2016 Project Abstract
For the Period Ending June 30, 2019

PROJECT TITLE: Game and Nongame Bird Pesticide Exposure
PROJECT MANAGER: Julia B Ponder, DVM MPH
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FUNDING SOURCE: Environment and Natural Resources Trust Fund
LEGAL CITATION: M.L. 2016, Chp. 186, Sec. 2, Subd. 03m

APPROPRIATION AMOUNT: $349,000
AMOUNT SPENT: $339,419
AMOUNT REMAINING: $9,581

Sound bite of Project Outcomes and Results
We documented neurobehavioral abnormalities in chickens from neonicotinoid exposure at doses compatible with what wild birds might ingest, as well as the availability of neonicotinoid-treated seeds on the agricultural landscape. We also identified changes in gene expression associated with exposure that may be useful in developing a non-lethal test for exposure.

Overall Project Outcome and Results
Neonicotinoids are the most widely used pesticides worldwide and are commonly applied as a seed treatment to corn, soybean, and wheat seeds, which compromise the majority of Minnesota's row crops. Previous risk assessments have suggested that wild birds may be exposed to large doses of neonicotinoids through the ingestion of treated seeds. Using chickens as a model species, we evaluated the impacts of oral neonicotinoid exposure on the immune and neurological systems. We also assessed availability of treated seeds to wild birds on the agricultural landscape and analyzed grouse carcasses for residues of exposure.

Accomplishments:

- We demonstrated neurological abnormalities in chickens exposed orally to imidacloprid, a commonly used neonicotinoid in seed-treatments
- We quantified seed spills on agricultural landscapes during spring planting season that may occur during loading or refilling seed hoppers
- We documented wildlife at neonicotinoid-treated seed spills with trail cameras and documented consumption of treated seeds.
- We documented neonicotinoid residues in the tissues of hunter-harvested grouse, indicating that those birds were exposed to the pesticides
- We identified 354 genes affected by imidacloprid exposure through RNA sequencing: 37 affected genes were detected in liver and 317 affected genes were detected in blood cells (which can be non-lethally collected, which may allow future development of detection assays)

The results of this project indicate that seed-eating birds in the wild may be exposed to seeds treated with neonicotinoids in the agricultural landscape through eating at seed spills. Ingestion of neonicotinoid-treated seeds by birds can produce neurological abnormalities that may impair survivability. Exposure can be evaluated through detection of pesticide residues in carcasses, as well as fecal pellets and blood cells. The results of this study may be used by the agricultural industry to reduce impacts to wild birds through education and process

change (reduce spillage), as well as state and federal governmental agencies reviewing appropriate and safe usage of these pesticides.

Project Results Use and Dissemination

Results of this project have been communicated to a large audience of stakeholders, including directly with industry colleagues through meetings with agricultural stakeholders; with federal and state agencies through public commentary response as well as requested webinars, presentations and conversations; and with the scientific community through publications (1 paper published, 1 submitted and 4 pending), conference presentations (4) and scientific posters (2). Details of all communications are provided in the final report. The results of our work show that wild birds are at risk of exposure to agricultural seeds treated with neonicotinoids and that ingestion of field-realistic doses causes significant behavior changes in chickens that were severe at higher doses and may impair survival of free-living gallinaceous birds. The adoption of practices that would reduce seed spills on the agricultural landscape would reduce the exposure risk to wild birds.

Environment and Natural Resources Trust Fund (ENRTF)
M.L. 2016 Work Plan Final Report

Date of Report: August 1, 2019

Final Report: August 1, 2019

Date of Work Plan Approval: November 4, 2016

Project Completion Date: June 30, 2019

PROJECT TITLE: Game and Nongame Bird Pesticide Exposure

Project Manager: Julia B Ponder, DVM, MPH

Organization: University of Minnesota dba The Raptor Center

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Location: Statewide

Total ENRTF Project Budget:		
	ENRTF Appropriation:	**$349,000**
	Amount Spent:	**$339,419**
	Balance:	**$ 9,581**

Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Subd. 03m

Appropriation Language:

$349,000 the second year is from the trust fund to the Board of Regents of the University of Minnesota to evaluate the potential risk to game and nongame birds from exposure to neonicotinoid-treated agricultural seeds. This appropriation is available until June 30, 2019, by which time the project must be completed and final products delivered.

I. PROJECT TITLE: Do neonicotinoids pose a risk to Minnesota's birds?

II. PROJECT STATEMENT: We propose to examine sub-lethal exposure of neonicotinoid pesticides in birds, using sharp-tailed grouse as a model. Neonicotinoid pesticides such as imidacloprid, thiamethoxam, thiacloprid, clothianidin are the most widely used pesticides worldwide. They are commonly applied as a seed treatment to most corn, soybean, sunflower, and wheat seeds. These crops comprise the majority of Minnesota's row crops. While their unintended impact on insect pollinators has caused the greatest amount of concern, recent studies have shown potential risk to birds. Risk assessments (American Bird Conservancy) have determined that the most likely route of exposure to large doses of neonicotinoids for birds is ingestion of treated seeds, although numerous other mechanisms exist (e.g., crops, soil, water, trophic transfer). Ingestion of a small number of treated seeds has been shown to be lethal to small birds. While larger birds are less likely to ingest a lethal dose through seed consumption, they may still be at risk for sub-lethal health impacts and may be exposed to multiple types of neonicotinoids. Sub-lethal effects found in the lab include behavioral abnormalities, declines in reproductive success, and immune suppression; but available studies have not adequately simulated field exposures nor provided tools to measure risk to wild birds.

Sharp-tailed grouse are a good model to understand risk to birds, as they utilize areas with high and low levels of agriculture in Minnesota; consume corn, wheat, and other crop types in which neonicotinoid-treated seeds would be available through spillage or after planting; and, are closely related to domestic chickens which are amenable to lab studies. Sharp-tailed grouse are also large making them less likely to consume a lethal dose, yet manifest detectable sub-lethal effects. Based on current knowledge, it is calculated that a grouse would need to eat 14 seeds for a sub-lethal dose and approximately 80 corn seeds for a lethal dose, the latter being unlikely in one feeding bout. Lastly, sharp-tailed grouse display at leks, an assembly area where multiple animals congregate for breeding displays and courtship. These leks are fairly stable in location among years, facilitating non-lethal collection of feces and blood from a large geographical area within and outside of agricultural areas, and allowing comparisons of naturally occurring low and high exposure groups.

The overall goal of this project is to assess whether birds are at risk from exposure to neonicotinoid-treated seeds in agriculture landscapes using sharp-tailed grouse as a model species. Our specific objectives are to:

- Assess exposure in wild grouse
  - Identify birds consuming neonicotinoid-treated seeds, quantify consumption per foraging bout, and measure neonicotinoid concentrations of seeds
  - Quantify grouse neonicotinoid residues in feces of breeding birds and tissues from hunter-harvested birds
  - Quantify the rate of seed spillage along roads and edges of agricultural fields (transect study)
- Establish exposure-response relationships in the lab
  - Assess impacts of exposure to neonicotinoid mixtures on the immune system in the lab using chickens as a surrogate
- Provide a means to link exposure to effect in field studies
  - Quantitatively link exposure to neonicotinoid mixtures, tissue residue concentrations (dose), and immune suppression in the lab to interpret tissue residue concentrations in wild birds

This study will provide preliminary data to evaluate the risk to Minnesota's birds from neonicotinoids by documenting access to neonicotinoid-treated seeds, comparing tissue residue in wild birds from agricultural areas and non-agricultural areas, establishing non-lethal methods of assessing exposure, demonstrating sub-lethal impacts of exposure, and assessing whether exposure to multiple neonicotinoids worsens their impact.

III. OVERALL PROJECT STATUS UPDATES:

Amendment request (10/18/2016):
Due to the inability to hire a post-doctoral candidate to oversee the laboratory studies, data collection and data analysis in the first year of this project, we are requesting to move one year of the budgeted salary for this

position to a graduate student classification. The only impact on the budget is to add a line for graduate student salary and adjust effort of post-doc, all within the original budget for personnel.

Amendment approved: 11/4/2016

Project Status as of: 30 January 2017
After an unsuccessful attempt to identify a qualified post-doctoral candidate, an alternate plan to utilize a graduate student for implementation of Activity 1/year 1 was developed and an amendment request submitted. A graduate student was successfully approved and funded for this project. Subawards have been put in place for Minnesota Department of Natural Resources (field work – Activity 2) and Southern Illinois University – Carbondale (sample analysis). Collection of field samples started in Fall 2016 and preparatory work for Activity 1 has been done. All activities are on schedule

Project Status as of: 30 July 2017
Activity 1 exposure and sample collection has been completed for four dosage groups and initial immune assays run. Initial analysis has been started of immune assay results. Tissue samples collected for residue analysis have been frozen pending shipment to SIUC.

For Activity 2, video was captured from 40 trail cameras placed on simulated seed spills to document animals that consumed treated seeds. Video analysis is pending. To quantify seed spills, a balanced sample of 50 townships with >50% of area planted in soybean, corn or wheat in 2014 were surveyed. Planting status was documented along with approximate size (e.g., area or count) of seed spills on roads, field edge or in field and crop type (where possible). Finally, fresh fecal pellets were collected from 46 sharp-tailed grouse leks and 27 prairie-chicken leks, and sent to SIUC for analysis.

A post-doctoral candidate has been hired for Activity 3 and is in the process of reviewing the protocols and research methodology.

Amendment request (9/08/2017):
Preliminary data analysis for Activity 1 indicates that there is not a statistically significant difference in immune function between the current treatment groups, although there is substantial individual variability. Based on this preliminary information and a power analysis of the current data, we are requesting the following changes to Activity 1:

- Reduction of dosing levels from six to five: based on preliminary findings, there is no value in assessing dosages lower than 3.3% LD_{50}
- Increase group numbers from 10 to 20: based on a power analysis of the preliminary results, this increase in sample size at each dose level is needed to detect a significant difference in immune function, if it does exist.
- Eliminate the clothianidin exposure groups: Clothianidin has the same mechanism of action of imidacloprid, so while the exposure dose needed to cause a clinical or subclinical effect may be different, the type of effect would likely be similar. By eliminating these groups, we would have the budget and resource capacity to increase the imidacloprid group sizes to 20, allowing us to fully evaluate if there is a statistical difference on immune function.

We are also proposing to amend Activity 3 based on the results of Activity 1. The use of genomics (RNA sequencing) to evaluate immune function is a more sensitive assay of immunotoxicity than the assays used in Activity 1 and is the basis for development of a biomarker for assessing exposure. We propose to reduce our exposure groups from four to three (0.25-20% LD_{50}) and add a second phase of RNA sequencing sampling to expand the potential for development of a biomarker.

In order to complete these efforts, we are requesting the following budget adjustments:

- Increase in budget for supplies from $4,250 to $15,900: the costs of supplies for immune assays and RNA sequencing was originally underestimated
- Increase in budget for research animal housing from $13,944 to $17,078 to reflect current pricing
- Increase budget for RNA sequencing from $12,600 to $38,600 to accommodate analysis of additional tissues
- Decrease in budget for analysis of neonicotinoid residues (SIUC) from $98,445 to $76,615 – we will evaluate residues in fewer tissues in Activity 3 as results from Activity 1 will provide adequate information on tissue distribution.

Amendment Approved by LCCMR 10/9/17

Project Status as of: 30 January 2018
Activity 1 data collection and analysis of immune assay results completed with final assessment of tissue residue analysis pending. Results are being written up for publication and have been presented at two scientific conferences.

All field work is complete for Activity 2 and final assessment of tissue residue analysis pending. Results have been presented at a scientific conference and discussed with both regulatory agency personnel (EPA) and industry representatives (Bayer).

Data collection for Activity 3 has been completed and samples processed for analysis (in process).

Amendment request (6/12/2018):
We are requesting a one year extension to our workplan (as allowed by appropriations language) in order to complete the previously approved activities, which behind schedule due to delays in receiving results from the Genomics Center. This will allow us to run our second phase of genomics sequencing, which has been postponed as it is dependent on the results of the first phase. As the genomics analysis and research animal costs were below budget projections, we are also requesting that those funds be moved to personnel to allow us to extend the post-doctoral fellow's appointment to facilitate completion of the genomics work and reporting. This requires the following budget changes:

- Activity 1: Reduce by $7,768, reflecting reduced research animal costs
- Activity 3: Reduce research animal costs ($9,850) and RNA sequencings costs ($5,339)
- Activity 3: Increase personnel costs by $22,957 (sum of above reductions)

Amendment Approved: [07/06/2018]

Project Status as of: 30 June 2018
All laboratory and field work has been completed, as has most data analysis. The final data analysis is pending completion of the second phase of genomic sequencing. Currently, three scientific manuscripts are in development with additional ones to come.

Information from this work was used in providing public comment on the technical merits of the EPA's recently released draft neonicotinoid ecological risk assessment. Additional communications with scientists, agencies and industry representatives have occurred.

Project Status as of: 30 January 2019
Statistical analysis of the clinical neurological signs has been completed for Activity 1 and a manuscript is in process with expected submission to peer reviewed literature in April 2019. The final data analysis for two

phases of genomic sequencing has been completed for Activity 3. Analyses and results are being written up for publication. Sequences and analytical codes have been uploaded to public domains.

Project Status as of: 11 April 2019
First manuscript has been submitted for publication for work done under Activity 2, a final draft is substantially complete for submission for work done under Activity 1 and a publication draft has been started for work done under Activity 3.

Amendment request as of 4/9/2019:
With our project substantially complete, we are requesting to use unused funds from personnel, travel and research lab services to do one additional round of laboratory analysis, looking for residues of neonicotinoid metabolites (products made by the liver from the original neonicotinoid exposure) to identify both possible presence of additional active ingredients in the body as well as potential new markers for exposure. In addition, we had an unexpected budget overrun on lab supplies and would like to adjust the budget to cover this.

- $5,567 moved from personnel salary and benefits
- $4,621 staying within laboratory and service contracts (moving from genomics analysis to metabolite analysis
- Activity 3 supplies increased by $1,200 ($912 from Activity 1 supplies, $1,188 from personnel salary/benefits) to cover overage
- $10,000 line added under service contracts for metabolite analysis of samples

Amendment Approved by LCCMR 4/30/2019

Overall Project Outcomes and Results:
Neonicotinoids are the most widely used pesticides worldwide and are commonly applied as a seed treatment to corn, soybean, and wheat seeds, which compromise the majority of Minnesota's row crops. Previous risk assessments have suggested that wild birds may be exposed to large doses of neonicotinoids through the ingestion of treated seeds. Using chickens as a model species, we evaluated the impacts of oral neonicotinoid exposure on the immune and neurological systems. We also assessed availability of treated seeds to wild birds on the agricultural landscape and analyzed grouse carcasses for residues of exposure.

Accomplishments:

- We demonstrated neurological abnormalities in chickens exposed orally to imidacloprid, a commonly used neonicotinoid in seed-treatments.
- We quantified seed spills on agricultural landscapes during spring planting season that may occur during loading or refilling seed hoppers.
- We documented wildlife at neonicotinoid-treated seed spills with trail cameras and documented consumption of treated seeds.
- We documented neonicotinoid residues in the tissues of hunter-harvested grouse, indicating that those birds were exposed to the pesticides.
- We identified 354 genes affected by imidacloprid exposure through RNA sequencing: 37 affected genes were detected in liver and 317 affected genes were detected in blood cells (which can be non-lethally collected, which may allow future development of detection assays).

The results of this project indicate that seed-eating birds in the wild may be exposed to seeds treated with neonicotinoids in the agricultural landscape through eating at seed spills. Ingestion of neonicotinoid-treated seeds by birds can produce neurological abnormalities that may impair survivability. Exposure can be evaluated through detection of pesticide residues in carcasses, as well as fecal pellets and blood cells. The results of this study may be used by the agricultural industry to reduce impacts to wild birds through education and process

change (reduce spillage), as well as state and federal governmental agencies reviewing appropriate and safe usage of these pesticides.

IV. PROJECT ACTIVITIES AND OUTCOMES:

ACTIVITY 1: Development of tools to assess neonicotinoid exposure and impacts in birds

Description:

Immune function is known to be altered by many factors, of which contaminants may be one. Immunology is increasingly being used to study toxicology in wild birds and immune function can be a sensitive indicator of contaminant exposure (Smits et al, 1999). A laboratory exposure study will be conducted at the University of Minnesota College of Veterinary Medicine (UM) to establish the neonicotinoid exposure concentration that impacts immunity. Using domestic chickens as a model species, we will determine what concentrations of imidacloprid (the most common neonicotinoid seed treatments used in Minnesota) effect immunity and what component of the immune system is most impacted by these exposures, providing specific data for Activity 3.

Domestic chickens will be used as our model species given their suitability to captivity and close taxonomic relationship with wild grouse. Using sub-lethal doses of imidalcoprid, we will expose chickens at five different dosages (plus controls) and run a panel of assays on each chicken to assess immune function. We will utilize assays that measure antigen-independent cell and humoral-mediated immune responses (Tier I assays), as well antigen specific responses (Tier II assays). Each of these assays is easily adapted to wild bird species and well-documented in the avian literature.

Summary Budget Information for Activity 1:

ENRTF Budget: $ 88,893
Amount Spent: $ 79,431
Balance: $ 9,462

Outcome	Completion Date
1. Lab exposure study and sample collection	30 JUN 2017
2. Laboratory analysis of samples for neonicotinoid concentrations	30 NOV 2017
3. Validate novel sensitive immune assay	30 NOV 2017

Activity Status as of: 30 January 2017

A graduate student has been funded to implement this activity. Study methodology has been refined and a detailed timeline established for the laboratory exposure study and sample collection. Supplies have been purchased in preparation for running the data collection part of the study. A proposal for animal care and use has been developed, submitted and is under review. Data collection for this activity is expected winter/spring 2017 (on schedule).

The subaward terms, conditions and deliverables have been finalized for Southern Illinois University – Carbondale (sample analysis) and the agreement completed.

Activity Status as of: 30 July 2017

Working in study groups of ten chickens, we have completed exposure and sample collection on four dosage groups (dosages 0.039mg/kg, 0.34mg/kg, 3.43mg/kg, and 10.41mg/kg of imidacloprid). On the samples collected, the following assays have been completed: Hemagglutination and hemolysis assay, phytohemaglutinin test, delayed hypersensitivity test. Preliminary data analysis indicates that there is not a statistically significant difference in immune function between the current treatment groups. There has been, however, a documentation of neurological effects in sublethally dosed birds ranging from mild depression to profound sedation.

Amendment request (9/08/2017):
Preliminary data analysis for Activity 1 indicates that there is not a statistically significant difference in immune function between the current treatment groups, although there is substantial individual variability. Based on this preliminary information and a power analysis of the current data, we are requesting the following changes to Activity 1:

- Reduction of dosing levels from six to five: based on preliminary findings, there is no value in assessing dosages lower than 3.3% LD_{50}
- Increase group numbers from 10 to 20: based on a power analysis of the preliminary results, this increase in sample size at each dose level is needed to detect a significant difference in immune function, if it does exist.
- Eliminate the clothianidin exposure groups: Clothianidin has the same mechanism of action of imidacloprid, so while the exposure dose needed to cause a clinical or subclinical effect may be different, the type of effect would likely be similar. By eliminating these groups, we would have the budget and resource capacity to increase the imidacloprid group sizes to 20, allowing us to fully evaluate if there is a statistical difference on immune function.

Activity Status as of: 30 January 2018
Data collection from live animals as well as the majority of laboratory analyses are complete. Groups of 20 domestic chickens were exposed to five doses of imidacloprid (0.04 mg/kg, 0.34 mg/kg, 3.44 mg/kg, 10.41 mg/kg and 15.62 mg/kg). One group of 20 chickens was used as a vehicle control group to represent normal immune function. Data collection and statistical analysis is complete for the following immune function assays: phytohemagglutinin-A (PHA) response test, delayed type hypersensitivity (DTH) test, antibody response to sheep red blood cells, and the microbicidal assay for *Staphylococcus aureus* and *Candida albicans*. There was no detectable immune suppression or stimulation in the five imidacloprid groups. Statistical analysis is currently being done on the complete white blood cell counts. The microbicidal assay using *Escherichia coli* is underway.

The chickens did exhibit significant, dose-dependent neurologic signs after oral imidacloprid exposure. Neurologic signs ranged from mild sedation to complete inability to stand and lack of response to external stimulation in the most severe cases. This data was used to calculate an estimated median effective dose (ED_{50}) that can be used in ecological risk assessments. Moderate clinical signs included moderate sedation, increased respiratory effort, ataxia and whole-body tremors. Additional statistical analysis methods are underway in order to gather more information regarding the potential risk imidacloprid treated seeds may pose to wild granivorous birds.

This portion of the study provides evidence that field realistic doses of imidacloprid may impair avian survival due to neurologic signs, but may not be immunotoxic. These results were presented in poster format at the Society of Environmental Toxicology and Chemistry (SETAC) conference in Minneapolis, MN in November 2017. Additional conference presentation opportunities are being pursued. A manuscript is currently in preparation for publication in the peer reviewed scientific literature.

Activity Status as of: 30 June 2018

Statistical analysis has been completed on the immunotoxicity and an estimated ED_{50} value has been calculated. As none of the immune assays showed detectable immune suppression, we did not identify a sensitive test (and therefore did not validate).

Activity Status as of: 30 January 2019

Additional statistical analysis has been performed and refined to thoroughly explain the clinical neurologic abnormalities observed in the study. Manuscript writing is well underway with anticipated submission to the peer reviewed literature in April 2019.

Project Status as of: 11 April 2019
Manuscript is in final stages of preparation for submission for publication.

Amendment request as of 4/9/2019:
We request to use leftover funds to further analyze our previously collected samples, looking for neonicotinoid metabolites residues.

Final Report Summary:
This activity demonstrated health impacts of neonicotinoid exposure to chickens, which were used as sentinels for wild, granivorous birds (seed/grain eating). Four groups of chickens were exposed to imidacloprid, a neonicotinoid pesticide commonly used in seed treatments for agricultural crops. Each group received a different dose of imidacloprid, given with food; chickens were treated daily for seven days. Assays were done to evaluate impacts on the immune system functioning and neurological/behavioral abnormalities post-exposure were observed, quantified and recorded. There were changes seen in immune system function assays evaluated in this study between the exposed chickens and the controls. There were significant neurological abnormalities seen in the chickens after ingestion of imidacloprid. The chickens presented dose-dependent abnormalities ranging from no responses through mild sedation to comatose. Additional clinical signs included increased respiratory effort, loss of neurological coordination and tremors. Neurological changes were temporary with chickens recovering within minutes to a few hours.

ACTIVITY 2: Establish risk to wild birds from neonicotinoid-treated agricultural seeds

Description: Using trail cameras, we will document any bird species that forage on spilled or recently planted seeds and the amount consumed. Trail cameras will be placed at the corners of recently planted fields to capture images of birds eating spilled or submerged seeds on tilled land in public ownership at twelve sites in highly agricultural areas. In addition, cameras will be put on simulated seed spills from these natural foraging areas to document the time it takes for birds to discover the spills and the number of seeds consumed in each foraging bout (per bird). Cameras will be placed in locations where risk of theft will be minimized by restricted access or opportunity for concealment.

Field observations of seed spills in recently planted fields will be used to quantify rate of seed spillage by field type (e.g., corn, soybean, wheat) from road-based transects in agricultural areas in the southern and western portions of the state. We will record locations and approximate number of seeds in spills near recently planted fields. To determine the proportion of seed spills that contain neonicotinoid-treated seeds, we will collect seeds from accessible spills and quantitatively assess for seven neonicotinoids.

Finally, feces and/or blood will be collected from grouse at leks in agricultural and non-agricultural areas and analyzed for neonicotinoid residues. Additional samples (ingesta and/or tissue) will be collected from 40-60 hunter-harvested grouse in the fall for analysis. Winter wheat is planted in September and October in Minnesota, so grouse might be newly exposed to treated seeds in the fall.

Summary Budget Information for Activity 2:		
	ENRTF Budget:	**$ 118,837**
	Amount Spent:	**$ 118,837**
	Balance:	**$ 0**

Outcome	Completion Date
1. *Camera study to document which species of birds consume spilled seeds*	03 June 2017

2. *Transect study to estimate seed spillage rates in Minnesota*	30 June 2017
3. *Analysis of grouse tissues for neonicotinoid residues*	30 March 2018

Activity Status as of: 30 January 2017
The subaward terms, conditions and deliverables have been finalized for the Minnesota Department of Natural Resources and the agreement completed. Thirty-one samples have been collected from hunter-harvested sharp-tailed grouse with an additional 19 samples from hunter-harvested prairie-chickens and pheasants. These latter samples will be considered for inclusion in this study (via amendment) if needed to complete deliverables for hunter-harvested samples to demonstrate exposure of wild birds to neonicotinoids (pending final numbers of grouse samples acquired). All samples have been sent to Southern Illinois University – Carbondale where they are pending analysis.

Position advertisements have been posted for seasonal technicians to collect field samples in spring 2017. These postings will close on January 30th and candidate selection finalized in February/March.

Activity Status as of: 30 July 2017
In spring 2017, we placed 40 trail cameras to capture video at simulated spills at each of 24 privately-owned fields and 16 WMAs. WMAs were selected to have food plots or Cooperative Farming Agreements (CFAs) and a land cover composition similar to that of the surrounding landscape based on the 2014 National Cropland Data Layer (USDA-NASS 2015). Spills were simulated with 1,000 wheat, corn, or soybean seeds. We checked cameras once weekly to replace batteries and data cards and deployed cameras in each location for 2 weeks. Videos will be examined in the upcoming months. Videos from our 2016 DNR-funded pilot study documented brown-headed cowbirds (*Molothrus ater*), red-winged blackbirds (*Agelaius phoeniceus*), Harris's Sparrow (*Zonotrichia querula*), American crows (*Corvus brachyrhynchos*), blue jays (*Cyanocitta cristata*), and brown thrashers (*Toxostoma rufum*) consuming treated seeds at spills.

To quantify seed spills, we drew a spatially balanced sample of 50 townships with at least 50% of the area planted in soybean, corn, or wheat during 2014 (USDA NASS 2015) and at least 50 miles of roads (DOT 2008). We surveyed the 38 most western townships selected due to a later start to planting during the spring of 2017. We began in the southern counties in late April and worked north as crops were planted. We recorded locations and approximate spill size near recently planted fields with the DNRSurvey mobile computer application. We recorded each quarter quarter-section in agricultural production, whether any part of it was recently planted (i.e., $\leq$early seedling stage), documented the size of seed spills on the road, field edge, or visible in the field, and crop type (when possible). Data will be analyzed for upcoming reports.

We collected fresh fecal pellets from prairie grouse leks during 2017, based on findings from our pilot study which indicated that feces were more reliable indicators of recent exposure to neonicotinoids than blood samples. We collected fresh fecal pellets from 46 sharp-tailed grouse leks and 27 prairie-chicken leks in 2017. Samples will be sent for analysis at SIUC.

Activity Status as of: 30 January 2018
In July 2017, we sent 182 samples to Southern Illinois University Carbondale for neonicotinoid analysis. These samples included 27 greater prairie-chicken fecal pellets, 47 sharp-tailed grouse fecal pellets, 7 sharp-tailed grouse livers, and 101 seed samples collected from seed spills or used in seed exposure experiments.

In December 2017, we sent 52 samples to SIUC for analysis of neonicotinoid concentrations. These samples included 17 greater prairie-chicken livers, 27 sharp-tailed grouse livers, 1 liver from an unidentified prairie grouse, 4 sharp-tailed grouse fecal pellet samples, and 3 gizzards with contents- 2 from sharp-tailed grouse and 1 from a greater prairie-chicken. The livers and gizzards were from hunter-harvested submissions in fall 2017. This was the last shipment of samples for analysis.

Videos of seed spills from the 2017 field season are still being reviewed. GIS Analysis is underway to quantify seed spill rates in 2017. Laboratory results have not yet been received for 2017 samples. Progress will continue and be included in the next report.

Activity Status as of: 30 June 2018

Final laboratory results were received from SIUC on June 29, 2018. Data analysis is underway on these results. Statistical analyses of all previous results are underway in preparation for manuscripts.

Activity Status as of: 30 January 2019
We plan to complete review of videos at seed spills in the next few weeks and are in process of preparing manuscripts for submission to peer-reviewed journals.

Project Status as of: 11 April 2019
All work completed and first manuscript submitted for publication.

Final Report Summary:
Activity 2 demonstrated the availability of pesticide-treated seeds to wildlife on the agricultural landscape, documented wild birds and mammals consuming these seeds and also documented neonicotinoid exposure in wild birds. Transect surveys were conducted throughout Minnesota's agricultural townships and both locations and approximate size of seed spills were recorded. All work was done from public roads. Seeds and seed spills were quantified on the soil surface after spring planting. Follow-up surveys were down to document what crops were planted at each survey point.

Forty trail cameras were used to capture video at simulated seed spills on privately-owned fields and wildlife management areas, and to document wildlife species eating the seeds. Over a dozen species of birds and mammals consumed seeds at these spills. Bird species included pheasants, geese, wild turkeys, doves, blue jays, brown thrasher, rose-breasted grosbeak, various sparrow species and blackbirds.

In order to document neonicotinoid exposure in wild birds, over 80 fecal pellets from grouse were collected from leks and analyzed for neonicotinoid residues. Pilot work for this project had previously demonstrated persistence of neonicotinoid residues in feces for up to two weeks after exposure. In addition to finding residues in fecal pellets from wild grouse, tissues from over 80 hunter-harvested birds (sharp-tailed grouse, prairie chickens and pheasant) were also found to have residues, documenting exposure in free-ranging game birds in two different ways.

ACTIVITY 3: Quantify impacts of sub-lethal exposure to neonicotinoid mixtures on the immune system
Description: Using the results of Activity 1, we will determine the quantitative relationship between neonicotinoid residues in tissues and immune function to provide direct information for field-based residue studies in wild grouse. The surrogate species, chicken, will be exposed to a single neonicotinoids (imidacloprid), and dose-dependent immune suppression will be measured using RNA sequencing and gene expression framework to evaluate immune function, which will be correlated with neonicotinoid residues in tissues. Our study will provide the necessary link between effects information ascertained via controlled laboratory experiments with field studies aimed at assessing exposure in wild grouse. Residues will be measured in liver tissue and excreta In addition, we will measure immunity using the most sensitive assay determined in Activity 1 (if a statistically significant impact is found) and gene expression in white blood cells acquired from a blood sample. Gene expression will allow us to identify biomarkers of exposure and effect in neonicotinoid exposed birds and will be assessed against immune function and residue concentrations to provide managers with non-lethal assays to understand exposure and effect in wild birds.

Summary Budget Information for Activity 3: **ENRTF Budget: $ 141,270**

Amount Spent: $ 141,151
Balance: $ 119

Outcome	Completion Date
1. Measurement of immune toxicity in exposed chickens	31 JAN 2018
2. Analysis of chicken tissue residues for neonicotinoids	31 JAN 2018
3. Complete data analysis of relationship between exposure and immune effects	30 JUN 2019

Activity Status as of: 30 January 2017
Other than finalization of subaward (SIUC), this activity is pending completion of Activity 1.

Activity Status as of: 30 July 2017
A post-doctoral candidate with expertise in ecotoxicogenomics and wild birds has recently been hired for Activity 3. He has reviewed the proposed protocols and methodologies, as well as the preliminary data from Activity 1 and suggested a revised protocol for Activity 3 (see amendment request).

Amendment request (9/08/2017):
We are proposing to amend Activity 3 based on the results of Activity 1. The use of genomics (RNA sequencing) is a more sensitive assay of toxicity than the assays used in Activity 1. We propose to add a second phase of RNA sequencing sampling (cryo-preserved organ tissues in addition to peripheral blood mononuclear cells). We are also proposing to remove the combined exposure groups as we will have no results for clothianidin exposure from Activity 1. The number of tissues evaluated for neonicotinoid residues will be reduced to two (liver and excreta) to allow budget space for the second phase of RNA sequencing as our residue results from Activity 1 are expected to be adequate for our analysis.

Activity Status as of: 30 January 2018
We completed our 23-day exposure experiments and collected RNA-quality blood and tissue samples. In total, we had 40 chickens divided into three treatments of imidacloprid exposures (2.72, 5.43, and 10.86 mg/kg - determined based on immune and behavioral functions in Activity 1) and one control group. We followed our time-series sampling strategy with blood samples were collected through 4 sampling occasions (day 7, 9, 16, and 23) and tissues were collected through 3 different end-points (12 chickens on day 9, 12 on day 16, and 16 on day 23). Laboratory work, including the isolation of peripheral blood mononuclear cells from whole blood samples and for the preservation of tissues was completed. RNA extraction and quantification from peripheral blood mononuclear cells and from preserved tissue samples is currently being performed. Because a statistically significant immune impact was not found in Activity 1, that assessment was not done for Activity 3.

Activity Status as of: 30 June 2018

RNA sequencing data has been generated from tissues collected during the exposure trials. Bioinformatics analysis was done to prepare sequences, which were then aligned with the Chicken Reference Genome and expressed genes in our samples recorded. A total of 24,881 expressed genes were detected in our 48 samples and preliminary pairwise differential analyses identified 499 genes having significant expressions. More than one third of the significant expressed genes belong to peripheral blood cells, which may be the basis for future development of a non-lethal exposure detection method.

Activity Status as of: 30 January 2019
Laboratory analysis of imidacloprid residues was completed. We found detectable levels of imidacloprid residues in livers that corresponded to dose group: none detected in the liver of the control group; 3 ng/g in liver of the 2.72 mg/kg treatment group (low dose); 8.8 ng/g in liver of the 5.43 mg/kg treatment group (medium dose), and 13.9 ng/g in the liver of the 10.86 mg/kg treatment group (high dose). Two phases of RNA sequencing data from 85 samples – including 58 peripheral blood mononuclear cell (PBMC—an assemblage of specific circulating

immune cells) samples, 16 brain tissue samples, and 16 liver tissue samples – was also completed. Bioinformatics analyses and gene expression analyses are completed. There were 24,881 genes observed in our samples. Based on a rigorous analysis of the current data, we detected:

- A total of 354 genes were affected by imidacloprid exposure.
- Specifically, the 58 blood samples had 317 significantly expressed genes which were distinctly different between groups and the number of affected genes increased with dose: 33 genes were changed in the control group; 55 genes in the low dose group; 84 genes in the medium dose group; and 145 genes in the high dose group.
- The 16 liver samples had only 37 affected genes detected. There was no gene which was significantly different in treated birds compared to control birds among all 16 tissue samples.
- The vast majority of the affected genes in PMBCs (259 of 317 genes, or 82%) were down-regulated, an affect that correlated with dose level. In contrast, 20 out of 28 expressed genes (71%) in livers were up-regulated.
- Statistical analysis showed that the 317 significantly expressed genes in PBMCs are different than the 37 affected genes in livers, in terms of their associated physiological functions, and in their correspondence to dose level (significance between treatment groups in PBMC genes, but not in brains or livers).

This portion of the study provides strong preliminary evidence that non-lethally acquired blood cells (i.e., PBMCs) constitute a sampling medium from which a molecular assay (a potential biomarker) may be developed for the detection of a bird's response to imidacloprid exposure. We have developed novel evidence indicating that the approach is *sensitive* to imidacloprid exposure, but do require further laboratory and field evidence for the *specificity* of the approach to imidacloprid/neonicotinoid exposure before field applications are warranted. In sum, the identification of a dose-dependent change in gene expression in non-lethally acquired immune cells is a significant contribution toward the goal of equipping scientists and managers with a means to detect a bird that has responded to imidacloprid exposure. Further work within the scope of this project will include the linking of the detected changes in gene expression to biological function as well as to doses known to alter bird behavior as reported in Activity 2 and that we detected through our field work in Activity 1.

Project Status as of: 11 April 2019
Manuscript in preparation for publication.

Final Report Summary:
In Activity 3, genetic analysis was used to identify potential biomarkers in chickens exposed to imidacloprid orally. RNA-sequencing was used to evaluate samples collected from exposed chickens to identify impacts on gene expression (the effect a gene has).

Four groups of chickens were exposed to imidacloprid doses identified in the earlier work (Activity 1). Two types of tissue samples (liver, brain) as well as peripheral blood cells were genetically sequenced. Liver and feces were also analyzed for residue analysis. Imidacloprid was detected in livers and feces in a dose-dependent manner. A total of 354 genes were affected by imidacloprid; 37 of these were found in liver samples and 317 were identified in blood samples, with the number of affected genes increasing with dose. The identification of significant gene expression alteration in blood cells may be the basis for future development of a non-lethal exposure detection method.

- Samples from chickens exposed to oral imidacloprid were evaluated for impacts on the expression of genes through RNA sequencing
- Imidacloprid residues were detected in livers in a dose-dependent manner. The liver samples had 37 genes affected by the imidacloprid exposure while peripheral blood cells (which can be collected non-lethally) had 317 affected genes.
- This information may be used in the future to develop an assay to identify non-lethal methods of detecting imidacloprid exposure in birds.

V. DISSEMINATION:

Description:

This study will help ensure that food plots and crops on state managed lands are planted with seed safe for wildlife. We will use outreach to inform stakeholders and partners managing for wildlife. This study would be among the first to examine exposure and consumption of these pesticides in wild birds, with broader impacts extending to population and pesticide management.

Our findings will be communicated with state (e.g. DNR) and federal (e.g. USFWS) land managers, as well as agencies tasked with agricultural regulation and environmental protection (MDA, USDA, EPA). Findings will be presented at state, regional, and national meetings (e. SETAC, TWS) as appropriate given the results. Publications will be produced for peer-reviewed journals, outreach newsletters, and annually for the DNR's Summaries of Wildlife Research Findings. Media outreach will also be pursued.

Status as of: 30 January 2017

No activity to date.

Status as of: 30 July 2017

Findings to date have been compiled as part of the Annual DNR Wildlife Research Summaries and will be posted online following internal review. In addition, an abstract for a scientific poster presentation has been submitted and accepted by SETAC (Society of Environmental Toxicology and Chemistry) for their annual conference.

Status as of: 30 January 2018

In November 2017, Drs. Roy and Franzen-Klein attended the meeting of the Society for Environmental Toxicology and Chemistry in Minneapolis. Dr. Roy gave a presentation on the results of Activity 2 in this study and Dr. Franzen-Klein submitted a poster on Activity 1 results. Feedback was good. A representative from the Environmental Protection Agency (EPA) Headquarters Office of Pesticide Programs, Environmental Fate and Effects Devision asked Dr. Roy to provide a webinar this winter for EPA staff and to provide public comments on draft risk assessment documents as appropriate. Representatives from Bayer Crop Science asked for recommendations to reduce seed spill rates. We suggested that they survey farmers to learn more about the problem and possible solutions.

In November 2017, Dr. Ponder attended the Raptor Research Foundation annual meeting in Salt Lake City, UT and presented on Exploring the Risk of Neonicotinoids in Wild Birds based on results from this study.

The results have also been presented at two internal seminar presentations at the University of Minnesota's College of Veterinary Medicine.

Status as of: 30 June 2018

Results to date have been summarized and submitted to the Environmental Protection Agency in response to their public commentary period for re-registration. In addition, Drs. Roy, Jankowski and Ponder participated in a webinar to share our results directly with employees in the EPA Office of Pesticide Programs in April 2018 and Dr. Jankowski led a presentation to the EPA Office of Environmental Review and Assessment in July.

In February 2018, Dr. Roy met with a group of Agricultural stakeholders representing the MN Farm Bureau and MN Crop Production Retailers. Dr. Ponder attended remotely to discuss options to clean up spills and reduce exposed seed in Minnesota. Internal communications include several presentations to Department of Natural Resource staff by Dr. Roy and a graduate student research seminar at the University of Minnesota by Dr. Franzen-Klein.

A scientific poster sharing the results of Activity 1 was presented at the International Conference on One Medicine, One Science in April 2018.

Status as of: 30 January 2019
The results of this study were presented to the clinical veterinary community at the ExoticsCon conference in September 2018. Other presentations completed in 2018 and planned in 2019 include:

1) Crop Production Retailers (stakeholder group), Dec 11 2018, Minneapolis
2) DNR Wildlife Managers on 4 dates at locations statewide (state managers), 2018
3) University of Minnesota- Mankato, Department of Biological Sciences, Nov 2 2018, Mankato
4) Minnesota Prairie Chicken Society (stakeholder group), Apr 21 2018, Glyndon
5) Minnesota Chapter of the Wildlife Society (state, federal and non-profit natural resource managers), Feb 20 2019, Duluth
6) Crop Production Retailers (stakeholder group), Mar 29 2019, Owatonna

In addition, the final report for Activity 2 is available online at:
https://files.dnr.state.mn.us/wildlife/research/summaries/forest/2016_neonictoids.pdf

Project Status as of: 11 April 2019
Manuscript submitted. Additionally, the work has been presented at The Wildlife Society (MN Chapter) and to seed distributors at UMN Extension Services training.

Final Report Summary:
Scientific papers: 1 published, 1 submitted, 4 pending:

- The first manuscript, *Multi-scale availability of neonicotinoid-treated seed to wildlife in an agricultural landscape during spring planting*, has been accepted for publication in Science of the Total Environment and is available on-line: https://www.sciencedirect.com/science/article/pii/S0048969719320212
- The second manuscript, *Evaluation of Neurobehavioral Abnormalities and Immunotoxicity in Response to Oral Imidacloprid Exposure in Domestic Chickens (Gallus gallus domesticus)*, has been submitted for publication.
- A third manuscript is in process of being finalized for submission

Oral presentations:

- Activity 2 results presented in November 2017 at the Society for Environmental Toxicology and Chemistry: *Neonicotinoids on the Landscape: Evaluating Avian Exposure to Treated Seeds in an Agricultural Region.*
- Initial results presented in *Effects of oral neonicotinoid exposure on domestic chickens (Gallus gallus domesticus)*, at the Raptor Research Foundation annual conference, November 2017
- Results presented at two internal seminars at the University of Minnesota
- Crop Production Retailers (stakeholder group), Dec 11 2018, Minneapolis
- DNR Wildlife Managers on 4 dates at locations statewide (state managers), 2018
- University of Minnesota- Mankato, Department of Biological Sciences, Nov 2 2018, Mankato
- Minnesota Prairie Chicken Society (stakeholder group), Apr 21 2018, Glyndon
- Minnesota Chapter of the Wildlife Society (state, federal and non-profit natural resource managers), Feb 20 2019, Duluth
- Crop Production Retailers (stakeholder group), Mar 29 2019, Owatonna
- ExoticsCon/Association of Avian Veterinarians Annual Conference 2018, Wildlife Track: *Potential Risks to Wild Birds from Neonicotinoid Pesticides*

- Prairie Grouse Technical Council (Bartlesville, OK), Nov 2019: *Neonicotinoids on the Landscape: Evaluating Avian Exposure to Treated Seeds in an Agricultural Region* (pending)

Other communications:

- Activity 1 results presented in poster format at the Society for Environmental Toxicology and Chemistry in November 2017: *Effects of oral neonicotinoid exposure on immune function in domestic chickens*
- Results submitted and comments shared in response to the Environmental Protection Agency public commentary period for re-registration process for neonicotinoids
- In Feb 2018, results were shared with various farming stakeholder groups
- The interim report for Activity 2 is available on-line on the DNR web site at: https://files.dnr.state.mn.us/wildlife/research/summaries/forest/2016_neonictoids.pdf
- A poster, *Effects of oral neonicotinoid exposure on immune function in domestic chickens,* was presented at the International Conference on One Medicine, One Science (April 2018).
- A poster, *Tracking the transcriptome: a non-lethal indicator of exposure to neonicotinoids in birds*, has been accepted for a poster in November, 2019 at Society for Environmental Toxicology and Chemistry annual conference.

VI. PROJECT BUDGET SUMMARY:

A. ENRTF Budget Overview:

Budget Category	$ Amount	Overview Explanation
Personnel:	$ 121,583	Post-doc (1 year, 1 FTE) responsible for project management, laboratory studies and data collection/analysis Graduate student (1 year, 1 FTE) responsible for laboratory studies, data collection and analysis. Lab technicians (1 year, .2 FTE) to run perform immune assays
Professional/Technical/Service Contracts:	$ 222,967	Subcontract to DNR for field collection of samples (200 samples), field observations around state and camera study (12 sites): $98,978 Subcontract to Southern Illinois University, Carbondale (SIUC) for laboratory analysis of neonicotinoid residues (350 samples), production of stock supplies for analysis: $98,978 Research animal housing for lab studies (Activity 1 – 28 days/130 chickens; Activity 3 – 28 days/48 chickens): $13,944 Research laboratory (UMN) for RNA sequencing (36 samples @$350): $12,067
Equipment/Tools/Supplies:	$ 4,450	Consumables for laboratory studies and immune assays (sample collection supplies, antigen for immune studies, plates for immune assays, chicken acquisition - 178)
TOTAL ENRTF BUDGET:	**$ 349,000**	

Explanation of Use of Classified Staff: This is not classified staff, but we need to contract with SIUC for sample analysis because there are not labs in Minnesota that will quantify residues in animal tissues. SIUC lab has established analytical methods and applied the methods to various projects in the past. Minnesota Department of Agriculture does to neonicotinoid assays, but their minimum level of detection is not sensitive enough and they have not established methods for detection in tissues.

Explanation of Capital Expenditures Greater Than $5,000: N/A

Number of Full-time Equivalents (FTE) Directly Funded with this ENRTF Appropriation: 2.2 FTE

Number of Full-time Equivalents (FTE) Estimated to Be Funded through Contracts with this ENRTF Appropriation: 1.67

B. Other Funds:

Source of Funds	$ Amount Proposed	$ Amount Spent	Use of Other Funds
Non-state			
	$	$	
State			
University of Minnesota	$ 182,552	$ 90,947	53% indirect rate
TOTAL OTHER FUNDS:	**$** 182,552	**$**	

VII. PROJECT STRATEGY:

A. Project Partners:

Dr. Julia Ponder, University of Minnesota, Avian and Conservation Medicine – PI, oversight of lab studies
Dr. Charlotte Roy, MN DNR, Research Scientist – co-PI, oversight of field studies
Dr. Da Chen, SIUC, Assistant Professor of Environmental Chemistry– co-PI, laboratory analysis of samples
Dr. Mark Jankowski, USEPA, Ecotoxicologist – consultant for lab study design and interpretation

B. Project Impact and Long-term Strategy:

This study will provide information about the safety of neonicotinoid seed treatments to birds, using sharp-tailed grouse as a model. It will provide information to assess the risk of consumption of seeds and evaluate whether other bird species are potentially at risk for exposure. This study would be the first to holistically examine exposure to mixtures of these pesticides in wild birds. We know insects are at risk from neonicotinoids, but the information gained will be important for more informed management of risk to vertebrates.

C. Funding History:

Funding Source and Use of Funds	Funding Timeframe	$ Amount
DNR pilot funding for camera work and small numbers of grouse samples for residue analysis to inform LCCMR study	July 2015 – June 2016	$ 96,500

VIII. FEE TITLE ACQUISITION/CONSERVATION EASEMENT/RESTORATION REQUIREMENTS: N/A

IX. VISUAL COMPONENT or MAP(S): Attached

X. RESEARCH ADDENDUM: Submitted

XI. REPORTING REQUIREMENTS:

Periodic work plan status update reports will be submitted no later than 30 January 2017, 30 July 2017, and 30 January 2018. A final report and associated products will be submitted between June 30 and August 15, 2018.

Environment and Natural Resources Trust Fund
Final M.L. 2016 Project Budget

Project Title: Game and Nongame Bird Pesticide Exposure
Legal Citation: M.L. 2016, Chp. 186, Sec. 2, Subd. 03m
Project Manager: Julia B. Ponder, DVM, MPH
Organization: University of Minnesota
M.L. 2016 ENRTF Appropriation: **$** *349,000*
Project Length and Completion Date: 2 Years, June 30, 2018
Date of Report: August 1, 2019

ENVIRONMENT AND NATURAL RESOURCES TRUST FUND BUDGET	Activity 1 budget	Amount Spent	Activity 1 Balance	Activity 2 budget	Amount Spent	Activity 2 Balance	Activity 3 budget	Amount Spent	Activity 3 Balance	TOTAL BUDGET	TOTAL BALANCE
BUDGET ITEM											
Personnel (Wages and Benefits)	$60,073	$60,073	$0				$78,900	$78,900	$0	$138,973	$0
TBD: Post-doc - 1 FTE, $56,010 (80%- salary, 20% benefits), 1 year											
TBD: Graduate student - 1 FTE, (55% salary, 45% benefits) 1 year = $56,010											
TBD: Technician - 0.2 FTE $9,563 (77.6% salary, 22.4% benefits), 1 year											
Professional/Technical/Service Contracts											
MN DNR: field collection of grouse samples over 2 seasons plus camera study and seed spillage documentation				$79,224	$79,224	$0				$79,224	$0
Southern Illinois University, Carbondale (SIUC): laboratory analysis of neonicotinoid residues	$2,980	$2,980	$0	$39,613	$39,613	$0	$34,022	$33,987	$35	$76,615	$35
UMN Research laboratory: RNA sequencings							$24,129	$24,129		$24,129	$0
D Chen Lab services - metabolite analysis	$10,000	$538	$9,462							$10,000	$9,462
University of Minnesota Research Animal Resources: research subject housing and oversight	$3,152	$3,152	$0				$819	$819	$0	$3,971	$0
Equipment/Tools/Supplies	$12,688	$12,688	$0				$3,300	$3,300	$0	$15,988	$0
Laboratory consumables ($4,000)											
Acquisition of research subjects (chickens) ($250)											
Travel expenses in Minnesota											
Mileage to pick up chickens	$0		$0				$100	$16	$84	$100	$84
COLUMN TOTAL	**$88,893**	**$79,431**	**$9,462**	**$118,837**	**$118,837**	**$0**	**$141,270**	**$141,151**	**$119**	**$349,000**	**$9,581**

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Multi-scale availability of neonicotinoid-treated seed for wildlife in an agricultural landscape during spring planting☆

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HIGHLIGHTS

- We quantified neonicotinoid-treated seeds on the soil surface after planting.
- Probability and density of soybean seeds on the soil surface were higher than corn.
- Neonicotinoids decreased rapidly on seeds on the soil surface but persisted 30 days.
- Over a dozen species of birds and mammals consumed seeds at simulated spills.
- Seeds on the soil surface should be considered in pesticide risk assessments.

GRAPHICAL ABSTRACT

ARTICLE INFO

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ABSTRACT

Neonicotinoid pesticides are applied to seeds and are known to cause lethal and sub-lethal effects in birds and mammals. Neonicotinoid-treated seeds could be available to wildlife through spillage or exposed seeds near or at the soil surface due to incomplete or shallow drilling. We quantified seed spills that may occur during loading or refilling the hopper at a landscape-scale using road-based surveys. We also quantified undrilled seeds in 1-m^2 frames on the soil in the center and corner of fields to obtain estimates at the field scale. We broadcast seeds on the soil surface of a tilled field and left them for 0, 1, 2, 4, 8, 16, and 30 days to quantify the decrease of neonicotinoids under field conditions. Lastly, we documented wildlife at neonicotinoid-treated seed spills with trail cameras. We estimated the number of spills during planting to be 3496 (95% CI: 1855–5138) and 2609 (95% CI: 862–4357) for corn, 11,009 (95% CI: 6950–15,067) and 21,105 (95% CI: 6162–36,048) for soybean, and 830 (95% CI: 160–1500) and 791 (95% CI: 0–1781) for wheat in 2016 and 2017, respectively. Exposed seeds were present at the soil surface in 35% of 71 fields. The probability that seeds were present on the soil surface was higher for soybeans (18.8 and 49.4% in the center and corners, respectively) than for corn (1.6 and 2.7%, respectively), and seed densities were also higher (1.04 vs 0.07 seeds/m^2, respectively). Neonicotinoids decreased rapidly on seeds on the soil surface but persisted as long as 30 days. Over a dozen species of birds and mammals consumed seeds at simulated spills, with an average time for birds to find spills of 1.3 ± 1.5 days

☆ This research does not reflect the official positions and policies of the United States Environmental Protection Agency (US EPA). Mention of products/trade names does not constitute recommendation for use by US EPA.This work was funded by the Minnesota Department of Natural Resources Wildlife Restoration (Pittman-Robertson) Program Grant and the Minnesota Environment and Natural Resources Trust Fund.

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and an average time to consumption of 4.1 ± 3.4 days. Seeds are abundant on the soil surface for wildlife to consume during the spring planting season and should be considered in pesticide risk assessments.

1. Introduction

Neonicotinoids, including imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX), comprise 25% of the global agricultural insecticide market, making them the most widely used pesticides worldwide, with imidacloprid comprising nearly half of this market (Jeschke et al., 2011; Mineau and Palmer, 2013; Goulson, 2013) until 2012 when thiamethoxam had the largest market share (Bass et al., 2015). Neonicotinoids are systemic pesticides that are commonly applied as seed treatments to important food crops like corn, soybeans, oilseed rape, sunflower, cereals, and beets. About 2–20% of the seed treatment is taken up by the plant as it grows and is distributed among the leaves, flowers, pollen, and nectar, at concentrations sufficient to control invertebrate pests (e.g., 5–10 μg per liter in sap; Sanchez-Bayo, 2014). Invertebrates are impacted at doses (0.82–88 ng active ingredient/insect) that are considered safe for vertebrates, because toxicity in vertebrates requires exposure to doses (14–5000 mg active ingredient/kg body weight) that greatly exceed the levels that produce effects in invertebrates (Goulson, 2013). Neonicotinoids bind very specifically to invertebrate nicotinic acetylcholine receptors, and because they bind less strongly to vertebrate receptors and are not as persistent in the environment as organochlorines, they have been considered much less toxic to vertebrates than pesticide options that predated the early 1990's (Tomizawa and Casida, 2005; Jeschke et al., 2011). This high specificity and systemic nature contributed to their widespread and rapid adoption beginning in 1994 with the registration of imidacloprid in the United States (FIFRA, 1996).

Importantly, demonstrated impacts of neonicotinoids on non-target invertebrates have been documented over the last decade (Krupke et al., 2012; Sanchez-Bayo, 2014; Goulson et al., 2015). Concerns for incidental impacts on pollinators (e.g., through availability in nectar and pollen) led the European Union to ban or place a moratorium on use of IMI, CLO, and TMX on flowering crops in 2013. In May 2018, the moratorium was expanded to include all outdoor use of IMI, CLO, and TMX by the end of 2018, based on the threat that these chemicals pose to pollinators due to their persistence in soil, solubility in water, transport away from the site of application, and uptake by other plants (Krupke et al., 2012; Main et al., 2014; Bonmatin et al., 2015; Morrissey et al., 2015). However, these pesticides are widely used in North America, and elsewhere in the world. Recent studies are now also documenting adverse effects of neonicotinoids that reach beyond pollinators to include vertebrates (see reviews in Mineau and Palmer, 2013; Gibbons et al., 2014). In the United States, neonicotinoids are currently under registration review by the Environmental Protection Agency (EPA), with risks to both pollinators and non-pollinators, including birds and mammals, under consideration.

Vertebrate toxicity is expected to occur at doses that exceed the levels available in crop plants consumed by humans and livestock (FIFRA 1996). Wild birds and mammals are most likely to be exposed to large doses of neonicotinoids through ingestion of treated seeds (Goulson, 2013; Gibbons et al., 2014), although numerous other exposure mechanisms exist (e.g., soil, trophic transfer; SERA, 2005; Douglas et al., 2015). The Minnesota Department of Agriculture (2014) stated that, "Although neonicotinoids are less toxic to vertebrates than to arthropods, direct consumption of neonicotinoid treated seeds may expose birds and other taxa to acute or chronic doses." Ingestion of a small number of neonicotinoid-treated seeds can be lethal to birds; for example, ingestion of a single treated corn kernel is lethal to a blue-jay sized (~85 g) bird (see reviews in Mineau and Palmer, 2013; Gibbons et al., 2014). However, toxicity varies by chemical and species, given differences in genetic and physiological factors including size, absorption, distribution, metabolic, and excretion processes (Bean et al., 2019). Differences among species in seed handling behavior could affect the ingested amount of chemical (Avery et al., 1997).

Sub-lethal effects in birds in the lab include hyporeactivity, lack of coordination, wing drop, immobility, disruption of migratory coordination, eggshell thinning, reduced egg hatching rate, impaired testicular function, and low weight in chicks (Cox, 2001; Lopez-Antia et al., 2013, 2014, and 2015; Tokumoto et al., 2013; Mineau and Palmer, 2013; Eng et al., 2017). Sub-lethal impacts in mammals include delayed sexual maturation, sperm deformities, premature deliveries, stillbirths, and offspring deformities (Rexrode et al., 2003; Anon, 2007). Yet, studies of neonicotinoid effects on vertebrates are overwhelmingly laboratory-based (91% of studies), which limits our ability to interpret the significance of findings in more natural settings (Gibbons et al., 2014).

Neonicotinoid-treated seeds could be available to wildlife through spillage during transport, reloading and refilling of the hopper or through seeds near or at the soil surface after planting (de Leeuw et al., 1995; Pascual et al., 1999; Lopez-Antia et al., 2016). The U.S. EPA estimated that ~1% of seeds remain accessible to granivores after planting (as reported by Goulson, 2013; Lopez-Antia et al., 2015). Higher densities of exposed seeds generally result in greater attraction of birds to fields (Murton et al., 1963; Feare et al., 1974). In Spain, 30 bird species were observed picking up treated seeds from cereal fields, and 3.1% of red-legged partridge (*Alectoris rufa*) gut contents collected by hunters tested positive for imidacloprid after planting of winter cereal crops despite insecticides not normally being used on winter cereal crops in the study area (Lopez-Antia et al., 2016). More recently in Texas, USA, 7 of 57 northern bobwhite (*Colinus virginianus*) livers had detectable concentrations of neonicotinoids (Ertl et al., 2018).

Given the toxicity to birds and mammals at the concentrations of neonicotinoids applied to treated seeds, consumption of treated seeds would be expected to produce lethal or sub-lethal effects in granivorous wildlife, yet poisoning incidents are infrequently reported. Dead and poisoned partridges have been found in agricultural fields in France following use of imidacloprid-treated seed (Berny et al., 1999; Mineau and Palmer, 2013; Millot et al., 2017). A few other pesticide poisoning incidents have been detected (Greig-Smith, 1987; Fletcher et al., 1995; de Snoo et al., 1999), but carcasses can be scavenged quickly (Ponce et al., 2010), may not be localized or may be inconspicuous if effects are not immediate (de Snoo et al., 1999), and may not raise suspicion of pesticides as the cause of death (Millot et al., 2017). Thus, seed consumption or sub-lethal exposure may be easier to detect in field settings than mortalities.

Field studies conducted in Spain have focused on availability and consumption of winter cereals (wheat, oats, barley, and triticale seeds) planted in the fall (Lopez-Antia et al., 2016). We therefore conducted a study to estimate availability and document wildlife consumption of neonicotinoid-treated seeds during the spring planting season in the Midwestern USA. Birds are initiating nests, laying eggs, and incubating nests during the spring, and mammals give birth and raise young, so sub-lethal reproductive effects related to consumption of treated seeds during the breeding season might be particularly long-lasting. Furthermore, we examined an agricultural landscape dominated by corn, soybeans, and wheat, which provided 3 sizes of seeds that may be ingested by birds with varied beak sizes and bill types, as well as mammals that consume beans and grains. Almost all corn planted in the

Midwestern USA has been treated with these pesticides (Stokstad, 2013); most soybean, wheat, and sunflower seeds are treated also; and neonicotinoids are applied as a foliar spray for several other crop types.

The overarching objective of our research was to determine whether wildlife may be exposed to potentially lethal or sub-lethal doses of neonicotinoids through treated seeds during the spring planting season. Specifically, we aimed to:

1- Quantify the rate of large seed spills during planting season at a landscape scale.
2- Quantify the availability of seeds on the soil surface in fields after planting.
3- Quantify the decrease of neonicotinoids (IMI, TMX, and CLO) on treated seeds left on the soil surface for up to 30 days.
4- Quantify the time for wildlife to find neonicotinoid-treated seed spills and determine whether wildlife consume treated seeds at simulated spills.

2. Materials and methods

2.1. Study area

We conducted our study in agricultural regions of western Minnesota. We quantified actual seed spills at the landscape-scale (Fig. 1a), seeds on the soil surface at the field-scale, and documented seed consumption at simulated seed spills (Fig. 1b) in the springs of 2016 and 2017.

2.2. Quantifying seed spills at a landscape scale

In the United States, all chemically treated seeds (e.g., neonicotinoids, fungicides, other pesticides) are unnaturally colored, as mandated by the Federal Seed Act. Treated seeds are highly visible and easily identified by their unusual color (e.g., pink, blue, green, purple), which is used to prevent accidental feeding to livestock and humans. We quantified the frequency of actual seed spills on the landscape by inspecting fields with visual access from roads in agricultural areas. This approach allowed for landscape-level seed spill quantification without requiring landowner notification that might bias behavior and compromise results. Because most spills likely occur during seed transport to fields for planting or during refilling and overfilling hoppers near field access points by roads, quantification of seed spills from roads should be minimally biased by visual access from roads. However, this assumes that spill rates are similar for fields adjacent to roads and fields non-adjacent to roads, which also have field access points and privately-owned access roads.

We identified 211 townships (i.e., 36 mi^2 or 9324 ha blocks in the U.S. Public Land Survey System) in the western third and southeastern part of the state of Minnesota, USA with ≥50 miles of roads and ≥50% of the area in corn, soybeans, and/or wheat production using the Minnesota Department of Transportation (MNDOT) Roads Layer (MNDOT, 2008) and 2014 Cropland Data Layer (USDA-NASS, 2015), respectively,

Fig. 1. a. Townships (9324 ha, $n = 76$) surveyed for seed spills during spring planting season in 2016 (dark gray), 2017 (light gray), and both years (light gray outlined with dark gray) in Minnesota, United States. b. Location of fields where seeds were counted on the soil surface after planting (left) and where cameras were placed at simulated spills (right) in Minnesota, United States during 2016 and 2017. Fields are indicated as larger than their actual size to show their relative locations at a statewide scale; thus, some fields cannot be distinguished separately from other nearby fields. Generally, the same sites were used for both objectives, but some differences occurred related to the stage after planting during our visits and the ability to return to sites to remove cameras.

in ArcGIS 10.2 (ESRI, 2015). These criteria were used to select townships with visual access to fields from roads while also not being so restrictive that the spatial distribution of the sample was constrained. We drew a spatially-balanced sample of 50 townships each year using a Generalized Random Tessellation Stratified (GRTS) design (Stevens and Olsen, 1999). However, we surveyed the 38 most western townships from the 50 selected each year, due to a later start to planting during the springs of 2016 and 2017, for a total of 76 townships surveyed during the 2 years of the study. We began in the southern townships and worked north as the soil warmed to temperatures suitable for planting during 18 April–23 May 2016 and 23 April–21 May 2017.

We recorded locations and approximate size (i.e., area) of seed spills near *recently planted* (i.e., based on row spacing and before the early seedling stage) fields with the DNRSurvey mobile computer application, a moving map software that allows digitization of aerial photography in real-time (Wright et al., 2015). Documenting only *recently planted* fields allowed for control in temporal variation in the timing of planting. For example, a field that has not been planted yet will not have a spill at the time of sampling, which is different from a spill not occurring during planting. Thus, by only including recently planted fields in our estimates, we measured spills during planting. We defined a "field" as a quarter of a quarter-section (i.e., 40 ac or 16.2 ha). We recorded each quarter of a quarter-section in agricultural row-crop production, whether any part of it was recently planted (i.e., before early seedling stage), documented the amount (number of seeds or approximate area) of spilled seed on the road, field edge, or visible in the field, and crop type (when known). When seed spills were accessible (e.g., along public roads and rights-of-way), we collected seeds to determine the proportion of accessible seed spills that contained neonicotinoid-treated seed. Seeds were sent to an analytical laboratory at Southern Illinois University Carbondale (SIUC) for quantitative determination of 7 neonicotinoids: IMI, TMX, CLO, thiacloprid (THIA), dinotefuran (DIN), nitenpyram (NTP), and acetamiprid (ACE).

After our survey of recently planted fields was completed in May, we repeated the survey for the same townships to identify the crops that were growing in fields. This allowed us to quantify spill rates per crop type planted during the time of our survey. We also noted additional spills observed during the second pass in 2017, but these spills were not included in spill rate estimates because surveys were conducted too long after most fields were planted.

2.3. Quantifying seeds on the soil surface after planting

To estimate the amount of seed at the soil surface of fields after planting, we used a 1-m^2 frame to define plots in recently planted fields and counted all treated seeds visible within the frame (Lopez-Antia et al., 2016). In each field, we sampled 5 plots in a randomly-selected corner and 5 plots in the center as estimated visually from field boundaries. Corner locations were randomly selected by flipping a coin twice. In each field corner, we paced 15 m and 30 m along each edge in an L-shape that had the field corner for a vertex to obtain a total of 5 measurements (i.e., 1 plot at a vertex, 2 plots at 15 m, and 2 plots at 30 m). We hypothesized that seed exposure would be greater at the end of rows where planters turn sharply than within rows. For field centers we paced 15 m in each cardinal direction to sample 5 plots, including the center. We counted all seeds on the soil surface within the frames, as well as documenting seeds observed on the soil surface while walking to plots, to get a better sense of whether our sampling intensity was sufficient to adequately characterize fields. We also recorded any seed spills that we observed in fields during our visits.

Fields included in our field-scale, post-planting surveys were comprised of 3 types; fields managed by the Department of Natural Resources (DNR) and farmed by DNR staff (hereafter, DNR fields), fields on lands managed by DNR but farmed by cooperating, private individuals in Cooperative Farming Agreements (CFAs), and privately-owned, privately-farmed fields (PVT). These surveys required permission to access privately-owned fields and thus private farmers were non-randomly selected by staff as individuals likely to cooperate with the study. We cannot exclude the possibility that farmers with prior knowledge of the study might have changed their seed stewardship behavior, but we attempted to minimize this through our selection of private farmers, and when landowner permission was not required (i.e., CFAs), participants were blind to the study. In 2016, we sampled plots in 10 DNR fields farmed by DNR staff, 36 CFA fields, and 2 PVT fields. In 2017, we sampled 6 CFAs and 17 PVT fields. In 4 cases, we included 2 PVT fields that were planted by the same farmer, but in 3 of these cases, the fields were planted to different crop types, with different planting equipment used for each crop type in cases where the equipment type used was known. Neonicotinoid-treated seed was no longer permitted on DNR-managed land beginning in 2017, but was not enforced in this initial year of implementation, so we continued to sample CFAs in 2017.

2.4. Quantifying availability of neonicotinoids on treated seeds on the soil surface

To estimate how long neonicotinoids may persist on seeds left on the soil surface, we broadcast hundreds of seeds on the soil surface of a tilled field by hand so that the seeds would experience UV, microbial factors, rainfall, and other ambient conditions in northern Minnesota. Experiments were conducted 5 May–4 June 2016 and 4 May–3 June in 2017. We exposed seeds to environmental conditions and collected 5–7 seeds of each type after environmental exposure for 0, 1, 2, 4, 8, 16, and 30 days to quantify the decrease of neonicotinoids. We noted daily precipitation and cloud cover during both years of the experiment, and measured exact rainfall amounts (mm) at the site of the experiment in 2017 with an Oregon Scientific RGR126N Wireless Rain Gauge. We conducted the experiment in 2016 and 2017 with 2 types of commercially available corn seed treatments (CLO and TMX) and commercially treated soybeans (IMI and CLO). After field collection, seeds were stored frozen (−18 °C or colder) until shipment to SIUC for neonicotinoid analysis.

2.5. Time for wildlife to find spills

We simulated treated seed spills in planted fields to estimate the time it takes for birds to discover spills and to identify wildlife species that consumed treated seeds. We selected CFAs on Wildlife Management Areas with a land cover composition similar to that of the surrounding landscape using the 2014 National Cropland Data Layer (USDA-NASS, 2015) in ArcGIS 10.2 (ESRI, 2015) and the available data on CFAs, which indicated there were 7420 ac (3003 ha) of row crops in 341 CFAs in southwest Minnesota and 2431 ac (984 ha) of row crops in 66 CFAs in northwest Minnesota (M. Benage and J. Williams, respectively, pers. comm.). We prioritized this portion of the study in 2016 because farmers and managers were prohibited from planting neonicotinoid-treated seeds on DNR-managed lands beginning in 2017. In 2016, we placed cameras at simulated spills at 11 CFAs, 3 DNR-farmed fields, and 2 privately-owned fields where we had obtained permission. In 2017, we placed cameras at each simulated spill in 16 CFA fields and 21 privately-owned fields.

Spills were simulated with 1000 treated corn, soybean, or wheat seeds. Seeds were counted with a SLY Automatic Seed Counter (Zhejiang, China), placed in separate bags, and stored away from sunlight. In 2016, we simulated 13 corn spills and 2 soybean spills. In 2017, we simulated 19 corn spills, 23 soybean spills, and 9 wheat spills. To simulate each spill, we buried a 25.4 × 50.8 cm seedling starter tray in the dirt, filled it with dirt, and placed the seeds in a thin layer on top of the dirt, so that we could account for any seeds that became submerged below the soil.

Camera locations at each site were selected along field edges to minimize risk of theft and to view a simulated seed spill in a recently

planted field. Bushnell® Aggressor Trophy Cam HD Cameras (Overland Park, Kansas) were deployed to capture 1 min of video when triggered by motion. We deployed cameras in each location for 3–6 weeks in 2016 and for 1–3 weeks in 2017, with weekly checks to replace batteries and data cards in 2017 after learning how quickly they needed to be replaced in 2016. Images were viewed to identify species at spills and document time until discovery of spills (i.e., when animals first arrived within 30 cm of a spill) and consumption of seeds by wildlife.

2.6. Analytical procedures for neonicotinoid measurement

Seed samples were ground into a powder and freeze-dried for 48 h. Approximately 0.01–0.02 g of dry samples were extracted with a mixture of acetone and hexane (1:1; v/v) using sonication. Prior to extraction, a mixture of isotopically labelled surrogate standards, including thiamethoxam-d_3, acetamiprid-d_3, clothianidin-d_3, imidacloprid-d_4, and thiacloprid-d_4 (purchased from CDN Isotopes, Quebec, Canada), was spiked with seed sample. The extraction was repeated 3 times (10 min each) and the resulting extracts were combined and concentrated to 20 mL. An aliquot of 1 mL of extract was cleaned through a gel permeation chromatography column (diameter: 1.5 cm; length: 40 cm) packed with 6 g of styrene divinylbenzene beads in a mixture of hexane and dichloromethane (1:1, v/v). The resulting extract was further purified through a 2-g Isolute ammonium silica cartridge. The cartridge was pre-conditioned with 10 mL of hexane and the concentrated extract was loaded and washed with 1.5 mL of hexane (discarded). Neonicotinoid analytes were then eluted with 12 mL of methanol/dichloromethane mixture (6:4, v/v). The final extract was concentrated and spiked with internal standard coumaphous-d_{10} (CDN Isotopes) prior to instrumental analysis.

Determination of neonicotinoids was conducted on an Agilent 1260 high performance liquid chromatography (HPLC) system interfaced with a 3200 QTrap triple quadrupole/linear ion trap MS (AB Sciex; Toronto, Canada). The HPLC was equipped with a ZORBAX Extended-C18 column (100 × 2.1 mm, 3.5 μm, 80 Å, Agilent Technologies). The mobile phase consisted of methanol (A) and water (B), both spiked with 0.1% formic acid (v/v). The mobile phase flow rate was 200 μL/min and the following gradient was employed: 10% B ramped to 70% B in 11 min (linear) and then ramped to 80% B in 6 min (linear), followed by a linear increase to 90% B in 2 min (held for 1 min) and then a change to 10% B in 1 min (held for 8 min). The MS was equipped with a TurboIonSpray® electrospray ionization (ESI) probe operated in the multiple reaction monitoring (MRM) mode.

2.7. Data analysis

To quantify seed spills at a statewide level, we first calculated the number of spills and the number of acres planted for each crop type in each surveyed township (i.e., a ratio estimator). We then calculated the ratio of sums across townships to calculate the mean $\hat{R}$ and variance of $\hat{R}$ ($var\hat{R}$) for the surveyed townships. We scaled up to the statewide level by multiplying these estimates by the number of acres for each crop type (i.e., a constant, A) in Cropland Data Layers for 2016 and 2017 [National Agricultural Statistics Service (USDA-NASS, 2017 and 2018)], and the variance of the statewide estimates were calculated as $var\hat{R} * A^2$. All means are expressed a $\mu \pm$ SD, except where noted otherwise. Confidence intervals (95%) were determined as $\mu \pm 1.96$(SD).

We examined predictors of exposed seeds on the soil surface in field plots after planting using the glmer function for generalized linear mixed models with field as a random effect. Our response variable was binomial (i.e., exposed seeds, or none) because our data were heavily zero-inflated with widely variable seed counts. However, we provide summary statistics of counts. We fit models for binomial responses using the R programming language (R Core Team, 2018) and the packages lme4, gplots, and AICcmodavg. Covariate predictors included seed type (corn or soybean), field type (i.e., DNR-farmed, private, CFA), field size, planting date, and plot location (i.e., field corner, center). We did not include wheat fields because our sample size was small ($n = 3$). Field size (in ha) was log transformed for a better distribution and planting date was rescaled to improve model convergence. To examine spills in the same fields, we used the glm function because we did not need to include a random effect for replicate plots when spill was the binomial response variable. (Spills were recorded anywhere in the field, not necessarily within plots). Similar predictors were included in models when the binomial response was 'spill' with the exception of plot location, which did not apply to spills.

3. Results

3.1. Quantifying seed spills at a landscape scale

We surveyed 429,269 ac (173,719 ha) in 2016 and 482,720 ac (195,350 ha) in 2017 during the spring planting season. Of the acres surveyed, 258,252 ac (60.2%) in 2016 and 112,389 ac (23.3%) in 2017 had been planted at the time of our surveys and could have had a spill. Planting in 2017 was later than in 2016 due to a very wet spring, with standing water in many fields during the planting season. At the time of our first pass of the road-based surveys in 2016, 79,752 ac (32,274 ha) of corn, 82,300 ac (33,306 ha) of soybeans, 73,205 ac (29,625 ha) of wheat, and 22,995 ac (9306 ha) of other crops were planted. In 2017, 40,111 ac (16,232 ha) of corn, 23,556 ac (9533 ha) of soybeans, 33,748 ac (13,657 ha) of wheat, and 14,973 ac (6059 ha) of other crops were planted during our first pass of the survey. We observed 211 large seed spills that were visible from the road during surveys in 2016 and 117 spills in 2017. In 2016, we documented 33 corn, 120 soybean, and 46 wheat spills, and 4 spills of other crop types, and 8 spills that could not be identified during the first survey. In 2017, we documented 13 corn, 61 soybean, and 23 wheat spills, 3 spills of other crop types, and 1 unidentified spill during the first pass, and in the second pass we discovered 2 corn spills, 13 soybean spills, and 1 unidentified spill. However, spills from the second pass were not included in our spill rate estimates because most planting had been completed weeks prior to the survey. Spill rates in the areas surveyed were calculated as 4 spills/10,000 ac corn, 15 spills/10,000 ac soybeans, 6 spills/10,000 ac wheat, and 2 spills/10,000 ac other crop types in 2016. Spill rates of 3 spills/10,000 ac corn, 26 spills/10,000 ac soybean, 7 spills/10,000 ac wheat, and 2 spills/10,000 ac of other crop types planted were calculated for 2017.

Extrapolating statewide required the assumption that spill rates visible in fields adjacent to roads were representative of spill rates in fields located elsewhere. If spills near roads were more likely to be cleaned up than those less visible to passersby, then this assumption may not have been tenable. Yet, we did not observe spills being cleaned up or covered during our surveys. Furthermore, most spills likely occurred during hopper refilling, and this often occurs near field access points along roads (96% of spills were detected <60 m from field edges), although we detected spills as far as ~200 m from the road based on distances calculated with aerial photos. Thus we think our assumptions were reasonable. Applying our spill rates across the acres farmed statewide (8,450,000 ac of corn, 7,550,000 ac of soybeans, and 1,321,000 ac of wheat were planted in Minnesota during 2016 [National Agricultural Statistics Service (USDA-NASS 2016 Cropland Data Layer); last accessed 5 June 2017], we estimated 3496 (95% CI: 1855–5138) corn seed spills, 11,009 (95% CI: 6950 –15,067) soybean seed spills, and 830 (95% CI: 160–1500) wheat seed spills statewide in 2016. In 2017, 8,050,000 ac of corn, 8,150,000 ac of soybeans, and 1,160,000 ac of spring wheat were planted (USDA-NASS, 2017 Cropland Data Layer; last accessed 5 March 2018), which scaled up to 2609 (95% CI: 862–4357) corn seed spills, 21,105 (95% CI: 6162–36,048) soybean seed spills, and 791 (95% CI: 0–1781) wheat seed spills statewide during the planting season. Spills increased as we moved from south to north, and the proportion of fields planted during our surveys also increased as we moved south

to north. Importantly, corn and soybeans are the most common crops in the southern part of the surveyed area, and soybeans and wheat are the most common crops in the north.

We collected samples from 107 actual spills of colored seeds on roadsides and right-of-ways, which were comprised of 26 corn, 58 soybean, 22 wheat, and 1 other bean spill. Of these spills, 77 (72%) tested positive for ≥1 neonicotinoid, with IMI being the most commonly detected (33%), followed by CLO (29%), and then TMX (26%). Corn was most commonly treated with TMX or CLO (50% and 62%, respectively), soybean with IMI (50%), TMX (19%), and/or CLO (26%), and wheat with IMI (19%) or TMX (19%). Fifteen spills contained seeds with 2 neonicotinoid treatments. When multiple seed treatments were applied to seeds in spills, TMX and IMI were most commonly applied together to soybean or wheat seeds. Other neonicotinoids (THIA, DIN, ACE, NTP) were not detected on seeds in spills. The geometric mean concentration on spilled seeds treated with each neonicotinoid and collected from roads and right-of-ways was 107.9 ± 7.6 μg/g for IMI (max: 890 μg/g), 85.9 ± 4.6 μg/g for TMX (max: 690 μg/g), and 209.6 ± 4.5 μg/g for CLO (max: 1120 μg/g).

3.2. Quantifying seeds on the soil surface

We documented exposed seeds on the soil surface in plots at 26 of the 71 fields (7 of 51 corn, 17 of 17 soybean, and 2 of 3 wheat fields) sampled in 2016 and 2017, and we observed spilled seed piles in 5 corn fields, 5 soybean fields, and 2 wheat fields. The average density of exposed seeds on the soil surface of centrally located plots was 0.04 (SE 0.03) corn seeds/m^2 (range: 0–5, $n = 255$ center plots), 0.6 (SE 0.2) soybean seeds/m^2 (range: 0–9, $n = 85$ center plots), and 7.8 (SE 5.0) wheat seeds/m^2 (range: 0–69, $n = 15$ center plots). The density of exposed seeds on the soil surface in corner plots was 0.10 (SE 0.06) corn seeds/m^2 (range: 0–15, n = 255 corner plots), and 1.5 (SE 0.3) soybean seeds/m^2 (range: 0–15, n = 85 corner plots), and 8.4 (SE 4.2) wheat seeds/m^2 (range: 0–51, n = 15 corner plots).

The most-supported model describing whether exposed seeds were detected on the soil surface in plots included additive effects of both field plot location (corner or center) and seed type (corn or soybean). No other competing models were identified with AICc <2.0 (Table 1). The probability of exposed seeds on the soil surface after planting was higher for soybean fields than for corn fields, and plots in the field corners had a higher probability of seeds on the surface than plots in the center of fields (Fig. 2).

The most-supported model for predicting the probability of a seed spill in the same fields included field type (CFA, DNR, or private, Fig. 3a), but 3 other models had AICc < 2.0 (Table 2). Two of these models contained field size, which was correlated with field type, because private fields were larger than fields that were farmed on publicly-owned land. Thus, we considered models with field size to be supported because they captured information already contained in the variable "field type." The remaining model also contained seed type, but the estimate was imprecise despite seed type having a large effect size (Fig. 3b).

Table 1
Comparison of support for generalized linear mixed models of binomial counts of exposed seeds on the soil surface (response variable) with field as a random effect and field location (corner or center), seed type (corn or soybean), field type (Cooperative Farming Agreement, DNR-planted, or private), field size in ha (log transformed), and survey date as predictors. Seed type and survey date are not in the same model because soybeans are planted after corn. Field type and Log(field size) are not in the same model because private fields are larger than public fields managed for wildlife.

Model	K	ΔAICc	Wt	Deviance
Location + seed type	4	0.00	0.79	300.2
Location + seed type + field type	6	2.67	0.21	298.8
Seed type	3	17.52	0.00	319.7
Log(field size) + seed type	4	19.39	0.00	319.6
Location + survey date	4	39.13	0.00	339.3
Location + field type	5	40.36	0.00	338.5
Location	3	51.03	0.00	353.3
Field type + survey date	5	53.06	0.00	351.2
Log(field size) + survey date	4	54.31	0.00	354.5
Survey date	3	56.58	0.00	358.8
Field type	4	58.01	0.00	358.2
Log(field size)	3	67.80	0.00	370.0

3.3. Quantifying availability of neonicotinoids on treated seeds on the soil surface

Neonicotinoids decreased on the surface of seeds quickly in both years, although initial concentrations were lower the second year after storage in an unheated outbuilding. The half-life of IMI was the longest, followed by TMX, and then CLO (Table 3). In 2016, rain fell on days 5, 6, 20, 23, 24, 26, 27, and 29 of the experiment, with sunny conditions dominating for 17 of the 30 days (Fig. 4a). In 2017, rain fell on days 12, 16, 17, 18, 19, and 24, with sunny conditions dominating on 20 of the 30 days (Fig. 4b). Concentrations exceeding 10 μg/g were present on all seeds after 16 days, and on IMI treated seeds after 30 days in 2016. We did not have a 30 day sample for CLO treated seeds in 2016 because no seeds remained on the soil surface, presumably due to wildlife consumption. In 2017, concentrations exceeding 10 μg/g were detected on IMI treated soybeans after 16 days and on CLO treated corn after 30 days. All treated seeds had low but detectable concentrations of neonicotinoids after 30 days in 2017, except CLO that was applied as a 2nd treatment on IMI-treated soybeans.

3.4. Time for wildlife to find spills

We reviewed images collected by trail cameras at simulated spills during spring 2016 ($n = 12{,}602$ videos) and 2017 ($n = 39{,}653$ videos). We documented ring-necked pheasants (*Phasianus colchicus*), Canada geese (*Branta canadensis*), American crows (*Corvus brachyrhynchos*), mourning doves (*Zenaida macroura*), wild turkeys (*Meleagris gallapavo*), blue jays (*Cyanocitta cristata*), brown thrasher (*Toxostoma rufum*), black-billed magpie (*Pica hudsonia*), rose-breasted grosbeak (*Pheucticus ludovicianus*), various species of sparrows (Emberizidae) and blackbirds (Icteridae), as well as white-tailed deer (*Odocoileus virginianus*), black bears (*Ursus americanus*), raccoons (*Procyon lotor*), rodents (mice and 3 species of squirrels), Eastern cottontails

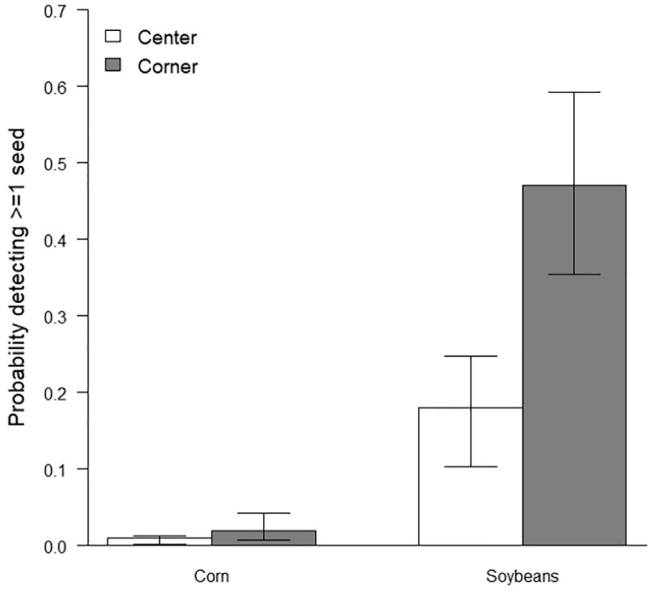

Fig. 2. The predicted probability of detecting ≥1 seed exposed on the soil surface after planting in 5 1-m^2 plots at the center and corners of corn and soybean fields in Minnesota, USA. Wheat fields were excluded due to small sample sizes ($n = 3$).

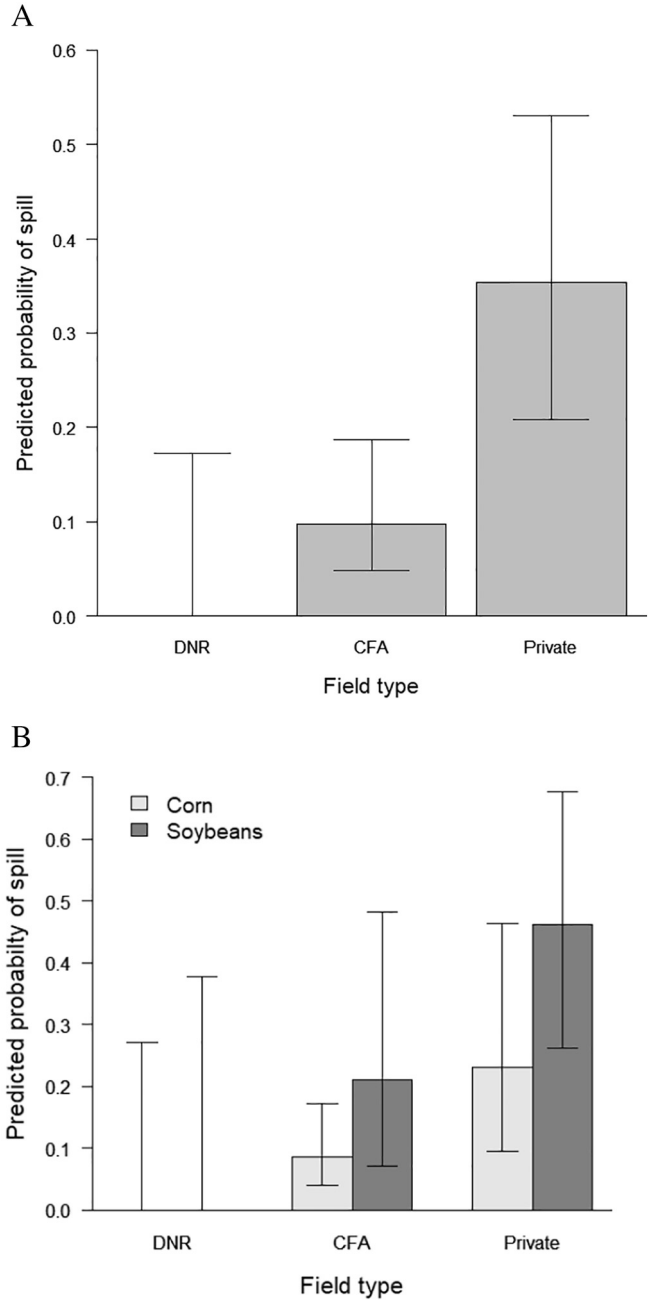

Fig. 3. a. The predicted probability of a seed spill of sufficient size to be visible from a distance occurring during farming operations in corn and soybean fields based on surveys after planting. Three field types were examined, 1) fields managed and farmed by staff of the Minnesota Department of Natural Resources (DNR), 2) publicly-owned fields farmed by private farmers with their own equipment in cooperative farming agreements (CFAs), and 3) privately-owned and privately-farmed fields. b. The predicted probability of a seed spill of sufficient size to be visible from a distance occurring during farming operations in corn and soybean fields based on surveys after planting. Three field types were examined, 1) fields managed and farmed by staff of the Minnesota Department of Natural Resources (DNR), 2) public fields farmed by private farmers with their own equipment in cooperative farming agreements (CFAs), and 3) privately-owned and privately-farmed fields.

(*Sylvilagus floridanus*), white-tailed jackrabbits (*Lepus townsendii*), red fox (*Vulpes vulpes*), striped skunk (*Mephitis mephitis*), and domestic cat (*Felis catus*) consuming treated seeds. The average time for birds to find spills (observed within 30 cm of a spill, but not necessarily consuming seeds) was 1.5 days (range 0–8 days, $n = 25$ spills) for corn, 0.9 days (range 0–3 days, $n = 18$ spills) for soybean, and 0.9 days (range 0–3 days, $n = 7$ spills) for wheat spills. The average time after a spill was established that birds were first observed to consume seeds at spills was 4.9 days (range 1–11, $n = 15$ spills) for corn, 5.0 days (range 0–11, $n = 6$ spills) for soybean, and 1.8 days (range 0–7 days, n = 6 spills) for wheat. The average time after a spill was established that mammals were first observed to consume seeds was 1.9 days (range 0–6, $n = 22$) for corn, 2.5 days (range 0–9, $n = 20$) for soybean, and 2.0 days (range 0–8, $n = 5$) for wheat.

Table 2
General linear models of binomial counts of seed spills and the predictors: seed type (corn or soybean), field type (Cooperative Farming Agreement, DNR-planted, or private), field size (log transformed), and survey date as predictors. Because corn is planted earlier than soybeans, survey date and seed type do not occur in the same models. Additionally, because privately owned fields were larger than fields on wildlife areas (DNR < CFA < private), we did not include field type and field size in the same models.

Model	K	ΔAICc	Wt	Deviance
Field type	3	0.00	0.22	48.29
Log(field size)	2	0.15	0.21	50.63
Log(field size) + seed type	3	0.21	0.20	48.50
Field type + seed type	4	0.73	0.16	46.76
Field type + survey date	4	2.17	0.08	48.20
Log(field size) + survey date	3	2.34	0.07	50.63
Seed type	2	2.83	0.05	53.31
Survey date	2	6.30	0.01	56.78

4. Discussion

We found that neonicotinoid-treated seed is common on the landscape during the spring planting season, both as seeds available on the soil surface and in seed spills. To our knowledge, this is the first study to document landscape-scale availability of neonicotinoid treated seed spills during the planting season, and the first to document availability of treated seeds on the soil surface after planting in North America. Furthermore, we also document that although neonicotinoids decrease rapidly under environmental conditions, wild birds and mammals find treated seeds at spills and consume the seeds within days, while chemical is still abundant on the seeds. Thus, wildlife may be exposed to doses of neonicotinoids that could potentially have sub-lethal or lethal effects. Our findings not only refute the idea that wild animals will not eat treated seeds, but unfortunately document that good seed stewardship practices were not always followed, despite clear warnings about dangers to wildlife on product labels.

Importantly, better seed stewardship could reduce the availability of neonicotinoid treated seeds on the landscape in the spring. We directly observed hundreds of large spills during our surveys, and estimated tens of thousands more spills occurring statewide, yet we never observed these spills being cleaned up or covered during our surveys. A seed spill large enough to be visible from the road is usually composed of thousands of seeds. Cleaning or covering spills could reduce the availability of seeds to wildlife. Chemical analysis of actual seed spills we found in our landscape-scale surveys had varying concentrations that would suggest that some spills had been left in fields for at least 5 days and not cleaned up or covered.

Outreach to farmers seems to indicate that many farmers do not read the product labels and are unaware of the dangers to wildlife (C. Roy, pers. comm.). This is borne out in our field-level sampling as well, with much higher spill rates in privately-owned, privately-farmed fields than publicly-owned fields managed by the DNR regardless of farmer (i.e., public staff or private citizen). Private farmers planting public lands with their own equipment had fewer spills than private farmers farming their own fields, which might indicate that wildlife awareness and a perceived expectation of seed stewardship may have impacted the number of spills left in fields (e.g., more careful hopper filling, refilling, or cleaning/covering spilled seed). Importantly, private farmers farming public land were blind to our study, but because we needed landowner permission to survey fields on private farms, farmers on private lands were aware of the study and our field visits. If this

Table 3
Half-lives ($t_{1/2}$), empirical rate constants (k), and equations for changes in neonicotinoid concentrations on soybean treated with imidacloprid (IMI) as the primary treatment and clothianidin as a second treatment (CLO2), and corn with either thiamethoxam (TMX) or clothianidin (CLO1). Seeds were placed on the soil surface in the environment for 30 days during May – June in 2016 and 2017 in Minnesota, USA. Equations for CLO1 in 2016 are not provided because no seeds remained on the soil surface after 30 days.

Chemical	Seed type	Seed color	$t_{1/2}$ (days)	k	Equation, R^2 both years	Equation, R^2 2016	Equation, R^2 2017
IMI	Soybean	Red	4.7	0.149	$729.66e^{-0.149x}$ $R^2 = 0.88$	$1006.6e^{-0.13x}$ $R^2 = 0.99$	$528.92e^{-0.168x}$ $R^2 = 0.97$
TMX	Corn	Red	3.6	0.193	$536.89e^{-0.193x}$ $R^2 = 0.80$	$794.66e^{-0.223x}$ $R^2 = 0.86$	$362.73e^{-0.163x}$ $R^2 = 0.76$
CLO1	Corn	Purple	2.0	0.352	$2195.8e^{-0.352x}$ $R^2 = 0.85$	NA	$689.17e^{-0.138x}$ $R^2 = 0.98$
CLO2	Soybean	Red	2.3	0.305	$39.464e^{-0.305x}$ $R^2 = 0.86$	$82.923e^{-0.354x}$ $R^2 = 0.86$	$18.782e^{-0.256x}$ $R^2 = 0.96$

awareness influenced the number of seed spills on private farms, it was not obvious in the direction we predicted (i.e., fewer spills). However, private fields surveyed in our study were larger than fields on public lands, and thus activities that lead to spills (e.g., refilling hoppers) may have been necessary more often on these larger fields. Fields with spills tended to be larger than fields without spills but the difference was not statistically significant (155.6 ± 191.1 vs 72.3 ± 137.9 ha, $t = -1.4$, $P = .09$). Regardless, we suggest that educating farmers about the importance of good seed stewardship could produce meaningful reductions in the seed available on the landscape in the spring. No spills were left on fields farmed by DNR staff, but staff were acutely aware of the dangers to wildlife and prioritized wildlife over other objectives.

Seeds available on the soil surface after planting were also very common, yet less likely to be easily improved through better seed stewardship practices. Although our estimated seed densities on the soil surface were an order of magnitude lower for corn and soybeans than that reported for winter cereals by Lopez-Antia et al. (2016) (11.3 ± 1.2 seeds/-m^2 in field centers and 43.4 ± 5.5 seeds/m^2 in the corners), they were still much higher than we anticipated with modern planting equipment for important row crops in the Midwestern United States. Winter cereal is normally standard drilled whereas corn and soybeans are precision drilled, and precision drilling produces fewer seeds on the soil surface (de Snoo and Luttik, 2004).

The differences we found in seed availability between the corners and centers of fields would support the interpretation that the equipment is more efficient at drilling seeds into the soil when moving straight along rows than when turning along the field edges, just as Lopez-Antia et al. (2016) reported for winter cereals seeds (i.e. wheat, oats, barley, and triticale seeds). de Snoo and Luttik (2004) reported the percentage of seeds on the soil surface of headlands was 3.5 times higher than in field centers for 8 different crops in The Netherlands. Innovations or farming practices targeted toward more efficient drilling of seeds at the end of rows might reduce the availability of seeds for wildlife. Importantly, many edge-dependent wildlife species (e.g., ring-necked pheasants, turkeys, white-tailed deer) tend to concentrate their activities nearer to field edges where seed is more available than in the center of fields.

Seed type was supported in statistical models of seed availability on the soil surface, with soybean being much more probable at the soil surface than corn. Corn is seeded at a lower rate than soybean; optimal corn seeding rates in Minnesota are 34,000–36,000 seeds per acre (University of Minnesota Extension Corn Seeding Rates), whereas optimal soybean seeding rates are 140,000 seeds per acre in southern MN, and 140,000–170,000 seeds per ac in central and northwestern MN (University of Minnesota Extension Soybean Seeding Rates). Corn is also planted deeper than soybean seeds (University of Minnesota Crop Production). Corn can have poor nodal root development at shallow depths, but soybean does not require deep planting for proper root development. Differences in seeding rates and planting depth between crops likely explain the difference in availability on the soil surface after planting. Spring wheat is sown at still higher seeding rates (1,300,000–1,400,000 seeds per acre) and can be planted shallowly, with deep seeding producing problems with emergence and vigor (University of Minnesota Extension Small Grain Seeding Rates). For these reasons, we would expect high wheat seed availability on the soil surface relative to corn, and our limited sample size seemed to support this.

We suspect that the probability of seeds on the soil surface was also influenced by the type of equipment used for sowing (Lopez-Antia et al., 2016), although we did not collect data on the type of planting equipment used. Planting at high speeds can also impact seed placement in the soil and increase necessary seeding rates (University of Minnesota Extension Planting Cautions). Because improper seed placement in the soil also produces fewer plants, farmers, in addition to wildlife, also benefit from proper seed placement in the soil. Nevertheless, some wildlife species [e.g, sandhill cranes (*Antigone canadensis*), greater prairie-chickens (*Tympanuchus cupido*), ring-necked pheasants] have been observed digging or pecking near the soil surface for newly planted seeds and/or foraging on new seedlings with the seed still attached (unpublished reports, numerous DNR Wildlife). Therefore, even the best seed stewardship and planting practices will not eliminate all seeds available to wildlife.

Seeds collected from spills had highly variable concentrations of neonicotinoids. Importantly, some seeds had concentrations below the detection limit, indicating that the seeds had treatments other than neonicotinoids (e.g., fungicides) for which we did not test. Furthermore, in many cases concentrations on seeds were well below the usual application rate, indicating either that the chemicals had leached off the seed surface (Smalling et al., 2018), decreased after exposure to environmental conditions, or that seeds might have been stored after purchase in previous years; chemical concentrations on seeds decreased by >20–50% in cold storage during the year between our 30-day seed experiments. Leaching of chemicals off the seed surface might occur after heavy rainfall events, however, during our 30-day seed experiments to measure changes in concentrations of neonicotinoids, rainfall events (usually drizzle or a light rain, and rarely more substantial) often occurred in between sampling dates. We did not detect a strong reduction in chemical concentrations as a result of rainfall during our experiment, however, more frequent collection of seeds would allow more precise quantification of rainfall impacts on concentrations. In summary, the factors affecting concentrations on seeds, in combination with seeds being exposed for varying amounts of time before discovery, makes it difficult to quantify doses that wildlife might ingest at actual seed spills.

Our study had several limitations that may have impacted our conclusions to some extent. First, we may have underestimated seed availability on the soil surface by not sampling at a high enough intensity. We observed many seeds on the soil surface in fields while walking to plots that were not captured by data from our 10 plots per field; 21% of surveyed fields had seeds detected incidentally outside plots but none detected within plots. We recommend future studies use a higher sampling intensity to obtain more precise estimates. Additionally, our landscape-level seed spill estimates assumed that spill rates in fields adjacent to public roads were similar to rates in fields non-adjacent to

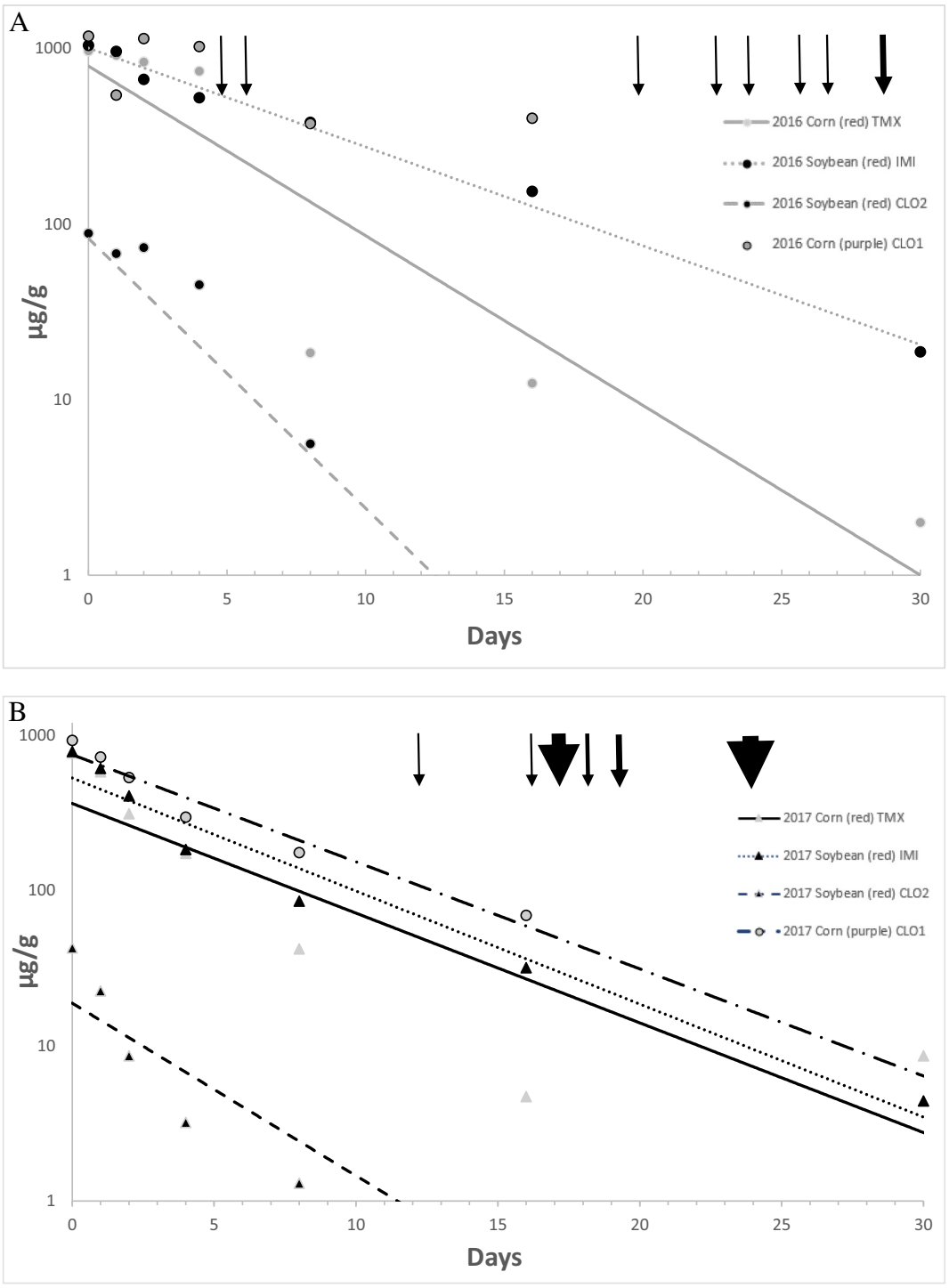

Fig. 4. a,b. Concentrations of neonicotinoid seed treatments on seeds left on the soil surface in northern Minnesota, United States for 0–30 days during May and early June 2016 (a, top panel) and 2017 (b, bottom panel). Clothianidin (CLO1) was a primary treatment on corn; imidacloprid (IMI) was a primary treatment on soybean, with clothianidin (CLO2) as a secondary treatment on the same seeds; and thiamethoxam (TMX) was a primary treatment on corn. Rainfall is indicated with arrows at the top, with drizzle and light rain indicated by a thin arrow and more substantial rain events indicated with a thick arrow. In 2017, we measured exact rainfall amounts (mm) and the amount of rainfall received (1–8 mm) is indicated by the size of the arrowhead. None of the rain events during the study would be expected to produce run-off.

public roads. We believe this assumption was reasonable because fields non-adjacent to roads are commonly accessed through privately-owned access roads, and we would expect similar spill rates near these access points because hoppers need to be refilled in these fields as well. However, if this assumption was not reasonable, then we may have overestimated the number of spills at the landscape level.

A second possible limitation was that nearly half of our cameras were placed on Wildlife Management Areas, and although we tried to select sites that were similar in landscape composition to the surrounding landscape (e.g., small WMAs in an agricultural matrix), wildlife may have been using these fields more often than other fields. If true, wildlife may have found spills on public lands more quickly than they might elsewhere. To examine this possibility, we compared the time to find spills on public land to the time to find spills on private lands. All simulated spills at both public and private fields were discovered by wildlife. Birds did find spills (defined as approaching the spill to within ~30 cm)

slightly sooner on public land than on private land (1.0 vs 1.6 days, $Z = -2.1$, $P = .03$), but the time to first consumption of seed was not different on public (3.8 days) and private land (4.5 days, $Z = -0.6$, $P = .6$, Wilcoxon-Mann-Whitney Test with package coin in R). Importantly, a delay in consumption shortly after a spill happens would make a much larger difference in the amount of neonicotinoid seed treatment remaining on seeds than a similar difference later (Fig. 4). However, when consumed, seeds left on the soil surface still had large concentrations of chemicals that could affect wildlife.

Another important limitation of our study was that knowledge of our visits may have influenced seed stewardship behavior. However, farmers with prior knowledge of our visits to examine seeds on the surface after planting were more likely to have spills, which was opposite to our concern that they would be more careful. We think it unlikely that seed stewardship behavior would be modified in the direction of waste and noncompliance with the law (i.e., FIFRA 1996), even if these farmers were cooperative with the DNR. Thus we think that despite these limitations, our study provides an important first look at neonicotinoid-treated seed availability for wildlife in the Midwestern United States.

Most of the previous research concluding that these chemicals are safe for wildlife are based on captive studies, but bird behavior in captivity does not necessarily replicate or resemble bird behavior in the wild. For example, captive red-winged blackbirds (*Agelaius phoeniceus*) given a choice between imidacloprid-treated rice and untreated rice chose untreated seeds more often (Avery et al., 1994), similar to red-legged partridges given a choice between imidacloprid-treated wheat and untreated wheat (Lopez-Antia et al., 2014), but wild birds are not presented with a side-by-side choice of food items. When red-legged partridges were presented with more unpredictable situations (i.e., more feeders to search as is more similar to field situations), treated seeds were consumed at higher rates (Lopez-Antia et al., 2014). Furthermore, red-winged blackbirds presented rice with 3 different treatment doses avoided only the highest treatment doses, but did consume imidacloprid-treated seeds at levels that produced ataxia and temporary illness (Avery et al., 1994), which Lopez-Antia et al. (2014) suggested as avoidance through post-ingestion distress in their study. Ataxia and temporary illness (Avery et al., 1993; Avery et al., 1994) to treated seed consumption could impair a bird's ability to escape predators and survive in the wild. Food availability and energy requirements are also unlikely to be similar between captive and field conditions, because wild animals must search for food, compete with conspecifics and heterospecifics, reproduce, and avoid predators. In one captive study where predators could attack but not reach red-winged blackbirds, the birds preferred to forage on treated seed nearer to cover than to forage on untreated seed farther from cover (Avery et al., 1994). In the same experiment, consumption of treated seeds was higher during colder temperatures, presumably due to increased food requirements (Avery et al., 1994). Wild birds likely have many additional factors influencing energetic requirements like reproductive behaviors, vigilance, and escape behavior that might impact their food choices.

5. Conclusions

This research provides evidence that treated seeds are consumed by wildlife, that seeds are not always drilled below the soil surface and are thus available for wildlife, and that packaging labels are insufficient to protect wildlife from seed spills. Seeds are abundant and widely available on the soil surface for wildlife consumption during the spring planting season. Soybeans were the most common seed available for consumption by wildlife on the soil surface and in seed spills, and is a seed type on which imidacloprid is still used in the United States. Imidacloprid is more toxic to vertebrate and invertebrate animals than other neonicotinoids. Corn and wheat spills were also documented in our study and due to their widespread agricultural importance and consumption by wildlife, may pose a substantial route of wildlife exposure to neonicotinoids. If the widespread-availability of treated seeds on the soil surface and in seed spills is not considered in pesticide risk assessments, they could pose a risk for sub-lethal and lethal effects to wildlife. We are exploring wildlife consumption of treated seeds in ongoing field research, but more field studies are needed.

Credit author statement

Charlotte Roy- Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, writing original draft.

Pamela Coy- data curation, formal analysis, investigation, methodology, review and editing.

Da Chen- conceptualization, funding acquisition, methodology, supervision, reviewing and editing.

Julia Ponder-conceptualization, funding acquisition, project administration, resources, review and editing.

Mark Jankowski -conceptualization, funding acquisition, review and editing.

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NEONICOTINOIDS ON THE LANDSCAPE: EVALUATING AVIAN EXPOSURE TO TREATED SEEDS IN AGRICULTURAL LANDSCAPES

Charlotte Roy, Da Chen[1], Julia Ponder[2], Mark Jankowski[2], and Pam Coy

SUMMARY OF FINDINGS

Neonicotinoid pesticides [e.g., imidacloprid (IMI), thiamethoxam (TMX), thiacloprid (THIA), clothianidin (CLO)] are commonly applied to agricultural seeds (e.g., corn, soybean, wheat, sunflower) and are known to cause lethal and sub-lethal effects in birds. Neonicotinoid-treated seeds could be available to wildlife through spillage or exposure to treated seeds near or at the soil surface after planting (de Leeuw et al. 1995, Pascual et al. 1999, Lopez-Antia et al. 2016). Using several lines of evidence, we examined sub-lethal exposure and the potential for exposure of wildlife to these pesticides in agricultural landscapes of Minnesota in 2016 and 2017. We documented exposed seeds at the soil surface in plots at 35% of 71 fields sampled after planting. We also quantified the rate of seed spills during planting season and documented 329 seed spills in the 76 townships surveyed in the spring. We documented birds and mammals eating treated seeds through field studies with trail cameras. We quantified consumption of treated seeds for 11 species of birds and 9 species of mammals, and in many cases we estimated that more than 25% of the LD_{50}—the amount of ingested substance to kill 50% of a test sample—was ingested. Seed exposure experiments conducted under environmental conditions indicated that neonicotinoids are persistent on the seed surface for as long as 30 days in the environment, so wildlife can ingest neonicotinoids on treated seeds for at least 30 days after planting.

We also conducted laboratory experiments using domestic chickens (*Gallus gallus domesticus*) to identify non-lethal and lethal sampling methods that could lead to measurement of individual- and population-level exposure, including residues in the excreta and blood of birds. Mean residue concentrations in chickens dosed in the lab were highest in the brain. In decreasing order of concentration, residues were also detected in liver, spleen, muscle, blood, kidney, then feces. Residues in chicken fecal samples collected in the lab had the highest frequency of detection in all tissues tested.

Finally, we collected field samples from prairie grouse leks and from hunter-harvested birds to evaluate whether wild birds were exposed to sub-lethal doses. Seventy-three of 82 (89%) liver samples collected from sharp-tailed grouse (*Tympanuchus phasianellus*) and 32 of 45 (71%) greater prairie-chickens (*Tympanuchus cupido*) contained concentrations above the Method

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Limit of Quantification (MLOQ) for at least 1 neonicotinoid. Similarly, 95 of 109 (87%) sharp-tailed grouse fecal pellets and 51 of 59 (86%) fresh greater prairie-chicken fecal pellets collected from leks have been analyzed and had concentrations above the MLOQ for ≥1 neonicotinoid. Most of the detected concentrations were <10 ng/g, which explains why earlier studies with higher detection thresholds than the current study concluded a more rapid clearance of neonicotinoids from vertebrates than we found. Only 3 greater prairie-chicken livers and 9 sharp-tailed grouse livers had CLO concentrations >10 ng/g, and 3 greater prairie-chicken and 7 sharp-tailed grouse livers had IMI >10 ng/g. Similarly, only 2 greater prairie-chicken pellets and 5 sharp-tailed grouse pellets had CLO >10 ng/g, and 9 greater prairie-chicken and 14 sharp-tailed grouse pellets had IMI >10 ng/g. These results show that wildlife were exposed to neonicotinoids through treated seeds, a large proportion of prairie grouse in Minnesota had quantifiable residues of neonicotinoids, and wildlife may have experienced both sub-lethal and lethal effects. Further research is necessary to evaluate individual- and population-level effects of these rates of ingestion of neonicotinoid-treated seeds.

INTRODUCTION

Neonicotinoids are the most widely used pesticides worldwide (Mineau and Palmer 2013), comprising 25% of the global agricultural chemical market. Their action is highly specific to invertebrates, with relatively low toxicities for vertebrates compared to pesticide options predating the early 1990's (Tomizawa and Casida 2005, Jeschke et al. 2011). This high specificity contributed to their widespread and rapid adoption beginning in 1994 with the registration of imidacloprid in the United States.

Recently, neonicotinoids have received a lot of attention because of their potential toxicity to bees and other pollinators and their possible role in colony collapse disorder. Several neonicotinoid treatments were banned or placed under a moratorium in Europe in 2013, and neonicotinoids are currently under registration review by the Environmental Protection Agency (EPA) in the United States. The Minnesota Department of Agriculture (MDA) recently conducted a special registration review of neonicotinoid pesticides with an emphasis on pollinators (MDA 2016). However, recent concern has not been limited to pollinators; the American Bird Conservancy called for research on the effects of neonicotinoids on birds and a ban on neonicotinoid seed treatments (Mineau and Palmer 2013). Evidence is accumulating that vertebrates are also adversely affected by these pesticides (see reviews in Mineau and Palmer 2013, Gibbons et al. 2014). MDA (2014) acknowledged that, "Although neonicotinoids are less toxic to vertebrates than to arthropods, direct consumption of neonicotinoid treated seeds may expose birds and other taxa to acute or chronic doses."

The most likely route of exposure to large doses of neonicotinoids for birds is ingestion of treated seeds (Goulson 2013, Gibbons et al. 2014), although numerous other mechanisms exist (e.g., soil, trophic transfer; SERA 2005, Douglas et al. 2015). Ingestion of a small number of neonicotinoid-treated seeds is lethal to birds; for example, a single treated corn kernel can kill a blue-jay sized bird (see reviews in Mineau and Palmer 2013, Gibbons et al. 2014). However, toxicity generally varies by chemical and species, given differences in genetic and physiological factors including size, metabolic, and digestive processes. Lethal impacts are rapid and difficult

to detect in the wild although a few pesticide poisoning incidents have been detected (Greig-Smith 1987, Fletcher et al. 1995, Berny et al. 1999, de Snoo et al. 1999). Sub-lethal exposure might be easier to detect in the wild than lethal exposure if mortality events are relatively small and carcasses rapidly removed by scavengers. Sub-lethal effects in birds in the lab include hyporeactivity, lack of coordination, wing drop, immobility, disruption of migratory coordination, eggshell thinning, reduced egg hatching rate, impaired testicular function, and low weight in chicks (Cox 2001, Lopez-Antia et al. 2013 and 2015, Tokumoto et al. 2013, Mineau and Palmer 2013, Eng et al. 2017). Avian reproduction can be affected by consumption of just 1/10th of a treated corn seed per day during egg-laying (Mineau and Palmer 2013).

Thirty bird species were observed picking up treated seeds from cereal fields in Spain, and 3.1% of red-legged partridge (*Alectoris rufa*) gut contents collected by hunters tested positive for imidacloprid after planting of winter cereal crops (Lopez-Antia et al. 2016). Dead and poisoned partridges have been found in agricultural fields in France following use of imidacloprid-treated seed (Berny et al. 1999). The EPA estimated that ~1% of seeds remain accessible to granivores after planting (as reported by Goulson 2013, Lopez-Antia et al. 2015). Use of neonicotinoid "treated articles," such as seed, is not currently tracked by the U.S. government due to the exemption in 40CFR §152.25(a). Yet, almost all corn planted in the Midwestern U.S. has been treated with these pesticides (Stokstad 2013); most soybean, wheat, and sunflower seeds are treated also; and neonicotinoids are widely used with other application methods for other crop types.

Studies of neonicotinoid effects on vertebrates are overwhelmingly laboratory-based (91% of studies), which limits our ability to interpret the significance of findings in more natural settings (Gibbons et al. 2014). Higher densities of exposed seeds generally result in greater attraction of birds to fields (Murton et al. 1963, Feare et al. 1974). Bednarska et al. (2013) identified a need for feeding rate information in the field to allow extrapolation of lab data to the field. Lopez-Antia et al. (2013) pointed to a "need for evaluation of real exposure to coated seed ingestion by wild birds, including feeding behavior analyses and estimation of food intake rates." Despite these calls for field studies and the time elapsed, the information deficits identified have still not been sufficiently addressed. Importantly, the U.S. still lags behind Europe (Berny et al. 1999, Lopez-Antia et al. 2013, 2016) in field-based studies focused on neonicotinoids and wildlife. We are therefore conducting a study to determine whether wild birds are exposed to neonicotinoid-treated seeds in agricultural landscapes in Minnesota. Preliminary data from our ongoing studies are reviewed below.

OBJECTIVES

The overarching objective of our research was to ascertain whether birds are exposed to neonicotinoid-treated seeds in agricultural landscapes. Specifically, we aimed to:

1- Quantify the rate of seed spillage and surface seed exposure after planting within fields.
2- Identify birds consuming neonicotinoid-treated seeds and quantify consumption per foraging bout.
3- Quantitatively link exposure and chemical residues in tissue, blood, and excreta to neonicotinoid concentrations in chickens (lab study).
4- Determine whether neonicotinoid exposure in wild prairie grouse can be detected from non-lethal sampling methods or from hunter harvested birds.

STUDY AREA

We conducted the field portions of our study in agricultural regions of Minnesota. Most field components were conducted in the agriculturally-dominated western portion of the state including the quantification of actual seed spills (Figure 1a), seeds on the soil surface and seed consumption at simulated seed spills (Figure 1b) in the spring. Field samples of prairie grouse came from the northwestern part of this region and also the east-central part of the state where agriculture was present but comprised a smaller proportion of the landscape (Figure 1c).

METHODS

Quantifying Seed Spills

All chemically treated seeds (e.g., neonicotinoids, fungicides, other pesticides) are unnaturally colored, as mandated by the Federal Seed Act. These seeds are highly visible and easily identified by their unusual color (e.g., pink, blue, green, purple), which is used to prevent accidental feeding to livestock. We quantified the frequency of actual seed spills on the landscape by inspecting fields with visual access from roads, field access points, and roadsides in agricultural areas. We hoped to avoid bias in spill rates that might result from obtaining permission to access privately-owned fields on foot, but this method makes the implicit assumption that spill rates associated with refilling and overfilling hoppers is similar for fields that are adjacent to roads and fields that are not adjacent to roads.

We identified 211 townships in the western third and southeastern part of the state with ≥50 miles of roads and ≥50% of the area in corn, soybeans, and/or wheat production using the Department of Transportation (DOT) Roads Layer (DOT 2008) and 2014 Cropland Data Layer (USDA-NASS 2015), respectively, in ArcGIS. These criteria were used to select townships with visual access to fields from roads while also not being so restrictive that the spatial distribution of the sample was constrained. We drew a spatially balanced sample of 50 townships and surveyed the 38 most western townships selected due to a later start to planting during the spring of 2016. In 2017 we selected 50 different townships and again surveyed the 38 westernmost townships due to a late start to planting. We surveyed a total of 76 townships during the 2 years of the study. We began in the southern counties and worked north beginning in late April as crops were planted.

We recorded locations and approximate number of seeds in spills near *recently planted* fields with the DNRSurvey mobile computer application. Documenting only *recently planted* fields allowed for control in temporal variation in the timing of planting. For example, a field that has not been planted yet will not have a spill at the time of sampling, which is different from a spill not occurring during planting. Thus, by only including recently planted fields in our estimates, we measured spills during planting. We defined a “field” as a quarter of a quarter-section (i.e., 40 acres). We recorded each quarter of a quarter-section in agricultural production, whether any part of it was recently planted (i.e., before early seedling stage), documented the amount (number of seeds) of spilled seed on the road, field edge, or visible in the field, and crop type

(when possible). To determine the proportion of seed spills that contained neonicotinoid-treated seed, we collected seeds from accessible spills (e.g., along public roads and rights-of-way) and quantified 7 neonicotinoids (Chen et al. 2014).

Quantifying Seeds on the Soil Surface

To estimate the amount of seed at the soil surface after planting, we used a 1-m^2 frame to define plots in recently planted fields and counted all treated seeds visible within the frame after planting (Lopez-Antia et al. 2016). We sampled 5 plots in a field corner and 5 plots in the field center as estimated visually from field boundaries while standing in the field. For corner locations we randomly selected 1 field corner per field by flipping a coin twice and paced 15 m and 30 m along each edge in an L shape that had the field corner for a vertex for a total of 5 measurements (i.e., 1 plot at vertex, 2 plots at 15 m, and 2 plots at 30 m). This approach incorporated sampling parallel and perpendicular to planting rows, and we suspected that seed exposure would be greater at the end of rows where planters turn sharply than within rows. For field centers we paced 15 m in each cardinal direction to sample for a total of 5 measurements, including the center.

In 2016, we sampled 36 fields on DNR-managed Wildlife Management Areas (WMAs) that were farmed by private individuals under contract through Cooperative Farming Agreements (CFAs), 2 privately farmed fields on private land where we had permission, and 10 fields farmed by DNR staff on WMAs. In 2017, we sampled 6 privately farmed fields in CFAs and 17 privately owned and farmed fields with landowner consent. During 2017, neonicotinoid-treated seed was not permitted on WMAs. When seeds were exposed, we could determine whether they were treated; however, we did not dig up seeds for confirmation. In 4 cases, 2 fields were known to be planted by the same farmer, but in 3 cases, the fields were planted to different crop types, with different planting equipment used for each crop type in 2 of 3 cases where equipment type used was known.

Quantifying Decay of Neonicotinoids on Treated Seeds on the Soil Surface

To determine how long neonicotinoids persist on the seeds left on the soil surface we distributed hundreds of seeds on the soil surface of a tilled field near Bemidji to experience UV, microbial, rainfall, and other ambient conditions. After environmental exposure for 0, 1, 2, 4, 8, 16, and 30 days, we collected 5-7 seeds of each type to quantify decay of neonicotinoids under environmental conditions. We recorded daily precipitation and cloud cover during the experiment. We conducted the experiment in 2016 with 2 types of commercially available corn seed treatments (CLO and TMX) and commercially treated soybeans (IMI). In 2017, we repeated the experiment, but also put out wheat seeds (CLO, but the seed treatment was applied locally rather than through an industrial application). After field collection, seeds were stored frozen until shipping to a laboratory at Southern Illinois University Carbondale (SIUC) for neonicotinoid analysis.

Documenting Consumption of Treated Seeds

In 2016, we selected 12 WMAs to place trail cameras to observe wildlife consuming seeds at simulated spills in planted fields. The available data on CFAs on DNR-managed land indicated 7,420 acres (3,003 ha) of row crops in 341 CFAs in Region 4 (southern region) and 2,431 acres (984 ha) of row crops in 66 CFAs in Region 1 (northwest region; M. Benage and J. Williams, respectively, pers. comm.). We selected WMAs with a land cover composition similar to that of the surrounding landscape using the 2014 National Cropland Data Layer (USDA-NASS 2015) in ArcGIS 10.2 (ESRI 2015). Working on WMAs minimized bias in farming activities that might result from prior knowledge of the study. Furthermore, neonicotinoid-treated seed has been commonly used by private farmers on WMAs and many DNR managers reported difficulty finding seeds that had not been treated. We prioritized this portion of the study in 2016 because farmers and managers were prohibited from planting neonicotinoid-treated seeds on WMAs beginning in 2017.

Camera locations were selected to minimize risk of theft and to view a recently planted field to document foraging at a simulated seed spill and on exposed or submerged seeds or seedlings. In 2016, spills were simulated with 1000 corn (n = 15 spills) or soybean seeds (n = 2 spills) to allow determination of the time it takes for birds to discover spills and the number of seeds consumed in each foraging bout by individual animals. Additionally, we placed cameras at 2 fields on privately-owned land where we had obtained permission. Cameras were deployed in each location for 3–6 weeks after planting. At each field, 2 motion-activated cameras were deployed—1 that captured 1 image/sec in still photos and 1 that captured 1 min of video when triggered by motion. The camera set for still photos also took photos at 5-min intervals between 0600–0800 hr and 1830–2030 hr to document birds foraging in fields during sunrise and sunset periods during the planting season. Images were examined to identify species of wildlife consuming seeds and the number of seeds consumed per foraging bout.

In 2017, we included more privately-owned fields, which were generally larger than fields planted on WMAs. We placed 1 camera at each of 24 privately-owned fields in addition to placing cameras at 16 WMAs. We simulated 20 more corn spills, 23 soybean spills, and 9 wheat spills of 1000 seeds each. Instead of capturing still images at simulated spills, which often produced ambiguous information about whether seeds were ingested, we instead set the cameras to record video only. Cameras were programmed to capture a 1 min video whenever the motion sensor was triggered. We checked cameras once weekly to replace batteries and data cards and deployed cameras in each location for 2–3 weeks. When we checked simulated spills, we restocked with an additional 1000 seeds of the same seed type if 25-50% of the seeds remained but switched to a different seed type (after removing any remaining seeds) if <25% remained.

Linking Field and Laboratory Exposure Concentrations in Birds

We quantitatively linked field sample concentrations to laboratory exposure concentrations through work with University of Minnesota-College of Veterinary Medicine (UMN-CVM) and SIUC. We determined how many days post-exposure that imidacloprid (i.e., the most common seed treatment in Minnesota, J. Zachmann, MDA, pers. comm.) was detectable in both non-lethally and lethally collected samples from dosed birds. A non-lethal method to determine sub-

lethal exposure would facilitate data collection during spring planting when spills would be expected to be most numerous.

At UMN-CVM, domestic chickens (*Gallus gallus domesticus*) were orally exposed to imidacloprid (IMI) for 7 days and serially sampled during and after the course of exposure to simulate repeated sub-lethal exposures. Chickens served as our model species given their suitability to captivity and close taxonomic relationship with wild grouse (Family Phasianidae). Small sample sizes are commonly used in dosing studies because the differences among treatment groups are expected to be very large and variability within groups low (e.g., Berny et al. 1999, Bednarska et al. 2013). We exposed chickens (n = 5) to 1%, 5%, and 20% of the LD_{50} (104.1 mg/kg IMI, Kammon et al. 2010) daily for 7 days by giving ~1.5 kg birds a daily IMI bolus of 1.04 mg/kg/day, 5.20 mg/kg/day, and 20.80 mg/kg/day (i.e., low, medium, and high dosage, respectively). The LD_{50} is the single dose that is expected to be lethal to 50% of test subjects. The LD_{50} would be reached if chickens ingested ~260–946 corn seeds (depending on application rate to seeds, which varies among seed companies). Stated differently, 3–10 seeds is comparable to the low, or 1%, LD_{50} dose. Thus, these were realistic doses. Prairie grouse (0.6–1.2 kg) are smaller than chickens and thus smaller doses (e.g., 104–780 seeds for the low-dose treatment, depending on bird weight) would be expected to produce similar results. Other neonicotinoids have a higher LD_{50} than IMI, so lethality would be expected at much higher seed ingestion levels for those pesticides.

The full laboratory experiment was completed only for chickens in the low- and medium-dosage groups because chickens in the high-dosage group were humanely euthanized on day 1 due to severe neurological and respiratory depression. Prior to exposure, baseline blood and excreta samples were collected. Sequential blood and excreta samples were collected on experiment days 1–21. Blood samples were collected at 0, 8, and 24 hours post-exposure and then on days 8, 14, and 21 post-exposure. Chickens that were considered at endpoint and euthanized had blood samples taken immediately before euthanasia. The low-dosage group was sampled for feces 1 day earlier than the medium group due to logistical challenges. Samples of internal organs (i.e., brain, kidney, liver, spleen) and muscle were taken from chickens that died during the treatment period or on day 21, whichever came first. Chickens were weighed on all days of sampling. Samples were sent to SIUC for residue analysis (Chen et al. 2014).

Descriptive statistics and graphing of the available data from these lab studies were performed to gain a preliminary sense of how IMI concentrations changed over time and in response to dose on a tissue-specific basis. According to best practices, we used geometric rather than arithmetic mean for chemical concentration data, which are typically lognormally distributed. Arithmetic mean is often biased high. Further statistical analyses will be conducted once we obtain the full dataset, including metabolites (i.e., neonicotinoids modified through metabolic processes) and feed concentrations.

Detecting Neonicotinoids in Free-Ranging Birds

We also collected samples from wild birds using both invasive and non-invasive methods to identify ways to assess exposure to neonicotinoids in the field. Fresh fecal pellets and blood

samples from trapped prairie grouse were collected during lek visits for a genetic study in spring 2015 and again in 2017 for this study. Samples were stored frozen until shipped to the lab at SIUC. Hunters also voluntarily submitted harvested prairie grouse in fall 2015, 2016, and 2017. Tissues and fecal pellets are being tested for thiacloprid (THIA), acetamiprid (ACE), thiamethoxam (TMX), IMI, clothianidin (CLO), dinotefuran (DIN) and nitenpyram (NTP).

DNR staff also assisted with lethal collections of granivorous birds observed foraging on treated seeds in the spring of 2016 under federal permit MB682323-0 issued to DNR. We are examining exposure to neonicotinoids using ingesta and tissue residue levels according to Chen et al. (2014) at SIUC.

RESULTS

Quantifying Seed Spills

We observed 212 large seed spills that were visible from the road during surveys in 2016 and 117 spills during surveys in 2017. However, we missed the peak of planting in many of the townships surveyed because both the springs of 2016 and 2017 were very wet and crops were planted later than usual. Planting in 2017 was later than in 2016, and we observed standing water in many fields during the spring planting season. At the time of our road-based surveys in 2016, 79,386 acres of corn, 82,341 acres of soybeans, 76,895 acres of wheat, and 21,427 acres of other crops were planted in the areas surveyed, amounting to 60.5% of the acres surveyed having been planted at the time of our survey. Spill rates in the areas surveyed were calculated as 4 spills/10,000 ac corn, 15 spills/10,000 ac soybeans, 6 spills/10,000 ac wheat, and 15 spills/10,000 ac other crop types. In 2017, 40,110 acres of corn, 23,556 acres of soybeans, and 33,749 acres of wheat, and 14,957 acres of other crops were planted during our surveys, or 23% of acres surveyed were planted at the time of our survey. Spill rates of 2 spills/10,000 ac corn, 27 spills/10,000 ac soybean, 7 spills/10,000 ac wheat planted were calculated. Extrapolating statewide requires the assumption that spill rates visible in fields adjacent to roads are representative of spill rates in fields located elsewhere. If spills near roads are more likely to be cleaned up than those less visible to passersby, then this assumption may not be tenable. Yet, we did not observe spills being cleaned up during our surveys. Furthermore, most spills occur during hopper refilling, and this often occurs near field access points along roads. Thus we think our assumptions are reasonable. Applying our spill rates across the acres farmed statewide (8,450,000 acres of corn, 7,550,000 acres of soybeans, and 1,321,000 acres of wheat were planted in Minnesota during 2016 [National Agricultural Statistics Service (NASS); last accessed 5 June 2017 National Agricultural Statistics Service], we estimate nearly 15,000 large seed spills statewide in 2016 and expect that if there is a bias, our estimates are biased low. In 2017, 8,050,000 ac of corn, 8,150,000 acres of soybeans, and 1,160,000 acres of spring wheat were planted (NASS; last accessed 5 March 2018 National Agriculture Statistics Service), which extrapolates to ~25,000 spills during the planting season. Spills increased as we moved from south to north, and the proportion of fields planted during our surveys also increased as we moved south to north.

Quantifying Seeds on the Soil Surface

We documented exposed seeds at the soil surface in plots in 25 of the 71 fields where we sampled 10 1-m^2 plots in 2016 and 2017, and when areas outside plots were included, 40 fields had exposed seeds at the soil surface (Table 3). Seeds were exposed in $\geq$1 centrally located plot in 20% of fields measured. Exposed seeds were detected in $\geq$1 corner plot of 30% of fields measured. The quantity of exposed seeds on the surface of fields was 0.47 seeds/m^2 (range: 0-69) in the center of fields and 0.77 seeds/m^2 (range: 0-51) in the edges of fields, which is an order of magnitude lower than that reported by Lopez-Antia et al (2016). Most (72%) of the fields we measured were planted to corn, 24% were planted to soybeans, and 4.2% were planted to wheat (Table 4). Most (73%) sampled fields were on public land but 81% of the sampled fields on public land were planted by private cooperating farmers with their own equipment. We suspect that spill rates are influenced by the type of equipment used for sowing (Lopez-Antia et al. 2016) and the seed type.

Quantifying Decay of Neonicotinoids on Treated Seeds on the Soil Surface

Neonicotinoids decayed on the surface of seeds relatively quickly, but concentrations exceeding 10 ng/g were present on all seeds after 16 days, and on IMI treated seeds after 30 days (Figure 2). We did not have a 30 day sample for CLO treated seeds because no seeds remained on the soil surface after 30 days, presumably due to wildlife consumption because the seeds were not removed from the tilled field by people.

Documenting Consumption of Treated Seeds

We reviewed images collected by trail cameras at simulated spills during spring 2016 (*n* = 188,399 photos and 12,602 videos) and 2017 (n = 39,653 videos). We documented ring-necked pheasants (*Phasianus colchicus*), Canada geese (*Branta canadensis*), American crows (*Corvus brachyrhynchos*), mourning doves (*Zenaida macroura*), wild turkeys (*Meleagris gallapavo*), blue jays (*Cyanocitta cristata*), brown thrasher (*Toxostoma rufum*), rose-breasted grosbeak (*Pheucticus ludovicianus*), various species of sparrows (Emberizidae) and blackbirds (Icteridae), as well as white-tailed deer (*Odocoileus virginianus*), black bears (*Ursus americanus*), raccoons (*Procyon lotor*), rodents, Eastern cottontails (*Sylvilagus floridanus*) and white-tailed jackrabbits (*Lepus townsendii*) consuming treated seeds. Consumption rates (seeds/min), the number of seeds eaten per 1 min video, and the total seeds eaten by an individual in consecutive videos are indicated in Table 1.

To estimate the toxicity of consuming neonicotinoid treated seeds, we estimated species-specific LD_{50} concentrations using standard metabolic scaling procedures (EPA T-REX[3]) with estimated toxicity values for surrogate species, the mass of surrogate species, and product-labeled concentrations of chemical on a treated seed (in mg/seed; Bayer Crop Science and Syngenta). Toxicity values (LD_{50} in mg/kg-bw) for surrogate species were acquired from EPA draft risk assessments or other documents (DeCant and Barrett 2010, Anon 2012, EPA_HQ-OPP-2011-0865-0242, EPA-HQ-OPP-2008-0844-1256, EPA-HQ-OPP-2011-0581-0093) to

[3] EPA T-REX guide

create the potential toxicity assessment (Table 2) for species observed consuming treated seeds in images. These metrics are useful for the assessment of risk in birds and mammals. In summary, potential exposure concentrations were much closer to estimated LD_{50} concentrations for birds than mammals.

Linking Field and Laboratory Exposure Concentrations in Birds

We collected 72 blood samples; 100 fecal samples; 15 samples of muscle, brain, liver, and kidney; and 103 eggs during laboratory IMI exposures of chickens. Based on a detection limit of 0.10 ng/g, IMI was detected more frequently and for a longer duration post-exposure in fecal samples (90.9%, $\leq$21 days post exposure) than blood (32.9%, $\leq$7 days post exposure; Table 5). Blood concentrations increased from the first samples taken at the start of the experiment (hr 0) to hr 8 and declined again at hr 24 (Figure 3); after this time, samples did not contain detectable IMI except for 1 sample taken on day 8. Fecal IMI concentrations followed a 3rd order polynomial pattern, increasing from the start of the experiment (day 0) until approximately day 6, decreasing until day 18 and holding steady or slightly increasing by day 21 (Figure 4). As expected, the low dose group tended to exhibit lower IMI fecal concentrations than birds in the medium dose group. IMI was rapidly removed from blood, but the change in concentrations varied 17,234-fold (c.f., 279-fold in feces; fold change is maximum detected concentration/minimum detected concentration across all groups and times), and thus blood may provide a more sensitive indicator of an acute exposure than feces. By contrast, fecal samples provided a more integrated, longer, and more consistent detection in exposed birds (Figure 3) and thus may be more applicable to field applications where time from chemical exposure will be more variable.

IMI was measured in internal organs which were collected on the final day of the experiment, depending on when birds were euthanized (Figure 5). Low- and medium-dosed birds were euthanized on day 21, whereas high-dosed birds were euthanized after showing clinical signs of distress on day 1. Detection frequency of IMI was highest in kidney, liver, and spleen (73.3%), although muscle and brain also exhibited similar detection frequencies (66.7%). Geometric mean tissue concentrations were highest in brain and lowest in the kidney (Table 6).

For analytical method quality assurance and control, we used matrix spiked recovery tests, procedural blanks, and recoveries of surrogate standards. IMI (25 ng) was spiked into muscle (n = 5) or blood (n = 5) and analyzed. Mean (± SD) recoveries were 86.7 ± 5.8% and 90.9 ± 4.9% in tissue or blood, respectively. One procedural blank was processed for every 10 samples, and no target compound was detected in any blanks. Good analytical performance was indicated by surrogate standards with recoveries ranging from 75% to 98%. Similar methods were used for THIA, ACE, TMX, and CLO and the method limit of quantification was calculated by multiplying the standard deviation from replicates with a Student's t-value appropriate for a 99% confidence level. Thus, the method limit of quantification (MLOQ) for IMI was 0.3 ng/g in tissue and 0.4 ng/mL in blood, for THIA was 0.7 ng/g and 0.6 ng/mL, for ACE was 0.7 ng/g and 0.8 ng/mL, for TMX 0.8 ng/g and 0.8 ng/mL, and for CLO was 0.7 ng/g and 0.7 ng/mL in tissue and blood respectively, Minimum detectable concentrations were lower and ranged 0.1–0.3 ng/g for the 5 neonicotinoids, but we took a more conservative approach for reporting and interpretation.

Detecting Neonicotinoids in Free-ranging Birds

Field-collected prairie grouse samples sent for neonicotinoid analysis included 61 sharp-tailed grouse fecal pellet groups and 34 greater prairie-chicken fecal pellet groups collected in 2015, and 46 and 27 pellet groups, respectively, in 2017 (no sample collection occurred in 2016). We also collected 5 blood samples from trapped sharp-tailed grouse, as well as 2 brains and 3 breast muscles from sharp-tailed grouse for which we had whole carcasses and sent them for neonicotinoid analysis. Hunters submitted livers from 11 prairie-chickens, 22 sharp-tailed grouse, and 3 prairie-chicken/sharptail hybrids during fall 2015, 17 prairie-chickens, 33 sharp-tailed grouse, and 2 pheasants during fall 2016, and 17 prairie-chickens and 27 sharp-tailed grouse during fall 2017.

Seventy-three of 82 (89%) livers collected from hunter-harvested sharp-tailed grouse, 32 of 45 (71%) greater prairie-chicken livers, and 3 of 3 sharptail/prairie-chicken hybrids from hunter-submitted samples had concentrations above the MLOQ for at least 1 neonicotinoid. Three of 3 blood samples analyzed tested negative for neonicotinoids. Dinotefuran and NTP were not detected in any samples. Neonicotinoids above the MLOQ in prairie-chicken livers included IMI (64%), CLO (27%), and THIA (2%) and in sharp-tailed grouse livers included IMI (79%), CLO (37%), THIA (5%), and ACE (1%). Maximum concentrations of neonicotinoids in prairie-chicken livers were 22.0 ng/g IMI, 15.0 ng/g CLO, and 1.1 ng/g THIA. (Note that ACE and TMX were reported in a previous report, but detected concentrations were below the MLOQ; 0.21 ng/g, ACE, and 0.43 ng/g TMX). Maximum concentrations detected in livers of harvested sharp-tailed grouse were 84.5 ng/g IMI, 21.0 ng/g CLO, 1.18 ng/g THIA, 0.71 ng/g ACE, and 0.5 ng/g TMX, again with TMX below the more conservative MLOQ. Similarly, 51 of 59 (86%) fresh prairie-chicken fecal pellets and 95 of 109 (87%) sharp-tailed grouse pellets collected from leks during springs of 2015 and 2017 contained concentrations above the MLOQ for at least one neonicotinoid. The most commonly detected neonicotinoid in the greater prairie-chicken fecal pellets was IMI (51%), followed by CLO (37%), and THIA (3%). Acetamiprid and TMX were not detected in feces, perhaps due to differences in the way they are metabolized or excreted. Maximum concentrations of IMI, CLO, and THIA in feces were 14.0 ng/g, 44.8 ng/g, 1.05 ng/g, respectively. In sharp-tailed grouse pellets, neonicotinoids above the MLOQ were IMI (62%), CLO (40%), and THIA (4%). Maximum concentrations were 39.7 ng/g IMI, 32.3 ng/g CLO, 0.9 ng/g THIA, with ACE and TMX below the MLOQ (0.2 ng/g and 0.5 ng/g, respectively). However, most of the detected concentrations were <10ng/g, which is below the detection limit in tissues in some other laboratories. Only 3 greater prairie-chicken livers and 9 sharp-tailed grouse livers had CLO concentrations >10ng/g, and 3 greater prairie-chicken and 7 sharp-tailed grouse livers had IMI>10ng/g. Similarly, only 2 greater prairie-chicken pellets and 5 sharp-tailed grouse pellets had CLO >10ng/g, and 9 greater prairie-chicken and 14 sharp-tailed grouse pellets had IMI >10ng/g.

Birds collected while foraging on treated seeds included 1 ring-necked pheasant, 5 red-winged blackbirds (*Agelaius phoeniceus*), 2 yellow-headed blackbirds (*Xanthocephalus xanthocephalus*), 4 brown-headed cowbirds (*Molothrus ater*), and 5 common grackles (*Quiscalus quiscula*). Two brown-headed cowbird livers tested positive for exposure to IMI and

CLO. One yellow-headed blackbird liver tested positive for IMI. Livers of all other birds collected while foraging on treated seeds tested negative for recent neonicotinoid exposure, indicating that this was either their first exposure or that previous exposures were not recent enough to detect.

DISCUSSION

We found that neonicotinoid-treated seed is common on the landscape during the spring planting season, both on seeds available on the soil surface and in seed spills. We also documented numerous avian and mammalian species consuming treated seeds at simulated spills, some of which ingested amounts that would be expected to produce lethal and sub-lethal effects. Samples obtained from wild birds during the fall hunting season also indicated recent exposure in a large proportion of harvested birds, which is consistent with consumption of treated seeds during planting of winter wheat in September and October in Minnesota. Indeed, several of the hunter-submitted sharp-tailed grouse carcasses contained wheat. These findings indicate a need for much more study into the exposure rates of wildlife to neonicotinoids. Population-level effects are possible based on the consumption rates, availability of treated seed, and persistence of neonicotinoids on seeds under environmental conditions that we observed. Thus, lethal and sub-lethal effects should receive more attention in wild populations, especially in granivorous species that consume seeds as part of their diet.

Field studies on neonicotinoids in vertebrates have been infrequent to date, in part due to methodological obstacles for field detection and in part due to the difficulty of isolating variables in field settings where variables cannot be easily controlled. We identified several methodological options that can be applied in field situations, including detection of residues in feces and tissues. Notably, fecal samples provide a non-invasive means to detect exposure in birds, which can be especially important for species of concern. Fecal samples also could be collected from the GI tract of live birds or from hunter-killed birds. For game species and more common species, internal organs like livers can also serve as an indicator of neonicotinoid exposure in lethal collections and livers are fairly easy for non-specialists to locate. Berny et al. (1999) reported that liver and kidney had the most consistent imidacloprid concentrations in fatally exposed wild birds, whereas crop and gizzard provided inconsistent concentrations. However, Lopez-Antia et al. (2015) reported that imidacloprid could be consistently detected in crops and livers of dosed partridges (*Alectoris rufa*). We had few ingesta samples, but our results also indicated that liver and kidney provide more consistent imidacloprid concentrations than other tissues.

Previous studies have demonstrated that neonicotinoids (e.g., thiamethoxam) are excreted primarily through the kidneys in mammals (Bednarska et al. 2013, Tomizawa and Casida 2005). Ongoing analytical work to measure metabolites of imidacloprid in feces and the uric acid wash in birds is expected to provide a more sensitive (i.e., higher fold concentration change) assay than current parent compound (i.e., imidacloprid unmodified by metabolic processes) data. Further work will be required to quantify how the potential environmental imidiacloprid exposure scenarios (concentration, duration, and frequency) influence the detection of parent compound and metabolites in feces and the uric acid wash in birds. Refining non-invasive collection is

necessary because UV light can and microbial degradation may degrade neonicotinoids (Lu et al. 2015; Lu et al. 2016; Ma et al. 2014). Thus pellet freshness is an important consideration. Most studies have suggested a rapid metabolism and elimination (~48 hours) of parent (i.e., unchanged) compound in the urine after *single* oral doses (Bednarska et al. 2013; Tomlin 2004). Other studies have had 10-fold lower detection thresholds in tissues, which explains the discrepancy between our study and others.

The highest concentration of IMI detected in livers of harvested prairie grouse (84.5 ng/g) was higher than that of chickens in the low and medium dose group at the end of the experiment. However, it was lower than the high LD_{50} group after early euthanization. Similarly, the highest concentration of IMI detected in field-collected feces (39.7 ng/g) was consistent with the 1% dose group, lower than the 5% dose groups within 3 days of exposure, and was generally higher than both dose groups 2 weeks post-exposure, although samples varied substantially. We cannot know if this indicates a higher initial exposure or how much the passage of time since exposure might have reduced these levels, but given that 1% LD_{50} (1.04 mg/kg) is comparable to the dose received after consuming 3–10 corn seeds and that IMI can be detected in tissues for as long as 21 days post-exposure, we consider it likely that this finding reflects a high initial exposure to IMI.

This research provides evidence contrary to several popularly held beliefs that wildlife do not eat treated seeds because they are unpalatable, that seeds are always drilled below the soil surface and are thus not available for wildlife, and that packaging labels are sufficient to protect wildlife from harmful effects. We encourage other researchers to replicate our study, and to pursue additional field studies of wildlife, to ensure that objective data are available to evaluate the risks of neonicotinoids to wildlife.

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Table 1. Birds and mammals documented eating seeds at simulated spills in Minnesota during 2016 and 2017 by seed type (corn, soybean, & wheat in separate sections of the table). Consumption rates (seeds consumed/min), the range of seeds consumed in 1 min videos, and the maximum amount of seeds consumed by an individual in consecutive videos.

Species	Scientific name	Corn Consumption Rate (seeds/min)	Sample size	Range (seeds eaten per 60 s video)	Max seeds eaten per feeding bout
Common grackle	*Quiscalus quiscula*	3.2	27	1-5	5
Blue jay	*Cyanocitta cristata*	27.7	4	2-4	6
Ring-necked pheasant	*Phasianus colchicus*	15.3	9	1-21	21
Red-winged blackbird	*Agelaius phoeniceus*	1.9	28	1-6	6
Brown thrasher	*Toxostoma rufum*	2.46	5	1-3	3
American crow	*Corvus brachyrhynchos*	28.1	16	1-24	24
Black-billed magpie	*Pica hudsonia*	12	1	2	2
Wild turkey	*Melagris gallapavo*	174.2	2	1-150	150
White-tailed deer	*Odocoileus virginianus*	54.2	8	5-111	650
13-lined ground squirrel	*Ictidomys tridecemlineatus*	7.7	24	1-13	22
Raccoon	*Procyon lotor*	11.9	32	4-21	268
Eastern cottontail	*Sylvilagus floridanus*	3.1	14	1-6	35
White-tailed jackrabbit	*Lepus townsendii*	3.8	5	3-5	43
Eastern gray squirrel	*Sciurus carolinensis*	3.1	4	1-4	23
Fox squirrel	*Sciurus niger*	3.3	9	2-6	48
Striped skunk	*Mephitis mephitis*	13	1	13	13
Red fox kit	*Vulpes vulpes*	2.1	5	1-3	3
Red fox adult	*Vulpes vulpes*	n/a	2	1-2	2

Species	Scientific name	Soybean Consumption Rate (seeds/min)	Sample size	Range (seeds eaten per 60 s video)	Max seeds eaten per feeding bout
Ring-necked pheasant	*Phasianus colchicus*	18.9	21	1-36	68
Canada goose gosling	*Branta canadensis*	33.6	2	3-7	9
White-tailed deer	*Odocoileus virginianus*	107.6	36	3-317	800
13 lined ground squirrel	*Ictidomys tridecemlineatus*	6.9	15	1-14	14
Raccoon	*Procyon lotor*	9.7	4	5-8	61
Eastern cottontail	*Sylvilagus floridanus*	9.4	12	1-14	14
Fox squirrel	*Sciurus niger*	1.0	1	1	1

Species	Scientific name	Wheat Consumption Rate (seeds/min)	Sample size	Range (seeds eaten per 60 s video)	Max seeds eaten per feeding bout
Red-winged blackbird	*Agelaius phoeniceus*	10.5	2	2-5	5
American crow	*Corvus brachyrhynchos*	29.8	4	4-30	61
Mourning dove	*Zenaida macroura*	16.2	32	1-31	73
Song sparrow	*Melospiza melodia*	1.6	6	1-2	2
Wild turkey	*Meleagris gallapavo*	199.7	5	153-215	700

Table 2. Estimation of potential avian and mammalian acute toxicity from different levels of treated seed consumption for focal species using surrogate species and metabolic scaling approaches as described in EPA's T-REX model. Mammalian scaling factor was 0.75 and avian scaling factor was 1.15. Neonicotinoid chemicals (CHEM) evaluated were clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (TMX).

CHEM	Focal species	Seed	Surrogate	Surrogate LD_{50} (mg/kg)	Estimated LD_{50} (mg/kg)	Max % of LD_{50}	Seeds (#) for LD_{50}	Time to LD_{50} (mins)
CLO	American crow	Corn	Bobwhite quail *Colinus virginianus*	200	174	38.2	63	2
CLO	Black-billed magpie	Corn	Bobwhite quail	200	200	7.0	29	2
CLO	Blue jay	Corn	Bobwhite quail	200	224	39.4	15	0.5
CLO	Brown thrasher	Corn	Bobwhite quail	200	228	21.9	14	6
CLO	Common grackle	Corn	Bobwhite quail	200	216	26.8	19	6
CLO	Red-winged blackbird	Corn	Bobwhite quail	200	239	56.6	11	6
CLO	Ring-necked pheasant	Corn	Japanese quail *Coturnix japonica*	423	271	5.5	379	25
CLO	Wild turkey	Corn	Japanese quail	423	221	12.5	1195	7
CLO	American crow	Wheat	Bobwhite quail	200	174	1.8	3384	114
CLO	Mourning dove	Wheat	Bobwhite quail	200	206	5.6	1300	80
CLO	Red-winged blackbird	Wheat	Bobwhite quail	200	239	0.9	571	54
CLO	Song sparrow	Wheat	Bobwhite quail	200	259	0.6	363	227

CHEM	Focal species	Seed	Surrogate	Surrogate LD_{50} (mg/kg)	Estimated LD_{50} (mg/kg)	Max % of LD_{50}	Seeds (#) for LD_{50}	Time to LD_{50} (mins)
CLO	Wild turkey	Wheat	Japanese quail	423	221	1.1	64457	323
IMI	Blue jay	Corn	House sparrow *Passer domesticus*	41	34	280	2	0.1
IMI	Common grackle	Corn	House sparrow	41	34	183	3	0.9
IMI	Red-winged blackbird	Corn	House sparrow	41	37	387	2	0.8
IMI	Ring-necked pheasant	Soy	Japanese quail	17	11	83.5	81	4
TMX	Blue jay	Corn	Mallard *Anas platyrhynchos*	576	804	11.0	55	2
TMX	Common grackle	Corn	Mallard	576	804	7.2	70	22
TMX	Red-winged blackbird	Corn	Mallard	576	889	15.2	40	21
IMI	White-tailed deer	Corn	Mouse *Mus musculus*	131	1063	1.0	65471	1208
IMI	13-lined ground squirrel	Corn	Mouse	131	233	66.6	33	4
IMI	Raccoon	Corn	Mouse	131	700	3.3	8098	681
IMI	Eastern cottontail	Corn	Mouse	131	384	8.7	401	129
IMI	White-tailed jackrabbit	Corn	Mouse	131	479	3.5	1216	320
IMI	Eastern gray squirrel	Corn	Mouse	131	297	20.8	111	36
IMI	Fox squirrel	Corn	Mouse	131	328	26.1	184	56
IMI	Striped skunk	Corn	Mouse	131	470	1.2	1105	85
IMI	Red fox adult	Corn	Mouse	131	595	0.1	3598	1799
IMI	White-tailed deer	Soy	Mouse	131	1063	0.2	374916	3484
IMI	13-lined ground squirrel	Soy	Mouse	131	233	7.4	189	27
IMI	Raccoon	Soy	Mouse	131	700	0.1	46375	4781
IMI	Eastern cottontail	Soy	Mouse	131	384	0.6	2296	244
IMI	Fox squirrel	Soy	Mouse	131	328	0.1	1052	1052

CHEM	Focal species	Seed	Surrogate	Surrogate LD_{50} (mg/kg)	Estimated LD_{50} (mg/kg)	Max % of LD_{50}	Seeds (#) for LD_{50}	Time to LD_{50} (mins)
CLO	White-tailed deer	Corn	Mouse	427	3466	0.3	228769	4221
CLO	13-lined ground squirrel	Corn	Mouse	427	759	19.1	115	15
CLO	Raccoon	Corn	Mouse	427	2282	0.9	28297	2378
CLO	Eastern cottontail	Corn	Mouse	427	1251	2.5	1401	452
CLO	White-tailed jackrabbit	Corn	Mouse	427	1562	1.0	4248	1118
CLO	Eastern gray squirrel	Corn	Mouse	427	967	5.9	387	125
CLO	Fox squirrel	Corn	Mouse	427	1070	7.5	642	195
CLO	Striped skunk	Corn	Mouse	427	1532	0.3	3861	297
CLO	Red fox	Corn	Mouse	427	1940	0.0	12573	6287
TMX	White-tailed deer	Corn	Rat *Rattus norvegicus*	1563	7135	0.1	470899	8688
TMX	13-lined ground squirrel	Corn	Rat	1563	1563	9.3	238	31
TMX	Raccoon	Corn	Rat	1563	4697	0.5	58247	4895
TMX	Eastern cottontail	Corn	Rat	1563	2575	1.2	2884	930
TMX	White-tailed jackrabbit	Corn	Rat	1563	3215	0.5	8744	2301
TMX	Eastern gray squirrel	Corn	Rat	1563	1991	2.9	796	257
TMX	Fox squirrel	Corn	Rat	1563	2203	3.6	1322	401
TMX	Striped skunk	Corn	Rat	1563	3154	0.2	7948	611
TMX	Red fox adult	Corn	Rat	1563	3994	0.0	25880	12940
TMX	White-tailed deer	Soy	Rat	1563	7135	0.0	3893016	36180
TMX	13 lined ground squirrel	Soy	Rat	1563	1563	0.7	1964	285
TMX	Raccoon	Soy	Rat	1563	4697	0.0	481541	49643
TMX	Eastern cottontail	Soy	Rat	1563	2575	0.1	23844	2537
TMX	Fox squirrel	Soy	Rat	1563	2203	0.0	10928	10928

Table 3. Exposed seeds on the soil surface after planting in 3 categories of field types in Minnesota during 2016 and 2017. Cooperative Farming Agreements (CFAs) are privately farmed areas on public land. Public fields were farmed by DNR staff with older planting equipment. Private lands were fields where we obtained landowner permission to survey fields after planting. We did not dig up seeds to determine whether they were treated, so if no seeds were on the surface, we did not know whether the seeds were treated.

Field type	# fields	Treated (T) or not treated (N)	Exposed seeds in center plots	Exposed seeds in corner plots	Exposed seeds outside plots	Spills
CFA (private equipment, public land)	42	18T, 2N	4 (10%)	4 (10%)	17 (40%)	4 (10%)
Public (old equipment, DNR staff)	10	3T, 4N	3 (30%)	5 (50%)	7 (70%)	0 (0%)
Private (pvt equipment)	19	13T, 4N	7 (37%)	12 (63%)	14 (74%)	8 (42%)
Total	71	34T, 10N	14 (20%)	21 (30%)	38 (54%)	12 (17%)

Table 4. Exposed seeds on the soil surface after planting by crop type in Minnesota in 2016 and 2017. We did not dig up seeds to determine whether they were treated, so if no seeds were on the surface, seed treatment was unknown.

Field type	# fields	Exposed seeds in center plots	Exposed seeds in corner plots	Exposed seeds outside plots	Spills
Corn treated	24	2	4	21	5
Corn untreated	1	0	0	0	0
Corn unknown if treated	26	0	0	0	0
Total (and %) corn fields	51	2 (4%)	4 (8%)	21 (41%)	5 (10%)
Soybean treated	9	5	8	8	4
Soybean untreated	8	6	8	8	1
Soybean unknown if treated	0	0	0	0	0
Total (and %) soybean fields	17	11 (65%)	16 (94%)	16 (94%)	5 (29%)
Wheat treated	1	1	0	0	1
Wheat untreated	1	0	1	1	1
Wheat unknown if treated	1	0	0	0	0
Total wheat fields[a]	3	1	1	1	2
Total (and %) all field types	71	14 (20%)	21 (30%)	38 (54%)	12 (17%)

[a]Due to low numbers of sampled wheat fields, percentages are not provided.

Table 5. Summary of imidacloprid detections in domestic chicken blood and feces in each of 3 dose groups at University of Minnesota- College of Veterinary Medicine in 2015. Note that birds in the high dose group were euthanized early, which may have limited the ability to eliminate imidacloprid in feces.

	Dose (mg/kg/day)	N	Percent detects	Fold change	Median	Geometric Mean	Minimum	Maximum
Blood (ng/ml)	1.04	6	20.0	4.2	1.7	1.4	0.5	2.1
	5.02	10	33.3	9.8	2.6	2.2	0.7	6.9
	20.80	8	61.5	2051.7	3270	805.6	4.2	8617
Feces (ng/g wet weight)	1.04	26	81.3	91.8	14.6	10.1	0.8	73.4
	5.02	39	97.5	278.9	19.1	14.1	0.7	195.2
	20.80	5	100.0	2.8	3.2	3.7	2.3	6.5

Table 6. Summary of tissue concentrations of imidacloprid in all laboratory-exposed domestic chickens for all dose groups combined at University of Minnesota- College of Veterinary Medicine in 2015.

Tissue	First detection (day)	Last detection (day)	Fold change	N	Percent detects	Min Conc[a]	Max Conc[a]	Median Conc[a]	Geometric mean conc[a]	SD
Feces	1	21	279	70	90.9	0.7	195	14.6	11.3	35.9
Kidney	NA[b]	NA	1681	11	73.3	0.5	823	1.7	13.4	276.5
Liver	NA	NA	19882	11	73.3	0.3	5766	6.7	64.6	2473.6
Spleen	NA	NA	30413	11	73.3	0.2	6387	16.8	63.6	2320.8
Brain	NA	NA	10410	10	66.7	0.6	5725	1212.7	76.7	2295.8
Muscle	NA	NA	3469	10	66.7	0.8	2775	382.3	62.8	1128.5
Blood	1	8	17234	24	32.9	0.5	8617	4.1	14.1	2389.5

[a] Conc = concentration (ng/g wet weight in tissues and ng/ml for blood).

[b] NA = Not applicable because tissues were collected when chickens were killed the last day.

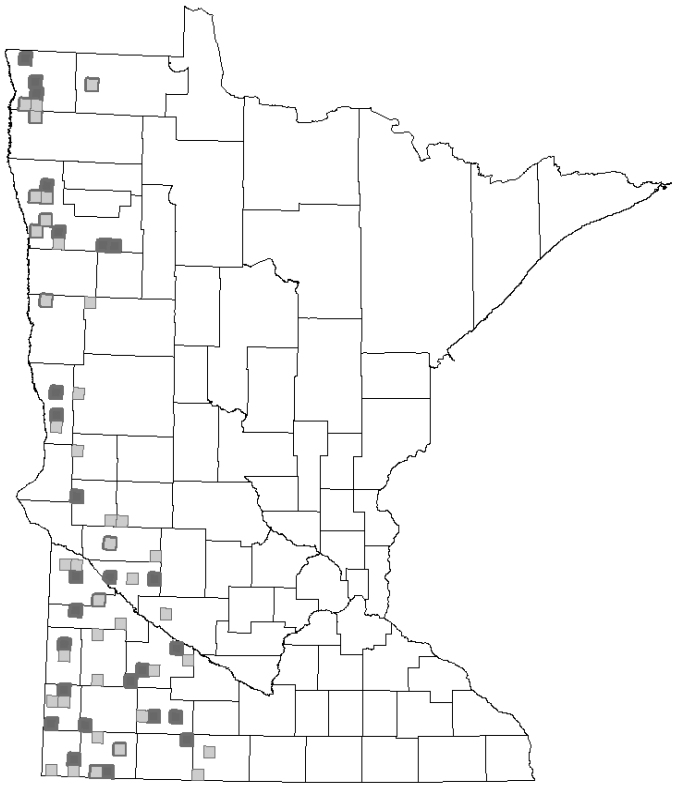

Figure 1a. Townships (*n* = 76) in Minnesota surveyed for seed spills during planting season in 2016 (dark gray), 2017 (light gray), and both years (light gray outlined with dark gray).

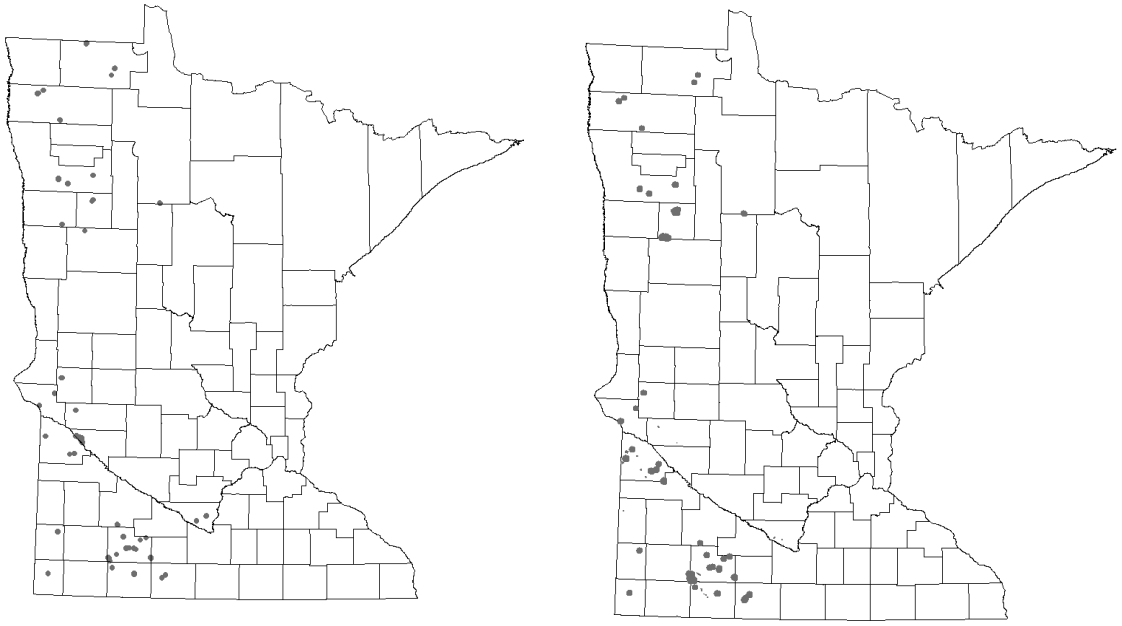

Figure 1b. Location of fields where seeds were measured on the soil surface after planting (left) and where cameras were placed at simulated spills (right) in Minnesota during 2016 and 2017. Fields are indicated as larger than their actual size to show their relative locations at a statewide scale; thus, some fields cannot be distinguished separately from other nearby fields (e.g., 17 fields on Lac Qui Parle Wildlife Management Area appear to be a single large site). Generally, the same sites were used, but some differences occurred related to the stage after planting during our visits and the ability to return to sites to remove cameras.

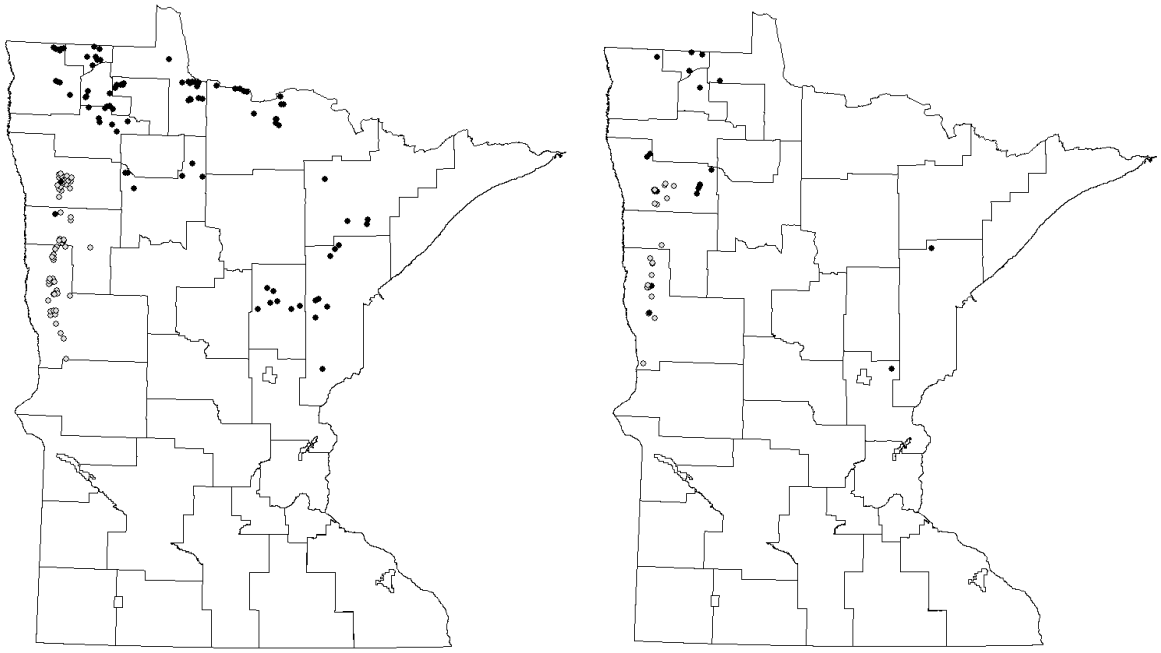

Figure 1c. Locations where sharp-tailed grouse (black) and greater prairie-chicken (gray) fecal pellet samples (left) and hunter-harvested birds (right) were collected in Minnesota during 2015, 2016, and 2017. No fecal pellet samples were collected during 2016.

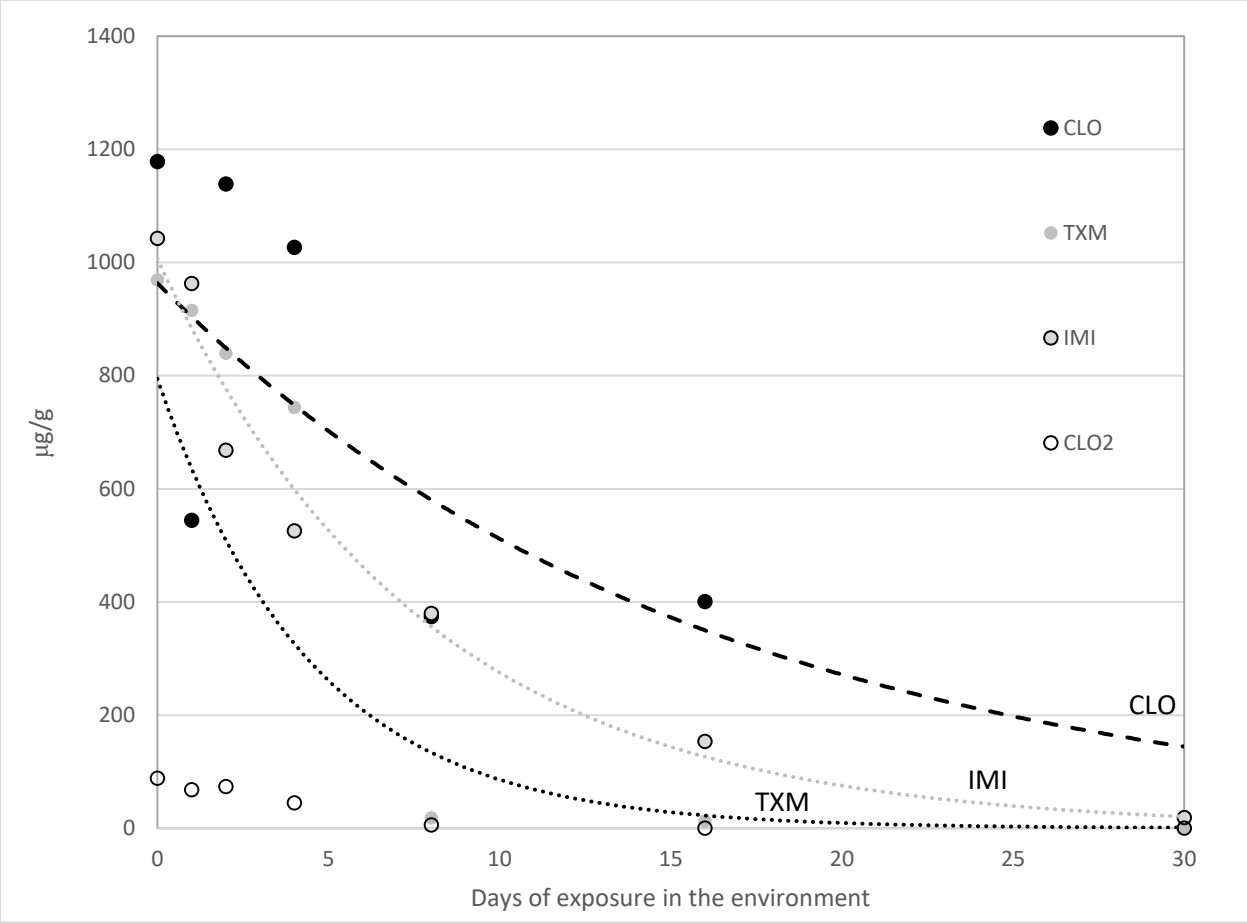

Figure 2. Concentrations of neonicotinoid seed treatments (Clothianidin -CLO, Imidacloprid -IMI, and Thiamethoxam -TXM) on corn and soybean seeds left on the soil surface for 0-30 days near Bemidji during 2016, according to an exponential decay model.

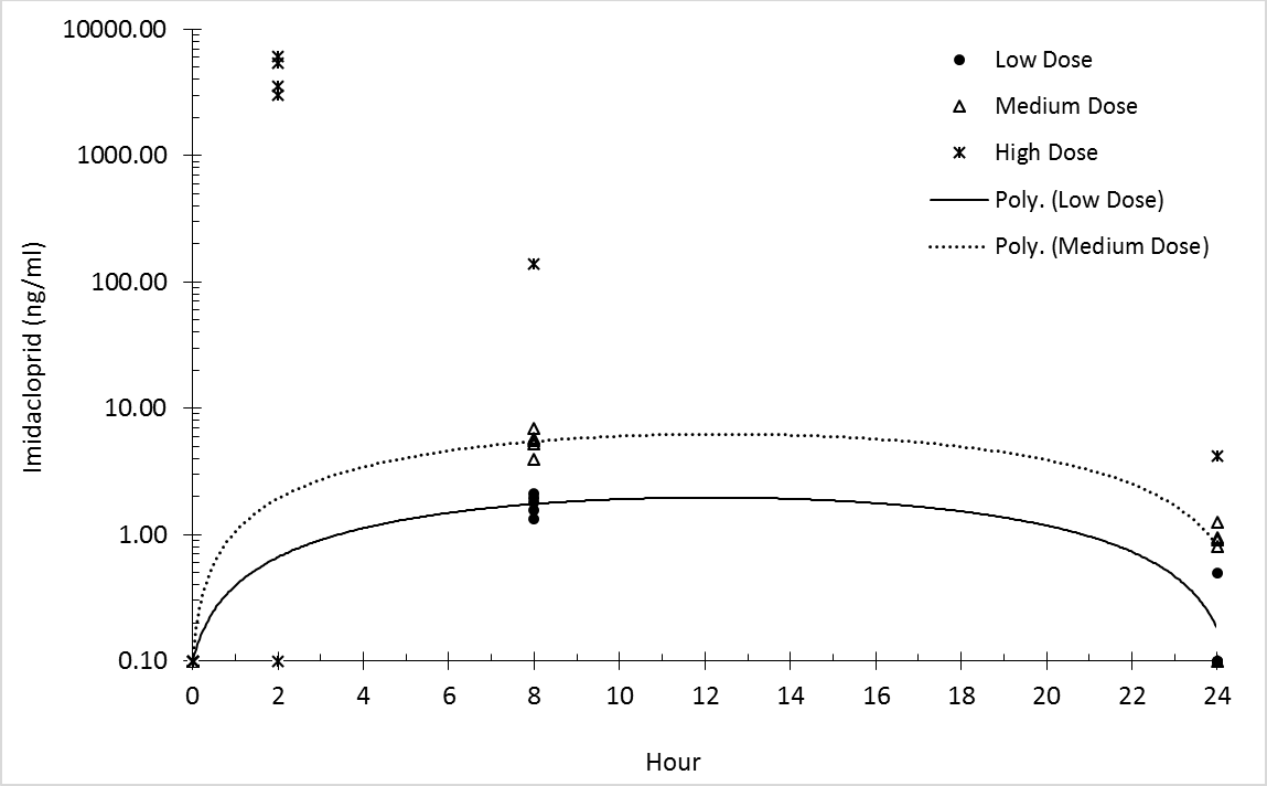

Figure 3. Changes in imidacloprid (IMI) concentrations in blood of dosed domestic chickens after 1 dose at the University of Minnesota - College of Veterinary Medicine in 2015. IMI doses were 1%, 5%, and 20% of a reported IMI LD_{50} for chickens (i.e., low, medium, and high dose groups, respectively). IMI detection limit is 0.10 or -1.0 $\log_{10}$ ng/ml in blood. Data points overlap when plotted on x-axis minimum value. A polynomial (Poly) trend line was fit for the low- and medium-dosed birds, but could not be fit to the data from high-dosed birds because chickens in this dose group were euthanized within 24 hours due to animal welfare concerns. Thus, the high dose group is not directly comparable to the other dose groups.

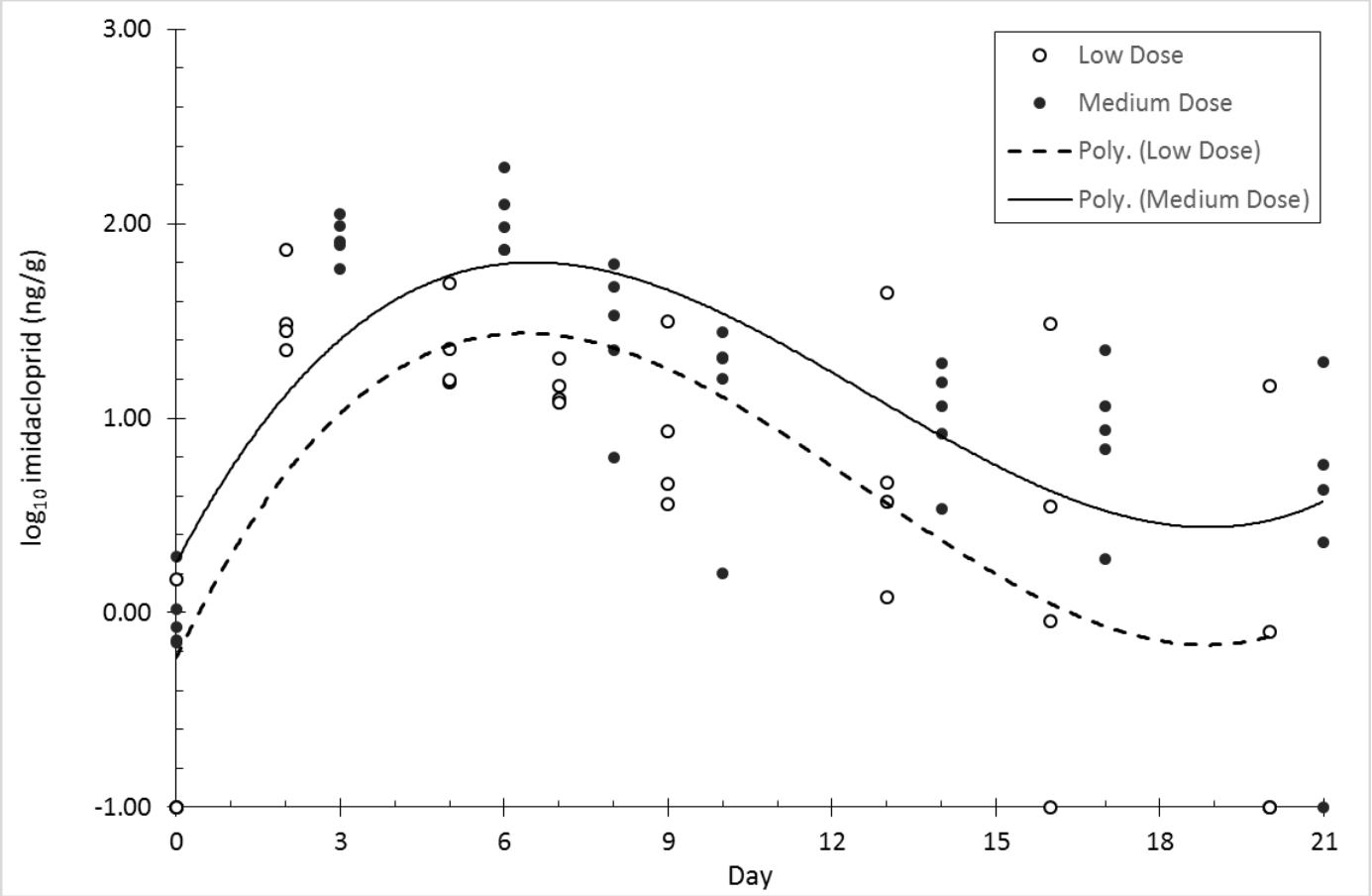

Figure 4. Changes in imidacloprid (IMI) concentrations in feces of dosed domestic chickens at University of Minnesota – College of Veterinary Medicine in 2015. Samples collected on day 0 were baseline samples, prior to exposure. Daily IMI dose for 7 days of 1% (low dose) and 5% (medium dose) of a reported IMI LD_{50}. The last day of dosing occurred on day 7 of the 21 day experiment. IMI detection limit is 0.10 or -1.0 log_{10} ng/g in feces. The high dose group is not included because samples were collected only on day 0, so no temporal trends could be determined. Chickens in the high dose group were euthanized within 24 hrs after dosing due to animal welfare concerns. Thus, the high dose group is not directly comparable to the other dose groups. Polynomial (Poly) trend lines were fit to the data for the low and medium dose groups.

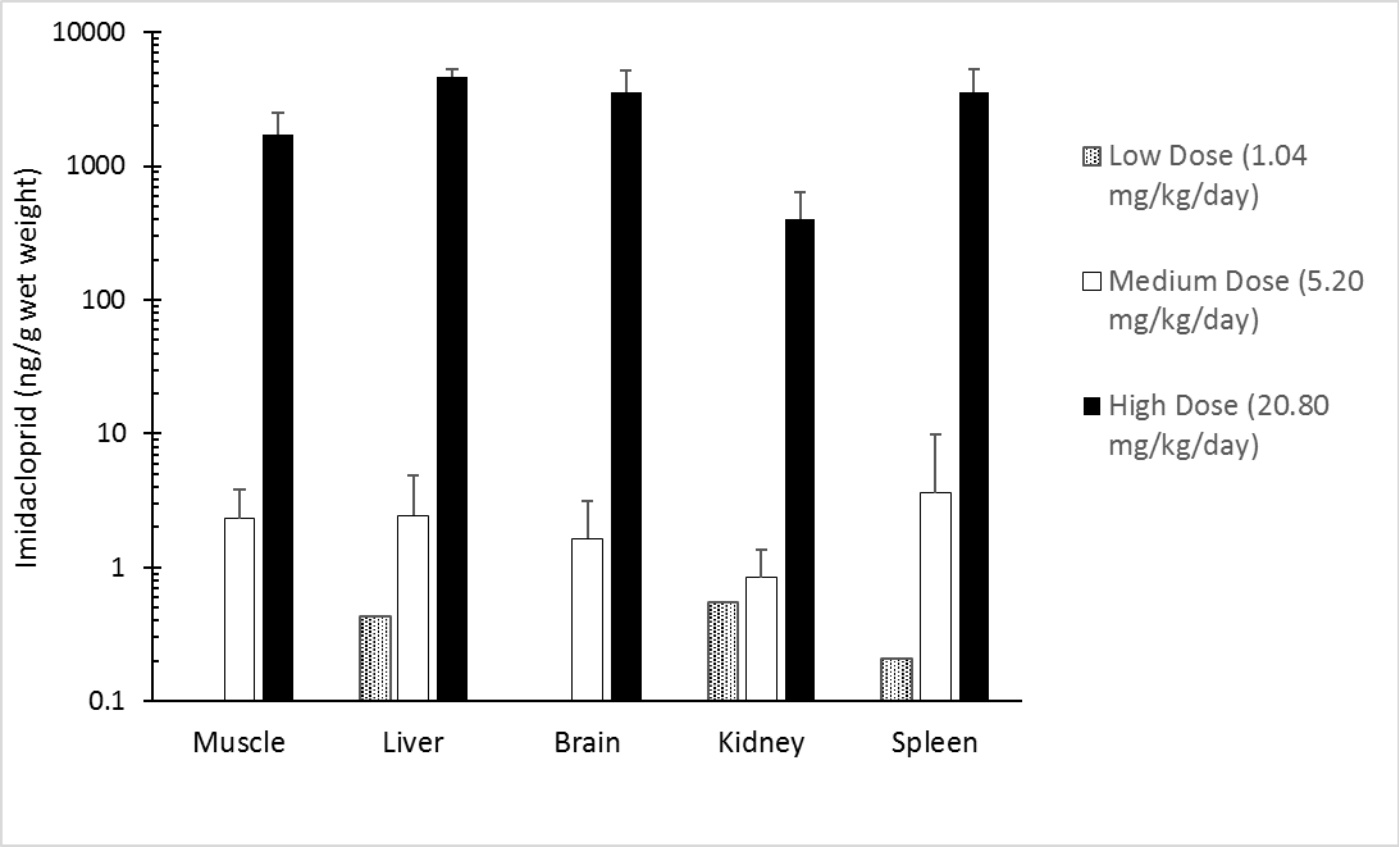

Figure 5. Concentrations of imidacloprid (geometric mean + SD ng/g wet tissue weight) in tissues of laboratory-exposed domestic chickens on experimental day 1 (high dose) or 21 (low and medium dose) at University of Minnesota - College of Veterinary Medicine in 2015. Data at the detection limit of 0.10 ng/g are not visible. Error bars represent the standard deviation of observations for a given group. No error bars are provided for the low dose group because bars represent only 1 individual with detectable concentrations.