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Effects of the Neonicotinoid Insecticide Clothianidin on Southern Leopard Frog (*Rana sphenocephala*) Tadpole Behavior

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Abstract

Neonicotinoid insecticides are highly water soluble with relatively long half-lives, which allows them to move into and persist in aquatic ecosystems. However, little is known of the impacts of neonicotinoids on non-target vertebrates, especially at sublethal concentrations. We evaluated the effects of the neonicotinoid clothianidin on the behavior of southern leopard frog tadpoles (*Rana sphenocephala*) after a 96-h exposure at 6 concentrations, including 0 (control), 0.375, 0.75, 1.5, 3.0, 6.0 µg/L. We quantified total displacement, mean velocity, maximum velocity, and time spent moving of tadpoles for 1 h post-exposure. Total displacement and mean velocity of tadpoles decreased with clothianidin exposure. Maximum velocity decreased linearly with concentration, but there was no relationship between time spent moving and clothianidin concentration. Our results suggest exposure to clothianidin at sublethal concentrations can affect movement behavior of non-target organisms such as tadpoles.

Keywords Amphibians · Behavior · Ecotoxicology · Exposure · Movement · Neonicotinoids

Neonicotinoids are systemic insecticides widely used around the world. Their relatively long half-lives in water (30–129 days, Lewis et al. 2016) and soil (7–6931 days, Goulson 2013) allow for long-term crop protection, and their water solubility improves the transport and translocation of the insecticide throughout the plant (Goulson 2013). However, they are also prone to leaching through soils and can contaminate waterways (Goulson 2013; Simon-Delso et al. 2015). Because of their widespread use and persistence in the environment, neonicotinoids have been detected in surface waters across a broad geographic range (Sánchez-Bayo et al. 2016). Observed neonicotinoid concentrations in aquatic ecosystems often greatly exceed benchmarks to protect against short term acute effects (< 0.2 µg/L) and long-term chronic impacts (< 0.035 µg/L) on aquatic invertebrate communities (Morrissey et al. 2015).

Non-target animals can be strongly affected by neonicotinoid exposure and it is largely unknown how they impact amphibians, which due to their life history and physiology are highly susceptible to contaminants (De Lange et al. 2009). Many amphibian species have complex life cycles and use aquatic habitats during their larval stage, and terrestrial habitats as juveniles and adults (Wilbur 1980). Thus, they have potential to be exposed to a variety of pesticides during their life cycle. Amphibians are most likely exposed to neonicotinoids through their highly permeable skin (Van Meter et al. 2014) or consumption of contaminated prey. Physiologically, after neonicotinoids have been absorbed or consumed, they can act to overstimulate the nervous system

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by mimicking acetylcholine and binding to the postsynaptic neuron receptors (Matsuda et al. 2001) which can cause behavioral changes (Goulson 2013).

In addition to direct toxicity, insecticides can affect non-target organisms in sublethal ways, including altered metabolism, endocrine disruption, developmental toxicity, immunotoxicity, and behavior (Köhler and Triebskorn 2013). Small changes in behavior can have large impacts on an organism's survival and development. Although the LC50 of amphibian larvae is high for neonicotinoids (100–219 mg/L, Feng et al. 2004; Sánchez-Bayo 2012; Anderson et al. 2015), sublethal concentrations could have a suite of effects contributing to amphibian declines (Carey and Bryant 1995). Some studies report no effect of clothianidin on tadpole mortality by predators, presumably indicating movement did not change (up to 0.35 mg/L, Miles et al. 2017), but others report tadpoles becoming sluggish and unresponsive (200–400 mg/L, Bayci 2011). Behavioral alterations could affect foraging and escape from predators, thus reducing tadpole growth and survival. Despite clothianidin being one of the most common neonicotinoids found in surface waters (Sánchez-Bayo et al. 2016), there is a lack of research on how clothianidin may affect non-target organism behavior at field-realistic concentrations. The current study quantified how clothianidin affects southern leopard frog (*Rana sphenocephala*) tadpole behavior after a 96-h exposure at concentrations of neonicotinoids observed in Missouri wetlands (0–0.970 µg/L, Kuechle et al. 2019). Direct mortality was not expected due to the high LC50s reported for amphibians (Feng et al. 2004; Moe 2017), but we hypothesized clothianidin exposure would affect tadpole total displacement, mean velocity, maximum velocity, and time spent moving.

Materials and Methods

Approximately 500 southern leopard frog tadpoles were collected from a small (~ 2 m diameter), isolated pond surrounded by untreated forest within the Baskett Wildlife Reserve near Ashland, MO, USA on 4 October 2016 and transported to a controlled animal care space at the University of Missouri Greenhouse in Columbia, MO, USA. Animals were slowly switched from source water to well water and the experiment was conducted at U.S. Geological Survey, Columbia Environmental Research Center (CERC) using methods modified from Calfee et al. (2014). An intermittent, proportional, flow-through diluter was maintained at 19°C and provided a control and 5 concentrations (0.375, 0.75, 1.5, 3.0, 6.0 µg/L) of clothianidin through 50% serial dilutions from a standard solution made with analytical grade clothianidin from Sigma-Aldrich International (Darmstadt, Germany) diluted in ultrapure Milli-Q water. The highest clothianidin concentration was set at twice the greatest value for aquatic ecosystems reported in the literature (Main et al. 2014) and the dilution series allowed for evaluation of concentrations measured in Missouri wetlands (Evelsizer and Skopec 2016; Kuechle et al. 2019). The diluter was divided into two spatial blocks with four replicate glass aquaria (28 cm × 13.5 cm × 25 cm) of each concentration in each block. This design resulted in eight replicates of each concentration, for a total of 48 experimental aquaria each with a 6 L volume.

At the beginning of the exposure on 13 October 2016, seven tadpoles (all Gosner stage 25, Gosner 1960, average mass ± SD = 0.071 ± 0.040 g) were randomly assigned to each replicate aquarium. The diluter system delivered about 250 mL of water to each replicate aquarium every hour resulting in a gradual replacement of the 6 L of test water each day. After 96 h, one tadpole from each replicate was moved to a test-water filled observational arena (10 cm diameter, 9 cm depth, ~ 370 mL volume) and recorded for 1 h using video cameras mounted directly above each arena. We wanted each video to be a true replicate, so we only recorded one tadpole from each replicate aquarium. All arenas were standardized for water depth, and were a smaller area than the replicate aquaria to effectively record tadpole movement. At the end of the experiment, tadpoles from the observational arenas were weighed and then euthanized using MS-222 (Leary et al. 2013). Each 1-h video was analyzed for movement metrics using the BioSense computer program (Patman et al. 2018). BioSense recorded observations at a rate of 28 times/s on tadpole location. We calibrated the program using the observation arena diameter (10 cm), which allowed us to convert pixels into real distances (nearest mm). We used greyscale thresholding to filter foreground objects from the background. All tadpoles remained in the field of vision during data collection, and more than 98% of observations were used in calculations. Two of the videos were unusable due to poor image quality or camera error, resulting in four fewer replicates for the 0 and 0.750 µg/L concentrations. Total displacement (cm), mean velocity (cm/s), maximum velocity (cm/s), and time spent moving (s) were calculated for each tadpole. An ANOVA model indicated that tadpole mass was not significantly different across the six clothianidin treatments ($F_{1,38} = 0.86$, $p = 0.36$), so we did not include mass as a covariate in any models.

We monitored diluter function, recorded tadpole survival, siphoned out waste, and measured temperature and dissolved oxygen (DO) daily during the experiment. During the experiment, tadpoles were fed approximately 1.6 (± 0.09 SD) mg per tadpole of 1:1 alfalfa:fish flakes on days 1, 2, and 3 to prevent tadpole-tadpole predation. We measured temperature and DO in two replicates each of the control, 0.375, 1.5, and 6.0 µg/L concentrations using a YSI handheld temperature and DO meter. On the first and last day of exposure, we measured temperature, DO, specific conductance, pH, ammonia, alkalinity, and hardness on a composite sample of each aforementioned concentration using standard methods. We collected five 800 mL water samples (0, 0.375, 1.5, 6.0, and 1.5 µg/L duplicate) from the test chambers for clothianidin concentration analysis on the first and last day of exposure. Samples were stored in 1 L glass amber bottles and chilled at 4°C until extraction. We subsampled 250 mL of water and shipped them in HDPE sample bottles to the U.S. Geological Survey, Organic Chemistry Research Laboratory in Sacramento, CA USA. A portion of each sample (25 mL) was analyzed via solid-phase extraction and liquid chromatography tandem mass spectrometry according to previously published methods (Hladik et al. 2014). The detection limit for clothianidin is 0.0062 µg/L (Hladik and Calhoun 2012), which is well below our lowest test concentration.

We checked for differences in survival by fitting generalized linear models in the ‘bbmle’ package (Bolker and R Core Development Team 2017) with a binomial distribution, logit link function, and concentration and block as predictors. We then used the ‘Anova’ function in the ‘car’ package (Fox and Weisberg 2011) to estimate *p*-values. We estimated how clothianidin exposure affected tadpole movement using the ‘rstanarm’ package (Goodrich et al. 2018) in R (R Core Team 2018). For each movement we tested both linear and quadratic responses to clothianidin with an additional effect of spatial block. For all models, we used weakly informative normal prior distributions with a mean of zero. We ran three chains for 2000 iterations and discarded the first half as warm-up to obtain 3000 simulations for analysis. We confirmed convergence using Gelman–Rubin statistic (R-hat < 1.01, Gelman and Hill 2006) and by examining traceplots. None of the models had influential outliers according to leave-one-out cross-validation (‘loo’) in the ‘rstan’ package (Stan Development Team 2018). We then assessed the relative weight of model fit using pseudo-BMA weights with Bayesian bootstrapping (Yao et al. 2018).

Results and Discussion

Tadpole survival was high across all treatments, with an average 89% survival. Mortality ranged from 0 to 29% in individual replicate aquaria, but based on the binomial model, neither clothianidin concentration ($p = 0.96$) nor block ($p = 0.85$) influenced survival. Tadpole total displacement was strongly negatively affected by clothianidin exposure (Fig. 1a, $b_1 = -1022$ [-1755, -190]), and the relationship was likely quadratic as the 95% credible interval only slightly overlapped zero ($b_2 = 120$ [-6, 240]). Mean velocity was similarly negatively affected by clothianidin (Fig. 1b, $b_1 = -0.19$ [-0.35, -0.03]) and also likely quadratic ($b_2 = -0.04$ [-0.002, 0.048]). Maximum velocity was likely negatively affected by exposure (Fig. 1c), although the credible interval overlapped zero ($b_1 = -0.28$ [-0.90, 0.37]), and there was high uncertainty in the model. The total time spent moving during the test period was very likely negatively related to exposure (Fig. 1d; $b_1 = -79$ [-167, 8]). The effect of block was negligible in all models, as indicated by the highly overlapping credible intervals (Fig. 1).

Water quality parameters remained relatively constant throughout the experiment. Water temperature and DO were consistent (19°C and 8.6 mg/L, respectively) across all treatments and dates. Conductivity, pH, alkalinity (as $CaCO_3$), hardness (as $CaCO_3$), and ammonia were similar between days 0 and 4 (Table 1). Ammonia was higher in the 0 and 0.375 µg/L than in 1.5 and 6.0 µg/L for unknown reasons, but well below the documented acute ammonia threshold of 10 mg/L for amphibians (Hecnar 1995). Mean clothianidin recovery was 75% of our original concentrations when analyzed a year after the experiment (Table 1) and ranged from 68% (0.375 µg/L) to 86% (6.0 µg/L treatment) of the original concentrations.

Despite generally high survival of amphibians exposed to the neonicotinoid clothianidin (Robinson et al. 2017), environmentally relevant concentrations of clothianidin altered multiple aspects of tadpole behavior, supporting our hypothesis. After a 96-h exposure, tadpoles moved less distance, were slower, and spent less time moving. Our study joins a growing body of evidence demonstrating that exposure to neonicotinoids can slow tadpole behavioral responses, even at concentrations as low as 0.25 µg/L (Moe 2017). Behavioral responses such as slower and reduced movements could cause tadpoles to be excluded from optimal foraging habitats and render them susceptible to predation (Griffiths et al. 1991; Boone and Semlitsch 2002).

Many North American amphibian species, including southern leopard frogs, use wetland habitats in the spring and early summer for breeding, which coincides with planting season and the subsequent movement of pesticides into nearby aquatic ecosystems (Main et al. 2014; Hladik et al. 2014). Tadpoles in wetlands adjacent to agricultural fields could be exposed to contaminants such as neonicotinoids (Swanson 2017) throughout their entire larval stage. Spring-breeding species would be present as eggs or tadpoles during the spring planting season and first influx of pesticides from agricultural fields. Amphibian sensitivity to stressors, such as pesticides and salts, varies across species and life stage (Degitz et al. 2000; Harris et al. 2000; Albecker and McCoy 2017). In addition, there may be life history differences in sensitivity within a species between spring breeding cohorts, that would rapidly develop and metamorphose in the summer, and fall breeding cohorts, which overwinter in ponds before metamorphosing the following spring. Thus, the timing, duration, and life history strategy may all be

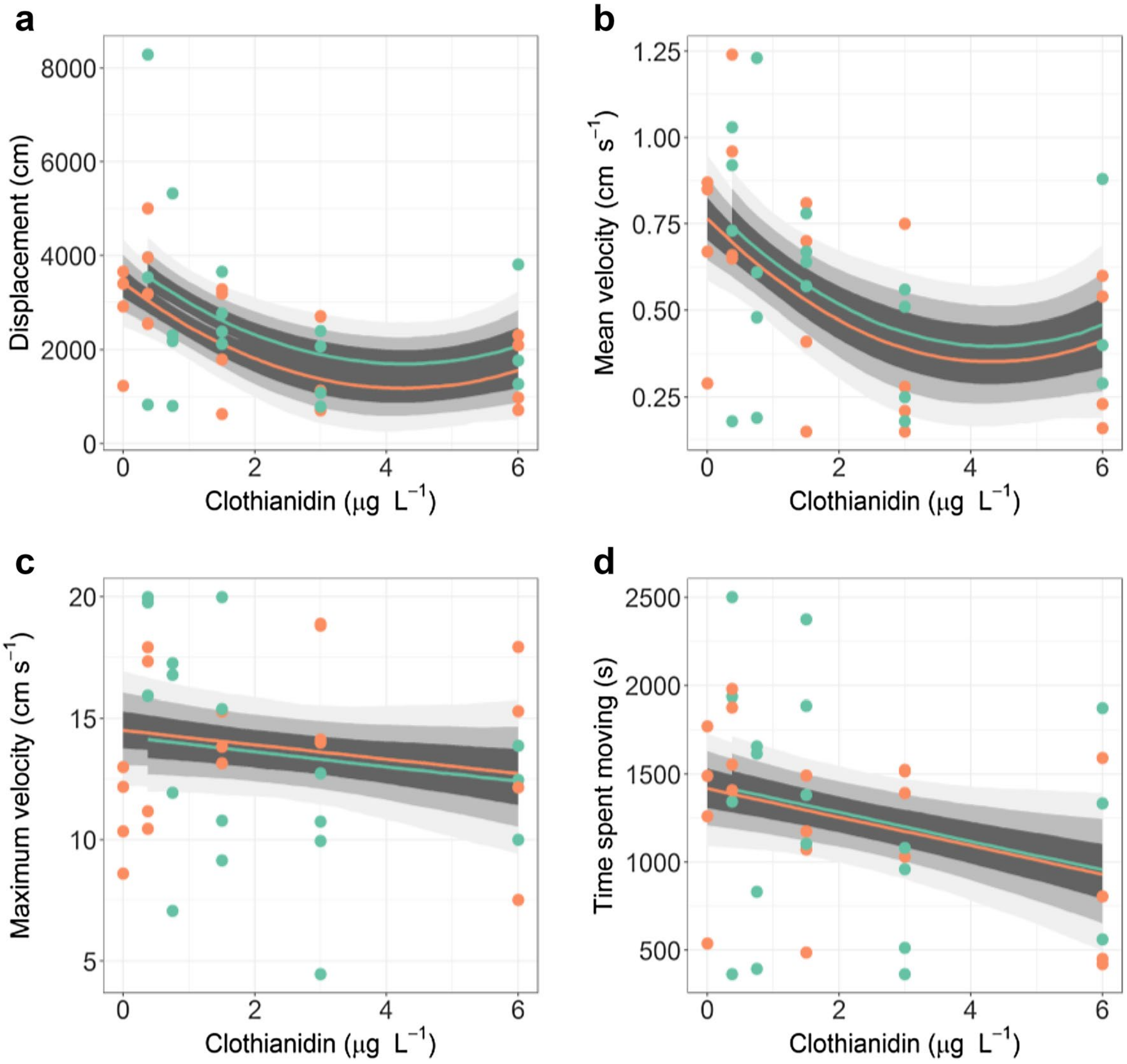

Fig. 1 1-h behavioral assays after a 96-h exposure to clothianidin: **a** total distance moved (displacement), **b** mean velocity, **c** maximum velocity, and **d** total time spent moving during the test period. Each point represents a tadpole. The orange color represents block one and the green block two. Best model fit is shown as regression line with credible intervals (CrIs) by block. Dark gray = 50% CrI, medium gray = 80% CrI, and light gray = 95% CrI

important factors in evaluating the sensitivity of amphibians to neonicotinoids.

Sublethal effects of pesticides can have important consequences for amphibian populations (Boone and Semlitsch 2002) and their surrounding ecosystems (Feng et al. 2004; Evelsizer and Skopec 2016). Our results, in conjunction with the research of Bayci (2011), Moe (2017), and Lee-Jenkins and Robinson (2018) support that sublethal, field-realistic concentrations of neonicotinoid insecticides can cause significant behavioral changes in tadpole movement, which could have substantial impacts on tadpole ability to forage, escape predation, and metamorphose before ponds dry. Neonicotinoids also have potential to alter community dynamics within aquatic ecosystems (Miles et al. 2017), and behavioral changes in tadpoles could affect their ecosystem roles as grazers and translocators of nutrients within and out of aquatic systems (Gibbons et al. 2006; Schmidt et al. 2017). Although vertebrates are generally thought to be insensitive to neonicotinoids, a growing body of literature has noted decreased activity (Crosby et al. 2015; Finnegan et al. 2017) and reduced growth (Hayasaka et al. 2012; DeCant and Barrett 2010) in exposed fishes.

The long half-lives (Goulson 2013; Lewis et al. 2016) of neonicotinoids mean that they can persist in surface waters for hundreds of days, and their low toxicity to vertebrates has made them an appealing alternative to other insecticides. Neonicotinoids are now widespread in surface waters (Sánchez-Bayo et al. 2016), and our study joins a growing body of work (Feng et al. 2004; Moe 2017) demonstrating negative effects of exposure at sublethal neonicotinoids

Table 1 Water quality parameters at the beginning and end of the 96-h exposure including the measured concentrations of clothianidin (ng/L and % recovery (measured/target × 100)]

Nominal clothianidin concentration (µg/L)	Conductivity (µS/cm)	pH	Total alkalinity ($CaCO_3$ mg/L)	Hardness (mg/L)	Total NH_3 (mg N/L)	Measured clothianidin (µg/L)	% Recovery
Beginning							
0	272.9 (0.8)	8.35 (0.03)	97 (1)	100 (0)	0.43 (0.03)	0	–
0.375	271.4 (0.3)	8.35 (0.01)	96 (0)	102 (3)	0.46 (0.03)	0.256	68
1.5	271.0 (0.5)	8.39 (0.01)	97 (1)	103 (2)	0.06 (0.04)	1.089 (0.0008)	73 (0)
6.0	273.2 (4.1)	8.41 (0.02)	98 (2)	104 (0)	0.07 (0.00)	4.883	81
End							
0	277.6 (0.7)	8.36 (0.01)	94 (5)	103 (1)	0.43 (0.20)	0	–
0.375	277.7 (0.2)	8.30 (0.02)	97 (4)	103 (1)	0.45 (0.61)	0.261	70
1.5	277.4 (0.3)	8.30 (0.02)	104 (1)	104 (2)	0.09 (0.11)	1.149 (0.0015)	77 (1)
6.0	270.5 (2.8)	8.33 (0.57)	99 (1)	105 (1)	0.05 (0.03)	5.144	86

Numbers in parentheses are the standard deviation (SD) of two measurements

concentrations. There is a need for more research on how non-target species are affected by chronic exposure to neonicotinoids at non-lethal concentrations.

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