid residues travelled up the food chain from soya beans to slugs to beetles (Table S2, Supporting infor-mation). As in our previous experiment, slugs fed upon thiamethoxam-treated soya beans were poisonous to ground beetles. Of the beetles that ate slugs, 84% in the high-insecticide treatment and 89% in the low-insecticide treatment were impaired the morning after slugs were introduced.

Neonicotinoids were also transferred from soya beans to slugs in the field and were detected in earthworms (Fig. 4). Soil, soya bean seedlings and slugs in thiamethoxam-treated plots had neonicotinoid residues several orders of magnitude greater than in control plots.

Neonicotinoids declined exponentially along the food chain, at a similar rate in the laboratory and field (Fig. 5; Site × Trophic Level: $F_{1,10} = 1.1$, $P = 0.32$). Concentrations of neonicotinoids were higher in the laboratory than in the field ($F_{1,11} = 22.2$, $P < 0.001$), and, for trophic

Table 1. Responses of plants, slugs and predators in a field experiment comparing soya bean plots planted with untreated (Control) or thiamethoxam and fungicide-treated (Neonic) seeds ($n = 6$ plots treatment^{-1})

Response (units)	Days after planting	Control mean ± SE*	Neonic mean ± SE*	Predicted effect[†]	%Δ_{ST}	η_p^2	η_{sp}^2	$F_{1,4}$	P[‡]
Potential slug predators (#/trap/72 h)	22	7.79 ± 0.57	5.38 ± 0.69	−	−31	0.80	0.42	15.9	**0.016**
Predation (prop. killed/24 h)	21	0.33 ± 0.04	0.22 ± 0.03	−	−33	0.85	0.34	24.1	**0.008**
Slugs (#/trap/72 h)	22	3.71 ± 0.71	6.21 ± 0.67	+	+67	0.85	0.40	23.1	**0.009**
Soya bean leaf damage (prop. area removed)	24	0.28 ± 0.01	0.24 ± 0.02	+	−14	0.53	0.17	4.46	0.10
Soya bean cotyledon damage (rating on 0–2 scale)	25	0.96 ± 0.04	0.89 ± 0.05	+	−7	0.38	0.07	2.42	0.19
Soya bean establishment (10 000 plants ha^{-1})	35	17.0 ± 0.23	13.8 ± 0.51	−	−19	0.94	0.76	63.8	**0.001**
Yield (t ha^{-1})	176	3.56 ± 0.08	3.37 ± 0.24	−	−5	0.97	0.05	118.4	**<0.001**

%Δ_{ST} is (mean$_{THX}$ − mean$_{Control}$)/mean$_{Control}$ × 100, the per cent change due to seed treatment.
η_p^2 is partial eta-squared, the per cent of non-block variation explained by seed treatment.
η_{sp}^2 is semi-partial eta-squared, the per cent of total variation explained by seed treatment.
The 'predicted effects' listed here for each response variable are illustrated in Fig. 1.
*We report raw means and standard errors, but F-tests were based on residual error once variation due to blocks was removed.
[†]The 'predicted effects' listed here for each response variable are illustrated in Fig. 1.
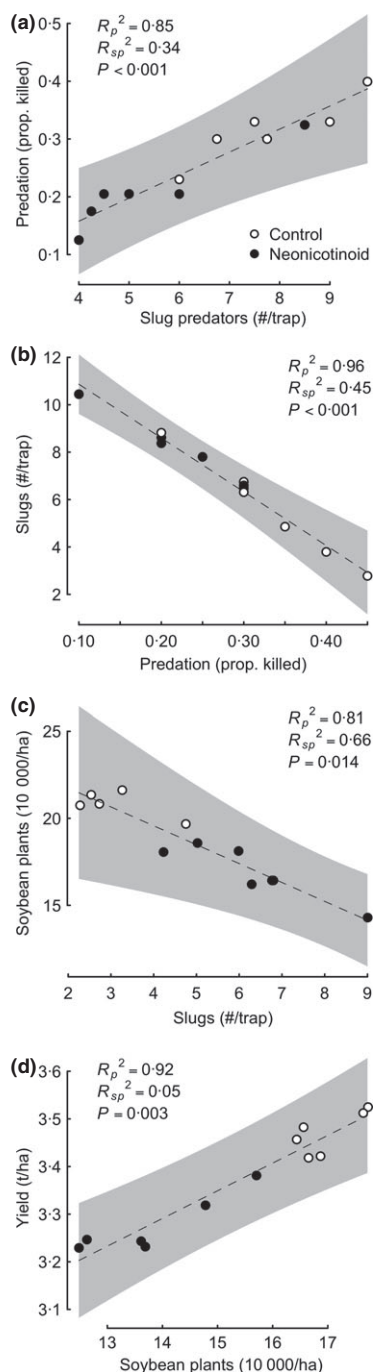[‡]Bold values are significant at $\alpha = 0.05$.

Fig. 3. Partial regression plots for relationships among organisms in soya bean plots planted with untreated ('Control') or thiamethoxam and fungicide-treated ('Neonicotinoid') seeds (n = 6 plots per treatment). *P* values test the significance of each partial correlation coefficient (Bonferroni-corrected α = 0·01). R_p^2 is the proportion of non-block variation explained by the predictor (squared partial correlation). R_{sp}^2 is the proportion of total variation explained by the predictor (squared semi-partial correlation). 95% confidence bands are shown in grey. (a) Activity–density of potential slug predators (number per trap) was positively related to predation on sentinel prey (proportion prey killed). (b) Slug activity–density (number per trap) was negatively related to predation on sentinel prey. (c) Soybean population (10 000 ha^{-1}) was marginally negatively related to slug activity–density (number per trap). (d) Yield (t ha^{-1}) was positively related to soya bean population.

level, the estimated slope (−3·25) suggests that neonicotinoids declined by ~96% per trophic level ($F_{1,11} = 363\cdot8$, $P < 0\cdot001$; Fig. 5). Even so, field-collected slugs contained 500 p.p.b. of neonicotinoids at the cotyledon stage (~13 ng slug^{-1}), dropping to 177 p.p.b. at the one-leaf stage (~6 ng slug^{-1}) and finally to non-detections by the end of the season (Fig. 4).

As expected, thiamethoxam was the dominant neonicotinoid residue in our samples, though we also found substantial quantities of its major degradates, especially clothianidin and related metabolites, in all sample types (Fig. 4). As neonicotinoids moved through the food chain, clothianidin and its metabolites became more prominent as a proportion of total neonicotinoids (Fig. 4, Table S2, Supporting information). Earthworms were the only organisms containing the neonicotinoid imidacloprid (25 and 23 p.p.b. in the two samples, respectively). Total neonicotinoid concentrations in earthworms were 54 and 279 p.p.b., corresponding to ~16 and 126 ng worm^{-1}.

Discussion

Neonicotinoid seed treatments are intended to maintain yield by protecting plants from target insect pests (e.g. *C. trifurcata*, *Delia platura*, scarab larvae), but we have discovered that they can also indirectly decrease yield by disrupting biological control of non-target pests. Moreover, one mechanism contributing to this trophic disruption appears to be a novel phenomenon of slugs passing neonicotinoids from treated plants to their predators. Our findings suggest that (i) benefits and costs of neonicotinoids for crop production are likely mediated by the relative importance of target and non-target pests in particular cropping systems and (ii) more broadly, neonicotinoids can move through soil food webs with important consequences for agriculture.

In our field experiment with heavy slug infestations, thiamethoxam seed coatings decreased predator activity–density and predation in the early season, increased slug activity–density and reduced soya bean establishment by 19% and grain yield by 5% (Table 1). Regressions among populations of predators, slugs and soya beans (Fig. 3) supported the hypothesis that seed treatments dampened a trophic cascade, fostering larger slug populations that hindered soya bean establishment and ultimately decreased yield. Given that insecticidal seed treatments enhanced slug populations and decreased plant populations, it may seem puzzling that leaf and cotyledon damage were similar in treated and control plots. We suspect this result was influenced by a small population (<1 per 10 plants) of bean leaf beetles (BLB) attacking plants in untreated plots. BLB damages leaves and cotyledons similarly to slugs, but should have been controlled by neonicotinoids in the treated plots (Johnson *et al.* 2008). The other important insect pest of soya beans in our area is soya bean aphid *Aphis glycines* Matsumura, but we did not quantify this pest because its densities at our site were

Fig. 4. Neonicotinoid concentrations (mean p.p.b. ± SE) in samples collected 12–169 days after planting (number of days for each sample noted in parentheses on the *x*-axis), from field plots planted with untreated (Control) or thiamethoxam and fungicide-treated (Neonic) soya bean seeds (*n* = 3 plots except for earthworms, where *n* = 2 plots, listed separately). Thiamethoxam (THX) was the active ingredient applied to the seeds, while CLO is clothianidin + clothianidin TZMU [*N*-(2-chlorothiazol-5-ylmethyl)-*N*-methylurea], metabolites of THX that are also insecticidal. Earthworms (Worms) were only sampled in Neonic plots.
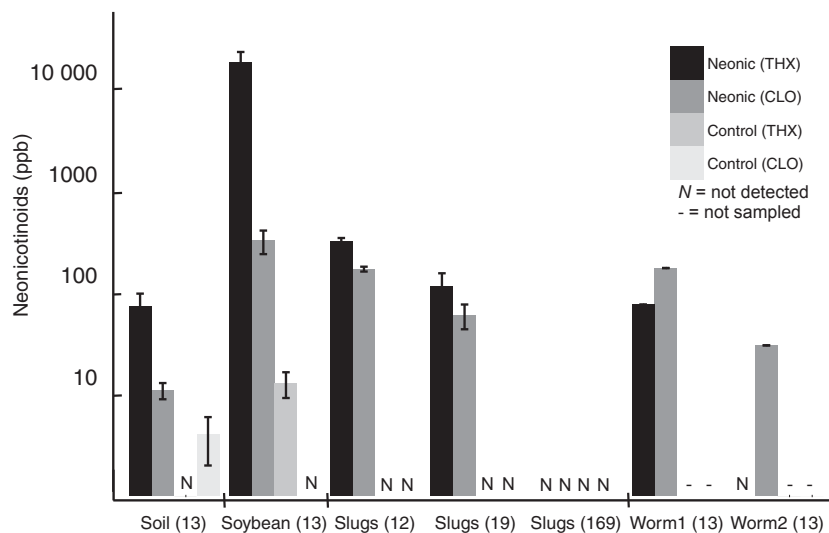

Fig. 5. Concentrations of neonicotinoids in soya beans, slugs *Deroceras reticulatum*, and ground beetles *Chlaenius tricolor* from laboratory and field experiments. Samples from the field were collected when soya beans were at the cotyledon stage. In the regression equation, 'Setting' represents the effect of experiment location (laboratory vs. field), while 'Level' represents the effect of trophic level (soya beans = 0, slugs = 1, beetles = 2).

very low. At a neighbouring site in the same year, soya bean aphid did not arrive until 8 August, and peak densities (150 ± 47 aphids plant^{-1}) were well below the economic injury level (McCarville *et al.* 2014), suggesting that this pest had negligible influence on yield. For slugs, our findings agree with previous evidence that slugs decrease soya bean yield mainly by killing plants and thus preventing their establishment, rather than by removing leaf tissue of already established plants (Hammond 2000). Peak slug activity–density in our experiment was roughly four times higher than that measured nearby in the previous 2 years (Douglas 2012), consistent with reports from

no-till farmers that slugs tend to be a problem in our region every 2–3 years (Douglas & Tooker 2012).

In the laboratory, thiamethoxam did not alter *D. reticulatum* survival or feeding behaviour, but slugs that fed upon thiamethoxam-treated soya beans were poisonous to the majority of *Chlaenius tricolor* individuals that consumed them, with symptoms ranging from poor coordination to death. These effects on beetles were not driven by fungicides because slugs from the fungicide-only treatment did not poison *C. tricolor*, consistent with previous toxicity data for mefenoxam and fludioxonil (Mullin *et al.* 2005). Concentrations of neonicotinoids in our laboratory experiment were high relative to the field; however, our laboratory experiment was fairly conservative in that predators ate only a single neonicotinoid-exposed slug. Rates of carabid food intake in the field are not well quantified, but many ground beetle species can eat close to their weight in prey each day (Thiele 1977), suggesting that generalist predators could be chronically exposed to neonicotinoids when slugs are abundant.

Some authors have argued that neonicotinoid seed treatments should have negligible influence on natural enemies, because the insecticide is 'targeted' in the plant, only reaching herbivorous species (Jeschke *et al.* 2011). Natural enemies, however, can encounter seed-applied neonicotinoids through omnivory (e.g. Seagraves & Lundgren 2012), and now we have found that they can be exposed via prey, consistent with previous studies where neonicotinoids were applied by other methods (e.g. Szczepaniec *et al.* 2011). Prey-mediated exposure through non-target pests could be especially disruptive to biological control, because it affects precisely those natural enemies that eat the pests.

Notably, tritrophic movement of neonicotinoids is similar to the mobility of the related plant toxin nicotine. Nicotine from solanaceous plant species is known to influence interactions between caterpillars and their

enemies, for instance, by reducing parasitoid fitness (Barbosa *et al.* 1986) and protecting caterpillars from some generalist predators (e.g. Kumar *et al.* 2014). The analogy to nicotine is intriguing and distribution of nicotine within plants and across natural systems may even suggest clues to the sustainable use of neonicotinoids, but it is important to recognize that effects of neonicotinoids may differ from nicotine because these synthetic compounds are far more toxic to insects (Jeschke & Nauen 2008).

The extreme potency of neonicotinoids helps explain why their tritrophic movement negatively influenced predators despite concentrations declining by ~96% per trophic level. Dietary toxicity of neonicotinoids to predators such as *C. tricolor* and other ground beetles is poorly characterized, but their toxicity to the similar-sized honey-bee (*Apis mellifera*) is well documented. Lethal doses (i.e. LD_{50}) of thiamethoxam and clothianidin are 4·4 and 3·5 ng bee^{-1}, based on acute oral exposure (Laurino *et al.* 2013), and doses of thiamethoxam as low as 1·34 ng bee^{-1} can impair foraging (Henry *et al.* 2012). For comparison, juvenile slugs in our field experiment contained up to 13 ng of neonicotinoids slug^{-1}. While not the focus of our study, our finding of neonicotinoids in earthworms (16–126 ng worm^{-1}) is also concerning for biological control, because earthworms are known to be important prey for generalist predators when other prey are scarce (Symondson *et al.* 2000). Notably, earthworms were the only organisms at our site that contained imidacloprid, which had not been used for at least 1 year. Given their burrowing behaviour, earthworms may be more likely to encounter and ingest neonicotinoid residues in soil, but this deserves further study.

An important implication of our study is that neonicotinoid seed treatments may worsen slug problems in managed ecosystems where neonicotinoids and molluscan pests overlap. *Deroceras reticulatum* is native to Western Europe and has invaded North and South America, Asia, South Africa and Oceania, causing economic damage to cereals, legumes, canola/rapeseed, strawberries, and myriad ornamental and vegetable species (South 1992; McDonnell, Paine & Gormally 2009). Neonicotinoids are commonly used in many of these crops and regions (Jeschke *et al.* 2011). While neonicotinoid seed treatments are currently suspended on bee-attractive crops in the European Union (EC 2013), they can still be used on some crops (e.g. wheat) that are prone to slug damage. We predict that neonicotinoids will most likely exacerbate slug problems when their use coincides with the small juvenile stage of slugs, because small slugs are acceptable to a wider range of predatory insects (Ayre 2001). Future research could explore whether neonicotinoids can also flow to predators through caterpillars, which are often not well controlled by neonicotinoid seed treatments (e.g. Kullik, Sears & Schaafsma 2011), or other insect pest species that tolerate neonicotinoids, including those that have evolved resistance. While our results appeared to be driven solely through top-down mechanisms, it is worth noting that herbivorous mites, another non-target herbivore, can be facilitated by neonicotinoids through both top-down and bottom-up ecological pathways (e.g. Szczepaniec *et al.* 2011).

Pesticide regulatory authorities, agricultural organizations, researchers and the public are struggling to weigh costs and benefits of neonicotinoid seed treatments both within and outside of agriculture. So far, these discussions have focused mainly on managed and wild pollinators and have only recently widened to include other wildlife such as aquatic invertebrates and birds (e.g. van Dijk, van Staalduinen & van der Sluijs 2013; Hallmann *et al.* 2014). Our findings highlight the importance of considering species that contribute to biological control, an ecosystem service conservatively valued at over $200 million per year for U.S. soya bean production alone (Landis *et al.* 2008). Effects on biological control may help explain why neonicotinoid seed treatments have only inconsistently improved yield in soya beans and other crops. For instance, neonicotinoid-fungicide seed treatments changed soya bean yield compared to an untreated control by −8% to +13% across 28 environments, but the factors producing this variability were unknown (Gaspar *et al.* 2014). Our research shows that a better understanding of ecological interactions among target and non-target pests and their natural enemies should allow us to better predict yield responses to neonicotinoid use.

Pest management scientists have long known that pesticides can impose trade-offs in agricultural production, and in fact, such discoveries were part of the impetus behind developing integrated pest management (IPM) as a knowledge-based alternative to the indiscriminate use of pesticides (Stern *et al.* 1959). In most cropping systems, neonicotinoid seed treatments are being used outside of an IPM framework (e.g. Gray 2012), and, as we show here, this indiscriminant use can have unintended consequences, with measurable costs for farmers. Using neonicotinoids only when and where they are needed, guided by a strong understanding of the underlying ecology, provides potential to harness their strengths and limit their weaknesses to achieve more sustainable pest control.

Acknowledgements

Data accessibility

Data available from the Dryad Digital Repository http://doi.org/10.5061/dryad.7s403 (Douglas, Rohr & Tooker 2015).

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Additional laboratory experiment with smaller slugs.

Appendix S2. Supporting results.

Appendix S3. Supporting methods.

Appendix S4. Supporting references.

Table S1. Activity-density of potential slug predators in plots planted with untreated or thiamethoxam and fungicide-treated soybean seeds.

Table S2. Neonicotinoid concentrations (p.p.b.) detected in samples from laboratory experiments.

Fig. S1. Slug survival when confined individually with *C. tricolor* after 7 days of feeding on soybeans with different seed coatings.

Fig. S2. Activity-density of slugs and potential slug predators over the season in plots planted with untreated or thiamethoxam and fungicide-treated soybean seeds.

Fig. S3. Predation on sentinel waxworm caterpillars in plots planted with untreated or thiamethoxam and fungicide-treated soybean seeds.

Video S1. A beetle (*C. tricolor*) ~12 h after eating a slug fed upon untreated soybean seedlings.

Video S2. Beetles ~12 h after eating slugs fed upon thiamethoxam-treated soybean seedlings.