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Chronic Neonicotinoid Toxicity and Wetland Limnocorral Approach

Community-Level and Phenological Responses of Emerging Aquatic Insects Exposed To
Three Neonicotinoid Insecticides: An In Situ Wetland Limnocorral Approach

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Abstract: Seasonal aquatic insect emergence represents a critical subsidy link between aquatic and terrestrial ecosystems. Early and late instar larvae developing in wetlands near neonicotinoid-treated cropland are at risk of chronic insecticide exposure. An in situ wetland limnocorral experiment compared emergent insect community responses to imidacloprid, clothianidin, and thiamethoxam. Over 15 weeks, 21 limnocorrals were dosed weekly for 9 weeks to target peak nominal doses of 0.0, 0.05 or 0.5 µg/L, followed by a 6-week recovery period. Thirty-nine aquatic insect taxa were recorded but 11 taxa groups made up 97% of the community composition. Principal response curves indicated that during the dosing period, community composition among the treatments resembled the controls. During the 6-week recovery period, significant deviance was observed in the high imidacloprid treatment with similar trends in the clothianidin treatment, suggesting that community effects from neonicotinoid exposure can be delayed. Non-biting midges (Diptera: Chironomidae) and damselflies (Odonata: Zygoptera) also emerged 18 to 25 days earlier in the imidacloprid and clothianidin neonicotinoid treatments, relative to controls. These data suggest that phenology and subtle community effects can occur at measured neonicotinoid concentrations of 0.045 µg/L (imidacloprid) and 0.038 µg/L (clothianidin) under chronic repeated exposure conditions. Synchronization and community dynamics are critical to aquatic insects and consumers; thus, neonicotinoids may have broad implications for wetland ecosystem function. This article is protected by copyright. All rights reserved

Keywords: Limnocorral, Insecticide, Neonicotinoid, Prairie wetland, Chironomidae
INTRODUCTION

Species-specific timing of life cycle events (e.g., metamorphosis, emergence, oviposition) inherently drives community dynamics (Nakazawa 2012), especially for insects emigrating from aquatic to terrestrial ecosystems. Aquatic insects transfer energy and nutrients between aquatic and terrestrial environments, supplying timely, abundant resources and ecosystem services (Batzer and Wissinger 1996). These linkages are paramount to numerous wetland dependent consumers. Aquatic insect life cycles are regulated by abiotic and biotic environmental variables, specifically temperature, photoperiod, food resources, and competition (Moore and Schlinder 2010), which dictate phenological synchronization of events. However, these environmental variables can be disrupted in areas of intensive agricultural production where there is an increased risk of sedimentation, drainage, and agrochemical contamination, including exposure to insecticides (Bartzen et al. 2010).

Among the many insecticides commonly applied over vast spatial scales are the neonicotinoids (Main et al. 2014). Neonicotinoid insecticides, specifically imidacloprid, clothianidin, and thiamethoxam, constitute some of the most heavily applied insecticides in the agricultural sector worldwide (Douglas and Tooker 2015; Simon-Delso et al. 2015). As nicotine agonists, neonicotinoids stimulate nicotinic acetylcholine receptor action and are highly toxic to pest and non-target insects alike (Tomizawa and Casida 2005). Simplified insecticide application methods (i.e., seed treatments) have greatly increased the amount of residual neonicotinoid active ingredient in the environment, particularly within soils (Jones et al. 2014) and surface waters (Morrissey et al. 2015). Episodic heavy rainfall events that promote insecticide runoff can shift and, in some cases, permanently alter aquatic insect community structure (Liess and Schulz 1999, Liess and Beketov 2011).

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Numerous single-species laboratory studies have shown that aquatic insects are extremely sensitive to neonicotinoid insecticides (Morrissey et al. 2015), especially under chronic exposure conditions. However, field-based studies examining the effects of neonicotinoids on aquatic insect communities are less common (van Dijk et al. 2013). The use of aquatic invertebrate mesocosm studies offer promise for evaluating the community-level risks of neonicotinoid exposure under more ecologically relevant conditions (Sánchez-Bayo et al. 2016). Based on lab studies, concentrations required to induce lethal effects to immature aquatic insect communities range from 1.0 µg/L (Sánchez-Bayo and Goka 2006; Hayasaka et al. 2012a; Hayasaka et al. 2012b) to 3.2 µg/L (Colombo et al. 2013). Studies of sub-lethal exposures under more natural conditions are required to fill the gap between species-specific laboratory and community-level field studies. Additionally, population endpoints such as aquatic insect emergence are more sensitive to neonicotinoid exposure than lethality and may cause indirect ecological impacts (Cavallaro et al. 2017).

Prairie wetlands are characterized as having unique hydrologic regimes and distinct aquatic insect assemblages associated with variable water retention time (van der Kamp 1995; Euliss et al. 2004), as well as frequent and chronic neonicotinoid detections (Main et al. 2014; Evelsizer and Skopec 2016). Long term contamination of prairie wetlands is largely driven by extended persistence in frozen soils and mobilization from snow melt into receiving wetlands (Main et al 2016) combined with recurrent use and persistence in surface waters (Main et al. 2015). Here, we conducted a 107-day (dosing and recovery) study to determine the comparative community-level and phenological effects of imidacloprid, clothianidin, and thiamethoxam on emerging aquatic insects. Exposures were at chronic, environmentally relevant concentrations within a typical prairie wetland using custom designed in situ limnocorrals. The effects of

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imidacloprid to laboratory organisms are well-represented in the aquatic toxicology literature, but data are lacking on other neonicotinoids and the responses of communities exposed under more natural conditions (Morrissey et al. 2015). Given the lack of field data on effects of imidacloprid, clothianidin and thiamethoxam relative to their wide-spread use in the agricultural sector, more data on those chemicals will better inform the current regulatory reviews and future policy decisions on their environmental safety.

MATERIALS AND METHODS

Study site

The experiment was conducted in a single class 5 (permanent) prairie wetland (Stewart and Kantrud 1971) at the St. Denis National Wildlife Area (NWA) roughly 40 km east of Saskatoon, SK, Canada. The St. Denis NWA serves as a model prairie wetland complex inhabited by representative species of waterfowl and wetland dependent fauna. The St. Denis NWA is under the management of Environment and Climate Change Canada where the surrounding land-use consists primarily of native prairie and hay fields. Intensive studies on hydrology and avian ecology over the past three decades provided an extensive, reliable dataset for selecting an experimental prairie wetland. The wetland was selected for its consistent inter-annual central water depth (1.0-1.3 m), characteristic prairie wetland water physicochemical parameters (e.g., pH, conductivity, dissolved oxygen), inflow from lands not under conventional production, and high insect secondary production. Emergent vegetation primarily consisted of *Typha latifolia* and *Alisma triviale*. Floating and submerged aquatic macrophyte composition within the study pond included a patchy distribution of *Lemna sp.* and *Ceratophyllum demersum*. Water quality testing prior to the study revealed no traces of neonicotinoid residues in the selected study wetland.
**Experimental design**

Twenty-one custom-built limnocorals (1.0 x 1.0 x 1.5 m) fitted with aquatic insect emergence traps were purchased from Curry Industries Ltd. (Winnipeg, MB, Canada; Figure 1). Limnocorals were fixed to Styrofoam floats encased with polyvinyl, and at the corners of each float, a nylon rope secured to a ring of ABS plumbers pipe (4” in diameter) was placed over the wooden stake; this allowed the floats secured to the limnocorals to rise and fall with the water level throughout the experiment (extra material in the walls allowed for expansion without pulling the bottom out of the sediment). Due to seasonal strong wind gusts and storms customary to the region, wooden stakes (8.0’ x 1.0” x 1.0”) anchored the floats and galvanized steel chains weighted the open bottoms of the polyethylene sleeves after firmly pressing them 12-15 cm into the sediment. The sediment seal along the sleeves was visually confirmed with the aid of an Aqua Scope II™. Emergence traps covered the entire limnocorral and featured a removable acrylic collection chamber which led to a polypropylene jar containing 70% ethanol. Glycol was added when evaporation rates accelerated in mid-summer from prolonged sun exposure and higher temperatures. Given the potential for biotic heterogeneity and seasonal changes in water depth, treatments were randomized across 3x7 blocks of limnocorals located in the center of the experimental wetland at a water depth of approximately 1 m.

**Abiotic measurements**

Temperature was monitored hourly inside and outside limnocorals throughout the dosing period with a HOBO Onset® temperature data logger (Bourne, MA, USA) and every two weeks during the recovery period. Physicochemical water quality parameters were also measured during the dosing and recovery periods. Dissolved oxygen (mg/L), conductivity (µS/cm), and pH.
were measured with a YSI ProPlus (YSI Inc., Yellow Springs, OH, USA) water monitoring
meter.

Water and insect sampling

All dosing and sampling were conducted by boat throughout the 107-day long (9-week
dosing and 6-week recovery) experiment to avoid disturbing the limnocorral sleeves and
surrounding sediments. Water samples were taken 4 days before the start of the experiment (day
-4), day 0 and immediately before and after each insecticide application during the dosing period,
and then weekly during the recovery period. Adult insects were collected from the polypropylene
jars every 3 to 4 days. To improve realism, and due to the small size of the limnocorrals, long
duration of the experiment, and potential for patchy larval distribution, emergence traps were
removed for 4 days every 12 days to allow for recolonization. Insects were collected by changing
the entire collection chamber and preserved and stored in 70% ethanol until identified and
counted. Adult Chironomidae were identified to at least the subfamily-level and all other insects
to the lowest possible taxonomic-level using dichotomous keys (Pinder 1995; Merritt and
Cummins 1996).

Neonicotinoid application and analysis

Limnocorrals were treated with imidacloprid, clothianidin, or thiamethoxam once weekly
during the exposure period to reach target nominal concentrations of 0.05 µg/L (low) or 0.5 µg/L
(high) relative to controls. Each neonicotinoid treatment had three replicate limnocorrals with the
addition of three controls, for a total of 21 limnocorrals. Several water sampling surveys have
identified the recurrent and prevalent distribution of neonicotinoids in prairie wetlands (Main et
al. 2014, 2015, 2016). The two exposure concentrations were selected to represent
environmentally relevant concentrations frequently detected in the region and were within the

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range of recently proposed and interim acute and chronic regulatory aquatic life guidelines (CCME 2007; RIVM 2014; Morrissey et al. 2015). For example, a seasonally structured water sampling effort in the Canadian Prairies (2012-2013) found that the average annual summed neonicotinoid concentrations (i.e., total imidacloprid, clothianidin, thiamethoxam, and acetamiprid concentrations) measured in wetland water ranged between 0.04 to 0.77 μg/L with peak concentrations up to 3.1 μg/L (Main et al. 2014). Similar water monitoring studies in Iowa, USA, found that mean clothianidin concentrations ranged between 0.17 to 0.52 μg/L in tile-drained wetlands over three sampling periods between May and June in 2014 (Evelsizer and Skopec 2016). Morrissey et al. (2015) also reported geometric means of 0.13 μg/L (averages) and 0.63 μg/L (maxima) in 29 surface water monitoring studies from 9 countries.

Imidacloprid (98.8% pure) and clothianidin (99.6% pure) were acquired from Bayer CropScience (Mississauga, ON, Canada); thiamethoxam (98.9% pure) was acquired from Syngenta Crop Protection (Guelph, ON, Canada). Stock solutions were prepared in 1-L volumetric flasks with reverse osmosis water (Barnstead® Diamond™ NANOpure, 18.2 MV/cm), then the appropriate volume was transferred into 250-mL bottles, transported in coolers to the study site, and poured directly into the appropriate limnocorral at each dosing event. Water in the limnocorral was gently stirred with a paddle to aid mixing. Further mixing was achieved through natural turnover within the pond from temperature-aided water density changes. The volume of each limnocorral was calculated by measuring the pond depth (i.e., L × W × H) and estimated to contain 1,000-1,300-L of wetland water. Individual volume measurements combined with predicted degradation rates, were used to adjust weekly dosing accordingly. In order to maintain the target exposures over the entire study period, we measured actual concentrations in the limnocorrals immediately post dosing, then 3 and 7 days later to obtain
This information allowed us to calculate the remaining concentration in the limnocorral and subsequently dose with the appropriate amount to reach our target nominal dose each week (Figure 2 and SI Table 1).

Limnocorral water samples for chemical analysis were collected by grab-style sampling at >10 cm below the surface of the water in the center of each limnocorral with 250-mL amber bottles and stored at 4°C. All water samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada in Saskatoon, SK. Methods adapted from Xie et al. (2011) allowed for simultaneous extraction of imidacloprid, clothianidin, and thiamethoxam when present through aqueous sample extraction, solid-phase extraction, and LC-MS/MS and are fully described in Main et al. (2014). Analytical standards of imidacloprid, clothianidin, and thiamethoxam were purchased from Chem Service (West Chester, PA, USA). Quality assurance/quality control (QA/QC) results provided recovery correction factors (RC %), limits of quantification (LOQ), and limits of detection (LOD). Mean (± SE) QA/QC were as follows: imidacloprid (RC=83.9 ± 4.4%; LOQ=6.5 ± 0.5 ng/L; LOD=2.2 ± 0.1 ng/L), clothianidin (RC=78.9 ± 6.6%; LOQ=10.0 ± 1.0 ng/L; LOD=3.3 ± 0.4 ng/L), and thiamethoxam (RC=91.3 ± 0.3%; LOQ=12.5 ± 1.5 ng/L; LOD=4.2 ± 0.5 ng/L). All concentrations were batch recovery corrected.

Data analysis

Univariate statistical tests compared all treatments and blocks within wetlands for physicochemical water chemistry parameters using a one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. To account for the potential influence by predatory taxa on emergence, total Zygoptera was also compared among treatments using Chi-square tests,
respectively (α = 0.05 level of confidence). Tests were performed using SigmaPlot™ Version 13.0 (Systat Software, Inc., San Jose, CA, USA).

The effects of the three neonicotinoids relative to controls on emerging adult insects were analyzed by the Principal Response Curves (PRC) method developed by van den Brink and Ter Braak (1999). These multivariate models are designed to evaluate community effects in experiments with repeated measures over time where the emphasis is on species composition. The PRC method executes a time-series partial redundancy analysis (pRDA) where the interaction between sampling time and treatment act as explanatory variables and sampling times as covariates (van den Brink et al. 2009). PRC results are interpreted by graphical representation with the sampling time on the x-axis and the first Principal Component (PRC1) of the treatment effects on the y-axis. In this format, spatial deviations from the mean of controls (center line) can be compared over repeated temporal sampling to determine community response, thus quantifying the effects of the insecticide treatment across multiple species simultaneously.

Significance of the PRC among treatments was evaluated by random Monte Carlo method (999 permutations) using the limnocorral taxa counts (log(x + 1)-transformed), obtaining an F-type test statistic based on the eigenvalue of the first Principal Component. The PRC method identifies the variance partitioning by reporting the explanatory content (in %) of first canonical axis (van den Brink and Ter Braak 1999). PRC scores for all species can be found in the supplementary file (Table S2) and those species with scores + or − 0.15 are displayed. The PRC analysis was performed using R version 3.4.1 (R Core Team 2017) package “vegan” (Oksanen et al. 2011), and a Dunnett’s test of the first principal component was used to determine whether significant differences between treatments and controls occurred at any sampling time points using package “multcomp” (Hothorn et al. 2008).

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In order to determine how timing of emergence varied among treatments, the cumulative proportional emergence of all taxa and for each of the 4 major taxa: Chironomidae, Zygoptera, Chaoboridae, and Limnephilidae was calculated as the number of insects emerging on each sampling day divided by the relative total abundance. Data were analyzed by fitting cumulative proportion curves with 95% confidence intervals for each treatment (control, 0.05, 0.5 µg/L imidacloprid, clothianidin, thiamethoxam) over time using nonlinear curve fits. Model selection (i.e., logistic, cubic, quadric, exponential) of curve fits was based on AICc weight and parameter estimates are shown for each best fit curve by treatment. The curve mean inflection point indicates the day which separates the curve into two equal regions of opposite concavity (i.e., 50% cumulative proportion). Parameter estimates of curves were statistically compared between treatments and controls using an analysis of means (ANOM). Significance was indicated by upper (later emergence) or lower (earlier emergence) exceedance of the generated 95% C.I.s (α = 0.05). An equivalence ratio was generated by comparing the rate of emergence in the control limnocorrals to each treatment (α = 0.05; default setting of a 25% change compared to the controls) where the control equivalence ratio is 1.00 and the C.I.s are 0.8-1.25 (i.e., 25% change from the control). Curve fitting and derivation of parameter estimates were performed in JMP®, Version 11.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Physicochemical water quality parameters

On average, temperatures (mean minimum and maximum; °C) inside and outside the limnocorrals during the dosing period ranged from 13.9-27.0°C and 14.2-25.9°C, respectively. Mean differences in temperatures between the inside and outside of the limnocorrals were not significant (Student’s t-test, p>0.05), indicating that the sleeves had minimal insulating effects.

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Temperature and depth decreased during the recovery period (Table 1). Mean (± SE) water physicochemistry measurements across all limnocorals during dosing were characteristic of a typical prairie wetland: dissolved oxygen (6.36 ± 0.17 mg/L), conductivity (2090.2 ± 19.83 µS/cm), pH (8.07 ± 0.02), and depth (1.27 ± 0.01 m), which were similar among treatments (Table 1). Water physicochemical parameters were also similar among the 3 experimental blocks (ANOVA; p>0.05) during both the dosing and recovery period.

Neonicotinoid exposure and degradation

Mean (± SE) measured high dose concentrations of neonicotinoids in water immediately after weekly dosing were 0.436 (± 0.062) µg/L imidacloprid, 0.384 (± 0.048) µg/L clothianidin, and 0.386 (± 0.106) µg/L thiamethoxam which was 87%, 77%, and 77% of the target nominal high dose (0.5 µg/L). Low dose concentrations were measured as 0.045 (± 0.006) µg/L imidacloprid, 0.038 (± 0.015) µg/L clothianidin, and 0.045 (± 0.018) µg/L thiamethoxam which was 90%, 76%, and 90% of the target nominal low dose (0.05 µg/L), respectively (Figure 2, SI Table 1). The control limnocorals remained below detection throughout the study. Although thiamethoxam can degrade to clothianidin, we only found 2 clothianidin detections in a 0.5 µg/L thiamethoxam treatment on day 49 and 56 and both were <LOQ (LOQ=10.0 ± 1.0 ng/L). To estimate dissipation between weekly insecticide applications, we calculated the mean percent active ingredient remaining seven days after the initial dosing (week 1) for a subset of imidacloprid, clothianidin, and thiamethoxam limnocorals; this was repeated for the final week of the exposure period (Figure 2). On average, seven days post-dosing, measured concentrations of the three neonicotinoids decreased by 37.6%, 35.7%, and 75.3% for imidacloprid, clothianidin, and thiamethoxam, respectively.

Insect abundance and diversity

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Eleven of the 39 insect taxa identified accounted for 97% of the total number of emerging adult insects collected and include the following: *Limnephilus infernalis* (27%), *Chironomus sp.* (26%), Tanytarsini sp. (13%), Chironomini sp. (8%), *Cricotopus sp.* (5%), *Ablabesmyia peleensis* (5%), *Chaoborus americanus* (4%), Orthocladiinae (4%), *Psectrocladius sp.* (3%), *Lestes disjunctus* (1.3%), and *Enallagma annexum* (0.7%). To characterize the potential role of predatory pressure on emergence, we determined the total proportion of predatory damselfly taxa among treatments ranged from 1.6% to 6.9%. One treatment (low thiamethoxam) had a greater proportion of predators observed ($\chi^2=20.65$, d.f. = 6, $p=0.002$) with a single limnocorral outlier of 15.1%.

The PRC analyses for imidacloprid treated limnocorral showed that 59.8% of the overall variance in the community composition dataset was explained by time (days) and 11.9% by treatment (neonicotinoid concentration in µg/L). The first canonical axis (PRC1) of the PRC explained 34.1% of the variance, whereas the variance in taxa composition between limnocorral replicates was 7.1%. PRC1 captured a significant portion of the variance in the insect community composition by the imidacloprid treatment (Monte Carlo permutation test, 999 permutations, F-ratio=22.55, $p=0.046$). Positive taxa weights were found for the hard-bodied flies. These taxa significantly increased in abundance in the high imidacloprid treatment during the recovery period relative to the controls (Dunnett’s test; $p=0.03$) at sampling day 71 (Monte Carlo permutation test, 999 permutations, F-ratio=2.70, $p=0.015$). The highest species weight was calculated for Ephydridae (0.2) followed by Canacidae and Musicidae. Negative taxon weights were found for primarily multivoltine ($\geq 1$ generation per year) groups (e.g., *Chironomus sp.*, *Ablabesmyia peleensis*, *Procladius sp.*, *C. americanus*, and Tantarsini sp.).

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The PRC analyses for clothianidin treated limnocorral showed that 57.1% of the overall variance in the community composition dataset was explained by time (days) and 9.4% by treatment (neonicotinoid concentration in \( \mu g/L \)). The first canonical axis of the PRC explained 29.3% of the variance (Monte Carlo permutation test, 999 permutations, F-ratio=12.74, p=0.74), whereas the variance in taxa composition between limnocoral replicates was 11.7%. Among the taxa with positive weights were three of the semi-aquatic parasitic wasp families, Diapriidae (0.06), Eulophidae (0.09), and Scelionidae (0.04). The highest positive taxon weight was calculated for Orthocladiinae (0.5). Similar to the imidacloprid treatments, the negative taxon weights were found for the multivoltine taxa with the highest negative taxon weight calculated for \textit{Chironomus sp.} (-3.4).

The PRC analyses for thiamethoxam showed that 55.5% of the overall variance in the community composition dataset was explained by time (days) and 12.3% by treatment (neonicotinoid concentration in \( \mu g/L \)). The first canonical axis of the PRC explained 32.3% of the variance (Monte Carlo permutation test, 999 permutations, F-ratio=19.22, p=0.26), whereas the variance in taxa composition between limnocoral replicates was 10.7%. Tanypodinae showed the highest negative taxon weight (-0.06). Many taxa weights in the thiamethoxam treatments were positive with the highest taxon calculated for \textit{Chironomus sp.} (2.8). This was followed by the caddisfly, \textit{Limnephilus infernalis} (2.1), and the chironomid subfamily Orthocladiinae (1.3).
**Proportion and timing of emergence**

A nonlinear curve fit function was performed to compare the emergence timing of all taxa combined, and the 4 most common taxa Chironomidae, Zygoptera, Chaoboridae, and Limnephilidae (Figure 4, Table 2). Equivalence ratios indicated significant differences in curve slope (i.e., >25% different from the set $\alpha$ of 0.05) for Chironomidae (imidacloprid high and low; clothianidin high) and Zygoptera (imidacloprid high; clothianidin high and low). The mean inflection point for all taxa was day 59; Chironomidae was day 44; Zygoptera was day 47; Chaoboridae was day 57, and Limnephilidae was day 92. Exceedance of the decision limits ($\alpha = 0.05$ level of confidence) indicated Chironomidae and Zygoptera exhibited significantly earlier (Lower) emergence with neonicotinoid exposure (Table 2). For example, Chironomidae, representing 64% of the total insect secondary production, emerged 18-19 days earlier in imidacloprid (high = day 38 and low = day 39) treatments relative to the controls (day 56) and 9-15 days earlier in the clothianidin treatments (high = day 41 and low = day 47). Zygoptera emerged 19-25 days earlier in clothianidin (high = day 36 and low = day 42) treatments relative to the controls (61) and 17 days earlier in the low imidacloprid treatment compared to the controls. Thiamethoxam was not observed to affect timing of emergence for any of the common taxa (Figure 4).

**DISCUSSION**

The results of this study indicate that low doses of imidacloprid and clothianidin, though not thiamethoxam, can have subtle but important effects on emerging aquatic insect community phenology and to a lesser extent community composition within a wetland. In this complex experiment involving *in situ* limnocorralss, we attempted to maintain nominal concentrations at 0.5 µg/L and 0.05 µg/L under semi-natural conditions – levels that are within the range of
proposed water quality guidelines for imidacloprid and, based on water sampling data, represent observed concentrations in natural prairie wetlands (Main et al. 2014). Since the neonicotinoids naturally dissipated during the 7 days between dosing, average exposures were lower and thus these results represent a conservative but realistic scenario. Recent preliminary risk assessments by the U.S. EPA (0.01 - 0.39 µg/L) and Health Canada (0.041 - 0.36 µg/L) are comparable to the water quality criteria proposed by earlier reviews (0.035 - 0.2 µg/L) based on all neonicotinoid compounds (Morrissey et al. 2015; U.S. EPA 2017). Our data suggest that phenological effects can occur at mean peak measured concentrations of 0.045 µg/L (imidacloprid) or 0.038 µg/L (clothianidin) and effects on aquatic insect communities emerging from wetlands may persist over time. No concentration-response was observed during the dosing period across all compounds tested. Once insecticide application ceased, aquatic insect communities displayed high variance among treatments during the recovery period with an observed significant change in community composition on Day 71 only in the high imidacloprid treatment (0.436 µg/L). Latent effects of chronic neonicotinoid exposure may alter the recovery trajectory of aquatic insect communities in Prairie wetlands, particularly where recolonization from uncontaminated areas may be less accessible.

Community alterations

Aquatic insects found in wetlands function as ecological generalists and can often occupy a range of wetland types (Wrubleski and Ross 2011). These wetlands can be characterized by varying hydroperiods, salinity levels, and plant communities (Stewart and Kantrud 1971) as well as aquatic insect assemblages (Driver 1977). Depending on climatic conditions, geographic location, surrounding land-use, and other landscape variables, aquatic insect communities can change drastically, or shift gradually, year-to-year even in the same wetland (Batzer 2013). This
variability produces high diversity in wetland ecosystems with species inventories reporting greater than 400 species in the Prairie Pothole Region (Euliss et al. 1999). Advanced tolerance for environmental fluctuation exhibited by wetland insect species, from daily to annual variability, has challenged wetland scientists, hence the lack of studies demonstrating clear ecological patterns influencing aquatic insect communities (Batzer 2013). A subtle decreasing trend in multivoltine species such as *Chironomus sp.*, *Ablabesmyia peleensis*, *Procladius sp.*, *Chaoborus americanus*, and Tantarsini sp. in the imidacloprid and clothianidin treatments was observed during the weekly pulsed-dosing period. These taxa are considered eurytopic and cosmopolitan due to their tolerance to a range of aquatic habitats (Pinder 1995). Specifically, *Chironomus sp.* were more abundant in the control limnocorrals, suggesting that imidacloprid and clothianidin potentially caused similar effects on emergence success. Similar to recent laboratory tests with *Chironomus dilutus* that describe the relatively equivalent toxicity of imidacloprid and clothianidin (Cavallaro et al. 2017; Maloney et al. 2017; Raby et al. 2018), the present study demonstrates that these two neonicotinoids may affect wild populations of *Chironomus sp.* under field conditions.

Separating the relative influence of a chemical stressor and normal environmental variation is a notable challenge in mesocosm studies. Some case studies cite habitat and physicochemical water parameters as having a greater impact on invertebrate communities than chemical stressors (Rico et al. 2016). Similar challenges exist in studies involving prairie wetlands. Chipps et al. (2006) reported greater prevalence of dominant multivoltine species (i.e., Culicidae and Chironomidae) under increased agricultural disturbance, suggesting concomitant effects from runoff and changes in physicochemical water parameters (Chipps et al. 2006). In the present study, experimental units were isolated within the same prairie wetland, thus creating a

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more uniform baseline and generally lower environmental variation. This indicates that the impacts of imidacloprid and clothianidin on emerging aquatic insect communities are chemically-induced and not from environmental disturbance or other physiochemical differences.

Previous neonicotinoid mesocosm studies feature exposure profiles varying from single to multiple pulses in lentic and lotic aquatic habitats (Sánchez-Bayo et al. 2016). Commonly faced with prolonged or repeated pesticide exposures throughout the growing season, previous studies indicate immature aquatic insect abundance and community structure are impacted by mean peak neonicotinoid concentrations greater than 1.0 µg/L (Sánchez-Bayo and Goka 2006; Beketov et al. 2008; Hayasaka et al. 2012b; Kattwinkel et al. 2016). Sub-lethal effects on feeding performance, leaf litter decomposition, and body length are documented at concentrations below 1.0 µg/L (Alexander et al. 2007, Alexander et al. 2008, Englert et al. 2012). In a similar lentic mesocosm study, a pulsed imidacloprid exposure scenario, ranging in concentration between 0.6 to 40.0 µg/L TWA (time weighted average), researchers found significant community effects at 5.2 µg/L TWA where Chironomidae represented the greatest abundance and diversity (Colombo et al. 2013). Columbo et al. (2013) also detected significant negative effects at much higher concentrations than this study at 12.0 µg/L TWA for abundance and 5.2 µg/L TWA for diversity. However, high variation in abundance among treatments occurred at concentrations ranging from 0.2 to 0.4 µg/L TWA, suggesting low-level concentrations acted as a stochastic stressor to the community. Similar variability was also observed in stream mesocosms after three weekly applications of imidacloprid at 1.63 µg/L where total benthic insect and dipteran density were slightly greater than controls (Pestana et al. 2009a). In the present study, high variation in abundance was observed at lower concentrations (<0.5 µg/L) suggesting general community

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stress. In an outdoor pond study, Ratte and Memmert (2003) observed Chironomidae and Baetidae as the most sensitive taxa after two applications of Confidor 200 SL (a.i. imidacloprid) with a NOEC (no-observed effect concentration) of 0.6 μg imidacloprid/L. In contrast, three single pulses of Admire at 2.0 and 20.0 μg imidacloprid/L revealed no effects on the Chironomidae community nor reductions in EPT taxa (Ephemeroptera, Plecoptera, and Trichoptera) (Pestana et al. 2009a). Differences in habitat, initial taxa composition, exposure duration, and concentration are some of the critical components to consider when interpreting variation in mesocosm responses.

Dispersal and recolonization are key components to external recovery of aquatic insect communities from pesticide exposure (Trekels et al. 2011). Due to the physical design of our limnocorrals and routine removal of the emergence traps, recolonization was occurring throughout the study. This approach may have influenced the overall sensitivity to neonicotinoid treatments and contributed to variable community responses among treatments. Previous studies have highlighted and quantified the value of recolonization to aquatic insect communities recovering from environmental stressors related to agriculture (Galic et al. 2013). The imidacloprid treatment was the only compound that showed a significantly different community composition from the controls, but only during the recovery period (Day 71). Given the patterns exhibited among all treatments, this suggests that the trends observed in community composition during the recovery period may be due to a combination of latent effects from neonicotinoid exposure or altered recolonization potential from nearby wetlands.

Imidacloprid is the most widely used neonicotinoid in both laboratory and mesocosm studies (Morrissey et al. 2015; Sánchez-Bayo et al. 2016). However, the paucity of available aquatic toxicity data (laboratory and field) for clothianidin and thiamethoxam has contributed to
assumptions of different neonicotinoids having similar toxicity. Here, imidacloprid and clothianidin appeared to have a slightly stronger effect on the emergent insect community structure and emergence timing than thiamethoxam. The more rapid dissipation of thiamethoxam over the 7 days between reapplication may have contributed to the difference. However, comparative studies of thiamethoxam to other neonicotinoids similarly report lower toxicity in terrestrial and aquatic insect species (Stamm et al. 2001; Jones et al. 2012; Cavallaro et al. 2017, Raby et al. 2018). In our laboratory studies on the model aquatic insect, *Chironomus dilutus*, 40-d (emergence inhibition) toxic equivalency factors for clothianidin (TEF = 1.62 ± 1.28) were similar to imidacloprid (TEF = 1.0) but thiamethoxam was approximately an order of magnitude less toxic (TEF = 0.11 ± 0.02). To date, there are no other published microcosm or mesocosm studies of thiamethoxam on aquatic insect communities. Though adverse community effects of clothianidin have been reported such as reduced abundance, diversity, and evenness, with a NOEC of 1.0 µg/L (dose range, measured concentrations, and LOEC unreported) (U.S. EPA 2011). As a known metabolic breakdown product of clothianidin, thiamethoxam is assessed under a surrogate approach by using clothianidin data (registered degradate CGA-322704) (U.S. EPA 2011). We found clothianidin and thiamethoxam displayed different community-level trajectories during the application and recovery period, and effects on emergence timing were apparent only for clothianidin. In contrast, imidacloprid and clothianidin had unique community and emergence patterns which suggests that effects vary by neonicotinoid compound and assumptions of equivalent toxicity may under or overestimate community responses.

**Impacts on aquatic insect phenology**

Emergence phenology represents a balance to optimize growth (i.e., biomass) and the probability of survival. To my knowledge, few studies have documented effects on emergence...
timing following neonicotinoid exposure. In one pulsed imidacloprid exposure study, mayfly emergence (*Epeorus* sp. and *Baetis* sp.) increased in response to water concentrations of approximately 0.1 µg/L (Alexander et al. 2008). Although we did not measure biomass or size of the insects in our study, reductions in size compared to control treatment have been previously observed (Alexander et al. 2008), which may potentially affect fitness and overall survivorship.

Similar pulsed applications of 100 µg thiacloprid/L to stream mesocosms caused two surges in chironomid emergence immediately after the two applications which the authors attributed to abundant food resources (Kattwinkel et al. 2016). While food supply can alter growth and development, ultimately leading to earlier emergence, it is unlikely that the seasonal availability of food sources would be solely responsible. Alternatively, chronic exposure to low levels of neonicotinoids as larvae may induce a stress response to accelerate development but may simultaneously disrupt processes related to optimal growth, metamorphosis, and survival.

In wetland environments that are highly variable, insects are adapted to increase the rate of emergence when environmental stressors are high in order to enhance survival probability. Enhanced predation threats can also affect the timing and synchrony of emergence events. A laboratory study specifically addressing the relative influence of predation risk and the neonicotinoid imidacloprid found adult *Chironomus riparius* emerged later in all treatments exposed to a high predation risk (Pestana et al. 2009b). The predatory taxa sampled from the emergence traps were restricted to a few odonate species, *Chaoborus americanus*, dytiscid beetles, and, to some extent, Tanypodinae chironomids. The taxa considered as prey items for *Chaoborus americanus* and Tanypodinae chironomids include copepods, cladocerans, and *Tubifex* sp. worms (Swift and Fedorenko 1975; Baker and McLachlan 1979). Dytiscid beetle larvae are cited as aggressive predators, but adult beetles were rare. Among all the limnocorral, 

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damselflies were the most abundant predatory taxa (ranging between 0.37 and 15.1% of the total abundance) but are known to select primarily microcrustaceans though some damselfly species can consume chironomid larvae (Thompson 1978).

In some insect mating systems, swarming is a critical lifecycle event that increases the probability of reproduction. Low-level neonicotinoid exposure may induce timing alterations to swarming events (i.e., premature swarming or futile searching for mates), adding stress to this time-sensitive mating tactic. Among the insect taxa that utilize swarms and exhibit protandry (males emerge slightly before females) are the Chironomidae. On average, neonicotinoid treatments were 13-days (± 2 S.E.) earlier in emergence compared to controls. Chironomid adults are short-lived and benefit from timing emergence events to enhance the probability of finding a mate. Natural and pesticide induced constraints on synchrony, paired with recent findings demonstrating skewed sex ratios from neonicotinoid exposure (Cavallaro et al. 2017), may exacerbate reproductive stress, potentially affecting population stability. Factors like wind (i.e., increased adult dispersal) and proximity to unaffected wetlands may be important to mitigate these risks.

One potential mechanism for the observed effects on emergence timing is that neonicotinoids may disrupt insect growth hormone homeostasis. Insect hormones regulate a number of functions critical to growth and development including molt and metamorphosis and successful transitioning from larva to adult (termed “ecdysis”) (Soin and Smagghe 2007). Failure to complete ecdysis and premature adult emergence are two common sub-lethal effects induced by insect growth regulating insecticides. For example, mosquito larvicides which mimic ecdysteroid agonists target mosquito larvae undergoing metamorphosis where, upon ecdysis, become trapped in their exuvia (Beckage et al. 2004). Similar effects were observed in
chironomids and culicids exposed to imidacloprid under controlled conditions (Song et al. 1997; Cavallaro et al. 2017), suggesting similar physiological complications arising from chronic imidacloprid exposure. It is also possible that neonicotinoid-induced acceleration of the developmental period may interfere with chitin synthesis. Cytochrome P-450, the main detoxification enzymes for neonicotinoids, plays a critical role during hydroxylation in the biosynthesis of ecdysteroids during the final immature larval instar stage (Rewitz et al. 2006).

Phenological plasticity is a fundamental trait for sustaining community-level interactions and supporting biodiversity (Ovaskainen et al. 2013). Changes in insect phenology can have consequences for insectivorous breeding birds in temperate regions, despite some adaptability of northern population to these changes in food resource availability (Charmantier et al. 2008; Gurney et al. 2017). However, a mismatch in peak insect abundance and the avian chick-rearing period may have detrimental long-term impacts (Hansson et al. 2014). Birds, especially long distance migrants, are likely most sensitive to alterations in insect emergence, such that incongruence of phenological events between insect and avian taxa (Both et al. 2009) may be further exacerbated by pesticides. Climate change and the neonicotinoid data presented in the current study indicate that both drivers can simultaneously advance the timing of insect development to the potential detriment of insectivorous consumers that may be unable to adjust their timing of breeding.

Collectively, the limnocorral data presented here indicate that emerging wetland insect communities are influenced by low-level, chronic imidacloprid and clothianidin exposure, albeit in a subtle manner. Like other studies ranking the relative toxic effects of neonicotinoid active ingredients, imidacloprid appeared to exert greater effects than clothianidin and thiamethoxam. Phenology and community structure may be compromised by imidacloprid and clothianidin.

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concentrations that exceed current chronic aquatic life benchmarks. The neonicotinoid mesocosm literature suggests that the threshold for significant lethal and severe sub-lethal impairment is approximately 1.0 µg/L. Here, we observed significantly advanced emergence events by Chironomidae and Zygoptera in imidacloprid and clothianidin treatments at concentrations between 0.038-0.44 µg/L. Significant variation was explained by PRC1 in imidacloprid-treated limnocorrals during the recovery period; suggesting effects on community composition may be delayed or persist after the chemical has degraded. Future studies should investigate the physiological relationship between the rate of aquatic insect emergence and sub-lethal neonicotinoid exposure as it relates to ecdysteroid hydroxylation, chitin synthesis, and metamorphosis. In addition, well-designed field studies are still needed to explore effects of widespread contamination of surface waters with neonicotinoid insecticides in combination with other agricultural and climatic stressors which may alter community dynamics across the wider ecosystem including aquatic and terrestrial insectivorous consumers.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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experimental and study site selection. No external parties influenced the experimental design, objectives, or results of the present study.

Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (christy.morrissey@usask.ca).
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Figure 1. Schematic (A) and photograph (B) of an individual, custom-built limnocorral unit manufactured by Curry Industries Ltd. (Winnipeg, MB, Canada) with an emergence trap.

Figure 2. Mean (± SE) measured, recover corrected concentrations for the high (0.50 µg/L) and low (0.05 µg/L) treatment for each active ingredient immediately post-application, seven days post-application (degradation), and recovery period. The dashed red line denotes the target concentration for each treatment post-application.

Figure 3. Principal response curves (PRC1) displaying the emerging insect community response of replicated limnocorrals (Y-axis determined by canonical coefficients Cdt) over time for three neonicotinoid insecticides, relative to the control limnocorrals (“0” line). Solid and dashed lines represent 0.5 µg/L (high) and 0.05 µg/L (low) treatments, respectively. The scores above 0.15 or below -0.15 on the species weight axis (right) are displayed for clarity. Values near “0” are considered more similar to the control community (i.e., taxa with positive values represent an increase in abundance relative to the controls, whereas negative values represent a decrease in abundance relative to controls). Grey shaded area indicates the 9-week pulsed-dosing period. * Significantly different from the control as determined by a Dunnett's test (p ≤ 0.05).

Figure 4. Nonlinear curve fits of the cumulative relative proportion emerged during the 9 week dosing period of the total 15 week (107-day) limnocorral experiment. Effects from imidacloprid, clothianidin, and thiamethoxam are displayed for three affected taxa; Chironomidae (A.), Chaoboridae (B.), and Zygoptera (C.). Solid lines represent the high (0.05 µg/L) treatment for each active ingredient and the dashed line represents the control treatment. Low (0.05 µg/L) treatments were removed for graph clarity.

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Table 1. Mean (± SE) measured water quality parameters in limnocorals among treatments and abiotic variables.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Imidacloprid</th>
<th>Clothianidin</th>
<th>Thiamethoxam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dissolved oxygen (mg/L)</td>
<td>5.8 (± 0.4)</td>
<td>5.9 (± 0.2)</td>
<td>6.0 (± 0.3)</td>
<td>5.7 (± 0.3)</td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>2209 (± 50)</td>
<td>2210 (± 40)</td>
<td>2252 (± 43)</td>
<td>2225 (± 43)</td>
</tr>
<tr>
<td>pH</td>
<td>8.14 (± 0.05)</td>
<td>8.10 (± 0.04)</td>
<td>8.13 (± 0.03)</td>
<td>8.10 (± 0.03)</td>
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<tr>
<td>Depth (m)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.27 (± 0.0)</td>
<td>1.04 (± 0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Inside limnocorals</td>
<td>20.46 (± 0.7)</td>
<td>15.54 (± 0.4)</td>
<td></td>
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<tr>
<td>Outside limnocorals</td>
<td>20.04 (± 0.7)</td>
<td>15.20 (± 2.2)</td>
<td></td>
<td></td>
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</table>

<sup>a</sup>Recorded on the inner and outer regions of the 3X7 limnocorral block design during the dosing and recovery period.
Table 2. Emergence timing from major taxa groups identified in limnocorral treated with high or low concentrations of imidacloprid, clothianidin, or thiamethoxam neonicotinoids relative to controls. Shown are the best estimates of the mean inflection point in experimental days (i.e., day when 50% cumulative proportion emergence was achieved) and the upper and lower decision limits of the 95% confidence interval (C.I.) around those best estimates. Curve fits were determined by greatest AICc weight; all curves were 2-parameter logistic curves. Equivalence ratios are relative to the rate (i.e., slope) of emergence displayed in control limnocorral. Bold text indicates significantly earlier than the mean inflection point ($\alpha = 0.05$ level of confidence).

<table>
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<tr>
<th>Taxa group</th>
<th>Curve-fit parameters</th>
<th>Control</th>
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<th>Clothianidin</th>
<th>Thiamethoxam</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>High</td>
<td>Low</td>
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<td>All taxa</td>
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<td>Lower</td>
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<tr>
<td></td>
<td>Equivalence ratio</td>
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<td>0.69</td>
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<td>Zygoptera$^b$</td>
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Chaoboridae

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Limnephilidae

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a*Chironomus sp.*, *Ablabesmyia peleensis*, *Psectrocladius sp.*, *Cricotopus sp.*, Tanytarsini sp., Tanypodinae and Orthocladiinae

b*Enallagma annexum* and *Lestes disjunctus*

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cChaoborus americanus
dLimnephilus infernalis and Philarctus quaeris
cAIC<sub>c</sub> weight = 0.962; Mean inflection point = 58.76
fAIC<sub>c</sub> weight = 0.998; Mean inflection point = 44.39
gAIC<sub>c</sub> weight = 0.931; Mean inflection point = 47.43
hAIC<sub>c</sub> weight = 0.513; Mean inflection point = 57.08
iAIC<sub>c</sub> weight = 0.994; Mean inflection point = 91.69
jDefault decision limits = (0.80-1.25); 25% difference from reference curve (i.e., controls)

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