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IMMUNOLOGY AND HOST-PARASITE INTERACTIONS - ORIGINAL PAPER

Sub-chronic exposure to a neonicotinoid does not affect susceptibility of larval leopard frogs to infection by trematode parasites, via either depressed cercarial performance or host immunity

Stacey A. Robinson[1] · M. J. Gavel[2] · S. D. Richardson[1] · R. J. Chlebak[1,2] · D. Milotic[3] · J. Koprivnikar[3] · M. R. Forbes[2]

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Abstract

Little information is available on the effects of neonicotinoid insecticides on vertebrates. Previous work using amphibians found chronic exposure to some neonicotinoids had no detrimental effects on fitness-relevant traits. However, there is some evidence of more subtle effects of neonicotinoids on immune traits and evidence that other pesticides can suppress tadpole immunity resulting in elevated levels of parasitism in the exposed tadpoles. The objective of our study was to assess whether neonicotinoid exposure affected tadpole immunometrics and susceptibility to parasitic helminths. We assessed northern leopard frog tadpole (*Lithobates pipiens*) levels of parasitism and leukocyte profiles following exposure to environmentally relevant concentrations of clothianidin and free-living infective cercariae of a helminth parasite, an *Echinostoma* sp. trematode. When comparing tadpoles from controls to either 1 or 100 μg/L clothianidin treatments, we found similar measures of parasitism (i.e. prevalence, abundance and intensity of echinostome cysts) and similar leukocyte profiles. We also confirmed that clothianidin was not lethal for cercariae; however, slight reductions in swimming activity were detected at the lowest exposure concentration of 0.23 μg/L. Our results show that exposure to clothianidin during the larval amphibian stage does not affect leukocyte profiles or susceptibility to parasitism by larval trematodes in northern leopard frogs although other aspects such as length of host exposure require further study.

Keywords Amphibian · Immunity · Leukocytes · Parasite · Neonicotinoid · Cercariae

Introduction

Environmental stressors and parasite infections can have measurable impacts on host populations (Marcogliese and Pietrock 2011). As occurrences of emerging infectious diseases are increasing at an unprecedented pace (Jones et al. 2008), understanding the relationships between environmental stressors, parasites and hosts has never been more critical. Parasites have a key role in shaping ecological communities (Poulin 1999). However, environmental stressors such as contaminants or thermal stress can influence parasite-host relationships, thus possibly contributing to host population declines or extinction events (Brunner and Eizaguirre 2016). The effects of environmental stressors on host-parasite interactions are not always unidirectional, rather, having both positive and negative effects and can affect hosts and parasites differently (Rohr et al. 2017). For example, while a stressor may increase host susceptibility to parasites, the parasite itself may experience detrimental effects when exposed to the same

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✉ Stacey A. Robinson
Stacey.Robinson@canada.ca

[1] National Wildlife Research Centre, Wildlife and Landscape Science Directorate, Environment and Climate Change Canada, 1125 Colonel By Drive, Ottawa, ON K1A 0H3, Canada

[2] Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada

[3] Department of Chemistry and Biology, Ryerson University, 350 Victoria Street, Toronto, ON M5B 2K3, Canada

environmental stressor, which is especially relevant for parasites with free-living infectious stages (Sures et al. 2017). Importantly, the joint effects of environmental stressors such as toxicants and parasites on hosts can be synergistic, antagonistic, or additive (Coors and Meester 2008). Thus, there is a need for consideration of multiple stressors (e.g. toxicants and parasites) when discerning effects on overall health of hosts. Also, researchers need to take into account how environmental stressors such as toxicants can affect parasites when considering the net effects on hosts (Rohr et al. 2008; Marcogliese and Pietrock 2011).

Amphibians are particularly important when considering the net effects of environmental stressors and parasites because they provide important ecosystem services, such as nutrient cycling, bioturbation and energy flow through insect predation or as prey themselves (Cortes-Gomez et al. 2015). However, since 1975, amphibians have been facing global population declines, with environmental stressors such as contaminants and infectious diseases implicated as possible contributing factors to extinction events (Carey and Bryant 1995; Carey 2000; Boone et al. 2007; Hayes et al. 2010; Blaustein et al. 2011, 2012). Among the pathogens and parasites of concern are the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) Longcore et al., 1999; ranaviruses; and helminth parasites such as the trematodes *Ribeiroia ondatrae* Looss, 1907 and *Echinostoma* sp. Rudolphi, 1809 (Blaustein et al. 2012; Koprivnikar et al. 2012). Amphibians can serve as excellent wildlife bioindicators because of their sensitivity to environmental stressors, particularly during their vulnerable aquatic life stages (Mason et al. 2013). Pesticides in particular have been found to have direct immunotoxic effects on some amphibian species (Hayes et al. 2006), as well as subtle sub-lethal effects such as immunosuppression (Christin et al. 2003; Mann et al. 2009). This can lead to increased vulnerability to parasite or pathogen infection, as well as reduced ability to tolerate or clear infections (e.g. Koprivnikar et al. 2007; Koprivnikar 2010; Pochini and Hoverman 2017b), but environmental contaminants can also affect amphibian parasites directly (e.g. Koprivnikar et al. 2006a; Hua et al. 2016). For example, echinostomatid trematodes can cause substantial mortality in their amphibian hosts (Koprivnikar et al. 2012) and have been listed as an emerging infection of concern due to anthropogenic induced environmental stress (Skelly et al. 2006). However, experimental exposure to an herbicide found that mortality of the free-living infectious stage (cercariae) was outweighed by an increase in host susceptibility such that the net effect was to elevate *Echinostoma trivolvis* infection in larval amphibians (Rohr et al. 2008). Therefore, it is important to quantify the cumulative impacts of environmental stressors that are concurrent for amphibians and their parasites.

Neonicotinoids are a widely used group of systemic insecticides that are persistent in soil and have a high potential for leaching and run-off into aquatic environments due to their high solubility (Anderson et al. 2015; Morrissey et al. 2015). Worldwide, neonicotinoid concentrations in surface waters have been found to range from 0.001 to 320 μg/L for first generation neonicotinoids such as imidacloprid, 0.003 to 3.1 μg/L and 0.001 to 225 μg/L for second-generation neonicotinoids clothianidin and thiamethoxam, respectively (Morrissey et al. 2015). This raises concerns over the potential for these insecticides to leach into agricultural waterways, commonly used by amphibians for breeding and subsequent larval development (Spolyarich et al. 2011; Starner and Goh 2012). A previous study found that the neonicotinoid imidacloprid can increase amphibian survival and can have minor negative effects on development (Robinson et al. 2017). Another study has found that neonicotinoids can increase parasite mortality when present at environmentally relevant concentrations (2 to 64 μg/L; Hua et al. 2016). Despite the high potential for exposure of amphibians concurrently to parasites and neonicotinoids, few studies have examined the effects of neonicotinoids on amphibian susceptibility to disease-causing organisms (Pochini and Hoverman 2017a; Hrynyk et al. 2018). Specifically, no studies have investigated whether neonicotinoids influence amphibian susceptibility to macroparasites such as helminths or whether these insecticides can influence parasite mortality and behaviour when exposed to concentrations measured in the environment. .

Our objective was to assess whether 16 days of exposure to the second-generation neonicotinoid clothianidin at 1 or 100 μg/L affected immunometrics and growth of larval northern leopard frog (*Lithobates pipiens* Schreber, 1782) or their susceptibility to parasitism by *Echinostoma* sp. We chose the lower concentration because it falls within ranges of clothianidin previously detected in the environment (0.003 to 3.1 μg/L; Morrissey et al. 2015) and our higher concentration because of the potential for clothianidin to accumulate in the environment (USEPA 2003). Specifically, we assessed whether exposure to technical grade clothianidin affected amphibian parasite infection levels, blood cell profiles and snout-to-vent length. We further assessed whether various concentrations (0.23, 1, 10, 50, 100 μg/L) of clothianidin affected *Echinostoma* sp. mortality and swimming behaviour. Based on past studies, we predicted that clothianidin would increase host stress levels, which would be reflected in their leukocyte proportions and subsequently increase their susceptibility to parasites. We also predicted that exposure to clothianidin would have no impact on *Echinostoma* sp. mortality or swimming behaviour, thus resulting in a

negative net effect with respect to infection in *L. pipiens*.

Methods

Animal collection and husbandry

Northern leopard frog (*L. pipiens*; formerly *Rana pipiens*) egg clutches from 12 breeding pairs were collected following an artificial breeding protocol (Trudeau et al. 2013; Vu et al. 2017). Twelve egg clutches were distributed evenly between four 60-L aerated plastic stock tanks, where three egg clutches were added to each stock tank. The stock tanks were housed in an environmental chamber at Carleton University (Ottawa, ON) that was on a 16:8-h light/dark cycle and set at 10 °C to simulate cool spring temperatures, slowing larval development to ensure susceptible developmental stages were available when sufficient numbers of free-living infective stages (cercariae) of trematodes became available. Once eggs hatched and tadpoles were free-swimming, they were fed kale (Europe's Best brand) and Ward's tadpole food three times a week ad libitum after 50% water changes using aged (≥ 48 h) City of Ottawa tap water (water aged to reduce chloramine concentration). Tadpoles were fed additional algal wafers (Hikari brand) once a week to sustain them over weekends. After three weeks at 10–12 °C, we increased the temperature daily by 1 to 23 °C. The environmental chamber was maintained at this temperature throughout the tadpole susceptibility experiment.

Nineteen aquatic snails (*Helisoma trivolvis* Say, 1817) infected with *Echinostoma* sp. were collected from several ponds in southern Ontario (collection sites and species identification described in (Milotic et al. 2018) and maintained in aerated glass aquariums containing aged City of Ottawa tap water in the same environmental chamber as the tadpole stock tanks (temperature = 23 °C). The snails were fed fresh baby spinach daily ad libitum, with 50% water changes every 48 to 72 h, as needed.

Experiment no. 1: tadpole susceptibility to parasitism

To test whether exposure to a neonicotinoid increases susceptibility of tadpoles to parasitic infection, we exposed Gosner stage 25 (Gosner 1960) northern leopard frog tadpoles for 16 days (i.e. sub-chronic) to 1 or 100 μg/L of technical grade clothianidin (98% purity; Lot no.: MH39115, AK Scientific, Palo Alto, CA, USA). We used technical grade product since some pesticide additives can have greater toxicity to non-target organisms than the active ingredient itself (Puglis and Boone 2011). Thus, by using a technical grade product, we aimed to better discern the effect of the active ingredient currently used in relevant formulated pesticides on amphibian-parasite relationships. Stock solutions were prepared in deionized water and stored in amber glass bottles in the dark at 4 °C between applications. Exposures were conducted in 9.5-L aerated aquariums (30.5 × 15.2 × 20.3 cm) that each represented a replicate and were filled with 5 L of aged tap water and 1 L of stock tank water. A total of 150 tadpoles were used in the experiment, where we had three treatment concentrations (0, 1, 100 μg/L of clothianidin), 5 replicates/treatment and 10 tadpoles/aquarium (i.e. 50 tadpoles/treatment). Tadpoles, all at Gosner stage 25, were haphazardly selected from the genetically diverse samples in stock tanks and haphazardly added to each treatment aquarium. Tadpoles were photographed for body measurements using ImageJ analysis software (version 1.48; US National Institutes of Health; Schneider et al. 2012) similar range in tadpole size across treatments; mean snout-to-vent length was 7.9 ± 1.3 mm (± standard deviation). Water changes and feeding followed the same protocol as for the stock tanks; however, the aquariums were dosed with the respective volume of stock treatment solution to re-establish exposure concentrations (i.e. static renewal exposures).

Water samples were collected before and 1 h after dosing to verify exposure concentrations throughout the experiment. Concentrations of clothianidin were measured by mass spectrometry using an AB Sciex API 5000 Triple Quadrupole Mass Spectrometer following an accredited method by the Canadian Association for Laboratory Accreditation, which is described in detail in Robinson et al. (2017) and Prosser et al. (2016). Samples were prepared and analyzed in duplicate, and standard reference material was analyzed with each set of samples. The method detection limit for clothianidin was 0.110 μg/L, and the method reporting limit was 0.340 μg/L. The mean standard reference material recovery was 99.9% ± 5.8% ($n = 7$), and the relative percent standard deviation for sample duplicates was 2.4% ± 2.1% ($n = 75$) which demonstrates good method accuracy and precision, respectively. We also monitored water quality (temperature (°C), dissolved oxygen (% and mg/L) and pH) from a sub-sample of randomly (using Random.org) selected aquariums representing each treatment prior to each water change (i.e. every 48 or 72 h) using the YSI Professional Plus (Pro Plus; YSI Incorporated, Yellow Springs, OH, USA).

After 16 days of exposure, we exposed the tadpoles to the *Echinostoma* sp. over two separate days (experiment day 17 and 19; day 0 was the day the first dose was applied), which provided a gradual ecologically relevant exposure to these infective parasitic stages (Ballabeni and Ward 1993; Torchin et al. 2005; Orlofske et al. 2013). Cercariae were stimulated for emergence from the snails by submerging the snails in water in a petri dish and placing the petri dish under a 60-W incandescent lamp (~ 30 cm between lamp and petri dish) for up to 1 h (Szuroczki and Richardson 2009; Milotic et al. 2017). Cercariae from individual snails were pooled to account for genetic variation. Cercariae were counted and added

Table 1 Measured concentrations of clothianidin (CLO) in tadpole- and cercariae-only exposure water. For experiment no. 2, there was only 1 day of exposure; thus, measured concentrations under days 0–14 represent only day 0. Nominal concentrations are represented in the treatment names (i.e. CLO_1 = 1 μg/L of clothianidin). Sample sizes (*n*) presented in parentheses

Treatments	Measured concentrations (mean ± SD; μg/L)		
	Days 0–14	Day 17[a]	Day 19[a]
Experiment no. 1: tadpole susceptibility			
Control	0 ± 0 (*n* = 8)	0 ± 0 (*n* = 5)	0 ± 0 (*n* = 3)
CLO_1	1.0 ± 0.05 (*n* = 8)	0.4 ± 0.02 (*n* = 5)	0.1 ± 0.02 (*n* = 3)
CLO_100	97.4 ± 2.9 (*n* = 8)	38.7 ± 1.75 (*n* = 5)	11.1 ± 1.55 (*n* = 3)
Experiment no. 2: cercariae longevity and activity			
Control	0 (*n* = 1)		
CLO_0.23	0.26 (*n* = 1)		
CLO_0.5	0.48 (*n* = 1)		
CLO_1	1.02 (*n* = 1)		
CLO_10[b]	–		
CLO_50	47.8 (*n* = 1)		
CLO_100	94.9 (*n* = 1)		

[a] Exposure concentrations are decreasing because of 50% water changes without clothianidin renewal between cercariae exposures

[b] Water sample used for CLO_10 cercariae exposure was lost during analysis

to the aquariums within 2 h of emergence from the snails. Water changes continued during the cercariae exposure, but without the renewal of clothianidin. As such, water samples were collected prior to cercariae being added to the aquariums to measure clothianidin exposure concentrations during the cercarial exposures (Table 1). To encourage contact between cercariae and tadpoles in the aquariums, the water was reduced to 5 L (i.e. 0.5 L/tadpole) and the aerators were turned off for the 24-h cercarial exposure period. *Echinostoma* sp. cercariae have a life span of ca. 24 h, where they are the most infective at 6 to 8 h post-emergence (e.g. Fried et al. 1997). Many other studies using larval amphibians and trematodes have used similar densities (0.5–1 L/tadpole) for experimental exposures of > 12 h to ensure high infection success (e.g. Johnson et al. 2012; Koprivnikar et al. 2019). Only 3 of the 5 replicate aquariums in each treatment received 70 cercariae/aquarium on day 17 and another 80 cercariae/aquarium on day 19 because an insufficient number of cercariae were emerging from the snails to allow for all aquariums to receive an adequate number of cercariae (i.e. only 9 of 15 aquariums received cercariae both days). By exposing tadpoles to a higher number of cercariae, we were more likely to get a wide range of infection intensity and thus an additional response variable other than infection status (i.e. infected/uninfected). Intensity of infection is an important measure to assess because it provides information on individual variation in susceptibility to multiple infections (Beldomenico and Begon 2010). Furthermore, as the virulence of parasites such as echinostomes is often dose-dependent (Beaver 1937), the intensity of parasitism can hold implications for tadpole fitness traits such as growth and survival (e.g. Fried et al. 1997; Orlofske et al. 2017). We define prevalence, abundance and intensity of infection following Bush et al. (1997), where prevalence is the percentage of hosts infected, abundance is the number of cysts in a single host and intensity is the number of cysts in a single infected host. Mean abundance is based on all individuals (infected and uninfected), and mean intensity is calculated using only infected individuals.

Tadpoles were maintained for 6 days following the final cercarial addition to allow cercariae to encyst (form metacercariae) in the kidneys and pronephroi and provided an opportunity for the host to mount an immune response (Martin and Conn 1990; Fried et al. 1997; Milotic et al. 2017). The tadpoles were then anesthetized (i.e. day 25 of the experiment) by immersion in 0.02% buffered MS-222 and euthanized in 0.2% buffered MS-222. All tadpoles were photographed for final body size measurements (i.e. snout-to-vent length) using ImageJ analysis software. Haphazard subsamples of 3 of the 10 tadpoles in each treatment replicate were then dissected, where the heart was removed and a heart blood smear was collected on a glass slide (following Gavel et al. 2019) providing a total of 45 blood smears as there were 15 replicates/treatment in total. The blood smears were air-dried, dipped in methanol and again left to air-dry. Once dry, slides were stored, stained at a later date with Giemsa stain (see Milotic et al. 2017) and observed for leukocyte profiles under × 40 magnification on a compound microscope until either 100 fields of view, or 100 leukocytes were examined,

Table 2 Parasitism and leukocyte profiles for northern leopard frog (*Lithobates pipiens*) tadpoles exposed to clothianidin and *Echinostoma* sp. cercariae. Treatments included a control and clothianidin at nominal concentrations of 1 μg/L and 100 μg/L represented by CLO_1 and CLO_100, respectively. Measures of parasitism are presented as prevalence (% infected), abundance (median (25–75% quartile)) and intensity (median (25–75% quartile)) of infection. For leukocyte profiles, mean values ± standard deviations are presented for each treatment with mean percent leukocyte differentials in parentheses. There were no significant differences among controls and either CLO_1 or CLO_100 in any of the parasitism or leukocyte profiles (see Table S3). Sample sizes (Number) are presented

Parasitism and leukocyte profiles	Control	Number	CLO_1	Number	CLO_100	Number
Prevalence	90%	30	90%	30	90%	30
Abundance	7.0 (4.0–11.0)	30	5.5 (3.3–10.0)	30	7.0 (2.3–12.8)	30
Intensity	8.0 (5.0–11.5)	27	7.0 (4.0–10.0)	27	7.0 (4.0–13.5)	27
Neutrophil count	4.4 ± 4.5 (4%)	13	5.7 ± 4.0 (5%)	15	4.4 ± 4.2 (4%)	14
Lymphocyte count	105.3 ± 10.5 (96%)	13	113.6 ± 12.4 (95%)	15	106.4 ± 8.7 (96%)	14
Eosinophil count	0.4 ± 0.7(0.3%)	13	0.6 ± 0.9 (0.4%)	15	0.1 ± 0.4 (0.1%)	14
Erythrocyte count	1623.6 ± 590.9	13	2155.0 ± 857.8	15	1959.1 ± 963.3	14
Leukocyte count	110.1 ± 10.5	13	119.9 ± 13.0	15	110.9 ± 7.3	14
Neutrophil:lymphocyte	0.04 ± 0.05	13	0.05 ± 0.04	15	0.04 ± 0.04	14
Leukocyte:erythrocyte	0.08 ± 0.04	13	0.06 ± 0.03	15	0.07 ± 0.05	14

following Davis et al. (2008) and as described in Milotic et al. (2017). Counts of white blood cells (WBC), including the specific eosinophils, neutrophils, lymphocytes and the red blood cells (RBC), were recorded (Hadji-Azimi et al. 1987). Ratios of total WBC to RBC and ratios of each specific type of WBC to total WBC were calculated.

All 10 tadpoles, including the three selected for heart blood smears, from each treatment replicate (total $n = 90$) were wrapped in methanol rinsed aluminium foil and stored at − 40 °C until necropsy. To quantify the number of *Echinostoma* sp. cysts that established within the kidney and pronephroi of the tadpoles, the tadpoles were thawed and the kidney and pronephroi were removed, placed on a microscope slide and observed under a compound microscope (following Milotic et al. 2017).

Experiment no. 2: cercariae longevity and activity

We assessed the direct toxicity of clothianidin on *Echinostoma* sp. to understand the sensitivity of this free-living parasitic life

Table 3 Results from the best-fit Cox proportional hazards model for time to death (overall Wald $z = 10.02$, $df = 6$, $P = 0.12$) and for the time to reduced swimming activity (overall Wald $z = 13.18$, $df = 6$, $P = 0.04$) for *Echinostoma* sp. cercariae exposed to various concentrations of clothianidin. Treatments included a control and clothianidin at nominal concentrations of 1 μg/L and 100 μg/L represented by CLO_1 and CLO_100, respectively. Exposure to clothianidin was not a significant predictor variable contributing to the time for *Echinostoma* sp. cercariae to die but did significantly influence the time to reduce *Echinostoma* sp. cercariae swimming activity. The hazards ratios are represented by exp(coef) and exp(−coef). Significant differences are in italics

Variable	Coef (= β)	exp(coef)	exp(−coef)	Robust se	z (Wald)	P
(a) Model(Time.Death) = Treatment						
Control vs CLO_0.23	0.33	1.39	0.72	0.28	1.20	0.23
Control vs CLO_0.50	− 0.02	0.98	1.02	0.28	− 0.08	0.94
Control vs CLO_1	0.07	1.07	0.93	0.28	0.25	0.80
Control vs CLO_10	0.09	1.09	0.91	0.27	0.33	0.74
Control vs CLO_50	− 0.32	0.73	1.37	0.28	− 1.15	0.25
Control vs CLO_100	− 0.41	0.66	1.50	0.28	− 1.48	0.14
(b) Model(Time.InActive) = Treatment						
Control vs CLO_0.23	− 0.67	0.51	*1.96*	0.32	− 2.11	*0.03*
Control vs CLO_0.50	0.37	1.45	0.69	0.30	1.23	0.22
Control vs CLO_1	− 0.34	0.71	1.40	0.31	− 1.10	0.27
Control vs CLO_10	− 0.31	0.73	1.37	0.30	− 1.04	0.30
Control vs CLO_50	− 0.23	0.80	1.26	0.30	− 0.75	0.45
Control vs CLO_100	− 0.36	0.70	1.44	0.30	− 1.20	0.23

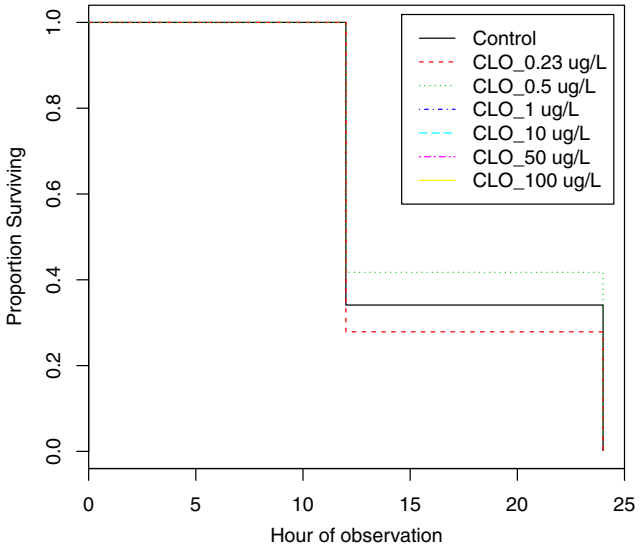

Fig. 1 Estimated survival functions for *Echinostoma* sp. cercariae exposed to various concentrations of clothianidin (i.e. CLO). There was no difference between controls compared to the clothianidin treatments in the proportion of cercariae surviving during the 24 h observation period. Nominal concentrations are represented in the treatment names (i.e. CLO_0.23 = 0.23 μg/L of clothianidin)

stage to clothianidin. Such information is useful in understanding parasite transmission dynamics in a polluted environment (Rohr et al. 2008) and provided insight to understand the measures of infection in our tadpole susceptibility experiment.

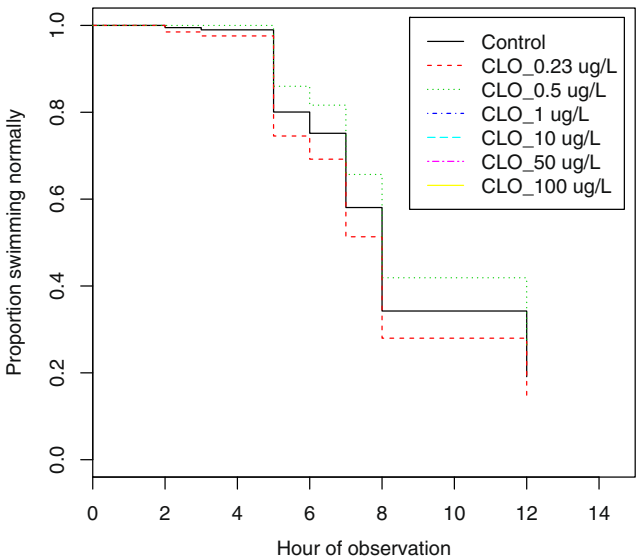

Fig. 2 Estimated survival functions representing time to reduced swimming activity for *Echinostoma* sp. cercariae exposed to various concentrations of clothianidin (i.e. CLO). There was a significant decrease in the proportion of cercariae with normal swimming activity in the CLO_0.23 treatment compared to controls during the 24-h observation period. Note: some cercariae died during the experiment before showing signs of inactivity; therefore, they were censored, and the *x*-axis has been truncated accordingly. Nominal concentrations are represented in the treatment names (i.e. CLO_0.23 = 0.23 μg/L of clothianidin)

On the day of the experiment, cercariae were stimulated to emerge from snails between 09:45 and 10:45 as described above in the tadpole susceptibility experiment. Within 2 h of emergence, the cercariae were distributed to individual wells in 96-well tissue culture plates with treatments randomly assigned to plates (using random.org), following procedures established in similar studies (e.g. Koprivnikar et al. 2006a; Rohr et al. 2008; Hua et al. 2016). Each well contained 100 μL of the respective clothianidin treatment water. We exposed cercariae to 0, 0.23, 0.5, 1, 10, 50 and 100 μg/L of clothianidin using the same technical grade product used in the tadpole susceptibility experiment (measured concentrations reported in Table 1). Each treatment had 5 replicates/plate and we replicated each plate 5 times; hence, 25 cercariae/treatment were assessed. Mortality and swimming activity (yes or no) were monitored at 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 h after cercariae were added to each well using a compound microscope (VanGuard, 1430BR). Normal swimming activity was defined as a consistent and continuous spiraling in the water; abnormal swimming activity was defined as slow circling and crawling on the bottom of the well (e.g. Koprivnikar et al. 2006a).

Statistical analyses

We used R statistical software for all analyses (version 3.4.0; R Core Team 2017). We used a generalized linear model with a binomial distribution and logit link function using the glm function (R Core Team 2017) to determine whether neonicotinoid treatment affected the prevalence of infection. The dependent variable was prevalence of infection, and treatment (control, clothianidin [1 μg/L and 100 μg/L]) and mean Gosner stage per aquarium were fixed effects. We used general(ized) linear mixed models (GLMMs) to determine whether infection abundance and intensity, total RBC, ratio of total WBC to total RBC, ratios of each individual WBC type (i.e. eosinophils, neutrophils and lymphocytes, respectively as a ratio of total WBC, as well as neutrophil to lymphocyte ratio) or final body size (i.e. snout-to-vent length) differed with neonicotinoid treatment (all fixed effects). We used GLMMs with aquarium (i.e. tank) as a random effect within the models to account for non-independence between tadpoles raised in the same aquarium. We used Gaussian distributions and log link function with packages lme4 (Bates et al. 2015) and lmerTest (Kuznetsova et al. 2016). We assessed dispersion to determine if appropriate distributions were used with the count data (i.e. abundance, intensity and total red blood cell count) and refit the model with a different distribution if overdispersion was detected (i.e. dispersion value > 1; Zuur et al. 2015). For the response variables abundance and intensity, we refit the models using a Poisson distribution using the glmmPQL function from the MASS package (Venables and Ripley 2002) to correct for overdispersion (dispersion values

were 29 with Gaussian distributions and were reduced to 1.06 and 1.07 with the Poisson distribution, respectively). Similarly, for the response variable total RBC count, we refit the model using a negative binomial distribution using the glmmADMB package (Fournier et al. 2012; Skaug et al. 2016) to correct for overdispersion (dispersion was 710,441.8 with Gaussian distribution and was reduced to 1.07 with the negative binomial distribution).

We applied data exploration techniques for all GLMMs following Zuur et al. (2009) and Zuur et al. (2010) to detect outliers, assess collinearity and visualize relationships between response variables and predictor variables. We selected the 'best-fit' model using Akaike Information Criterion (AIC; Nakagawa and Cuthill 2007) model selection procedures, where the model with the lowest AIC score was selected (see Supplemental Data, Table S1). Each model was validated by examining residual plots following Zuur et al. (2009) and Zuur et al. (2015). For example, homogeneity of variance was assessed by plotting residuals versus fitted values and model misfit was examined by plotting residuals versus each covariate. Model parameters for the best-fit model were reported following Zuur et al. (2009) and Zuur et al. (2015).

We then used Cox proportional hazards regression analyses to test for the main effects of clothianidin treatment on the following: the likelihood of cercariae to survive in a treatment (control, 0.23, 0.5, 1, 10, 50 and 100 μg/L) and the time elapsed until death and the likelihood of cercariae to maintain normal swimming activity, using the Survival ('survfit') package (Therneau 2015) and the 'coxph' function. Using this package and function, we were able to account for individuals that did not change their swimming activity during the observational period (i.e. right-censored data) and were able to include co-variables in the model (Fox and Weisberg 2011). We compared several Cox proportional hazards models (see Table S1) and tested the assumption of proportional hazards for each model using the 'cox.zph' function. We also considered plate as a clustering variable because cercariae exposed on the same plate (albeit in independent wells) could be considered non-independent observations. We selected the best-fit model by comparing each models AIC scores and selecting the model with the lowest AIC (Nakagawa and Cuthill 2007).

Results

For the tadpole susceptibility experiment, we found measured concentrations of clothianidin ranged from 97 to 100% of the expected nominal concentration for each treatment from day 0 to 14 of the exposure and that no clothianidin was detected in the control aquariums (Table 1). The concentration of clothianidin decreased by day 17 and 19 because of the 50% water changes with no renewal of clothianidin between the cercariae exposures. For the cercariae longevity and activity experiment, measured concentrations were also similar to expected nominal concentrations, where the percent recovery ranged from 95 to 113% (Table 1), and there was no clothianidin contamination in the control sample. For simplicity, all treatment comparisons are presented using nominal concentrations. Throughout the exposure, water temperature, dissolved oxygen and pH were similar between treatments (Table S2), and all measures were within optimal ranges for amphibian husbandry (OECD 2009).

We found 81 of the 90 tadpoles exposed to cercariae contained at least one metacercaria (cyst). There was no difference in the prevalence of infection between controls and the 1 or 100 μg/L treatments (GLMM, $P > 0.05$; Table 2; Table S3), where 90% of the tadpoles were infected in each treatment, including the controls. We also found no significant differences between controls and the two clothianidin treatments in abundance or intensity of infection (Table 2; Table S3). For the leukocyte profiles, we first determined if there was any differences between control tadpoles that had not been exposed to clothianidin or parasites and the tadpoles that had been exposed to parasites only (i.e. Control_P), clothianidin only (i.e. CLO_1_NoP, CLO_100_NoP), or clothianidin and parasites (i.e. CLO_1_P, CLO_100_P) and found no significant differences in any of the leukocyte ratios (WBC:RBC, neutrophils:WBC, lymphocytes:WBC, or neutrophils:lymphocytes) or in total counts of RBC (Table S4). Therefore, parasite-exposed and unexposed tadpole leukocyte profiles were combined within their designated neonicotinoid treatment (i.e. control, CLO_1 and CLO_100) for further analyses. Even with the larger sample size, there were still no significant differences between leukocyte profiles or total RBC counts between controls and CLO_1 or CLO_100 treatments (Table 2; Table S3). We were unable to statistically test for differences in eosinophil:WBC ratios because only 12 of the 42 blood smear counts detected eosinophils (i.e. $n = 4$ tadpoles from controls; $n = 6$ tadpoles from CLO_1; and $n = 2$ tadpoles from CLO_100) with a range of 1 to 3 eosinophils detected per tadpole (Table 2).

We also assessed if clothianidin affected final body size (i.e. snout-to-vent length) of the tadpoles during the experiment and found no significant differences between control and CLO_1 or CLO_100 (Table S3). We found no differences in snout-to-vent length between control tadpoles unexposed to parasites or clothianidin compared to tadpoles exposed to either parasites or a combination of clothianidin and parasites (Table S4). Hence, we used all 147 tadpoles from the experiment to determine whether there were differences in final snout-to-vent length with exposure to clothianidin at 1 or 100 μg/L.

Finally, we assessed if clothianidin was directly toxic to the free-living cercariae by assessing survival and swimming activity. We found no difference between controls and any of the clothianidin treatments in the likelihood of cercariae to die and

the time it took to die within the 24 h observation period (overall Wald $z = 10.02$, $df = 6$, $P = 0.12$; Fig. 1; Table 3). However, we did detect a minor reduction in the likelihood of cercariae to continue normal swimming activity and the time it took for the change in swimming activity in the CLO_0.23 μg/L treatment compared to controls (overall Wald $z = 13.18$, $df = 6$, $P = 0.04$; Fig. 2; Table 3).

Discussion

We found no significant differences in any aspect of parasitism between our controls and either clothianidin treatment, indicating that sub-chronic exposures to sub-lethal concentrations of clothianidin do neither increase susceptibility of larval *L. pipiens* to infection by the *Echinostoma* sp. trematode nor were there any effects on tadpole leukocyte profiles. Our results contrast with multiple studies that have found amphibians exposed to pesticides are more susceptible to parasitism (Rohr et al. 2008; Pochini and Hoverman 2017b). For example, Rohr et al. (2008) found that larval green frogs (*Lithobates clamitans* Latreille, 1801) exposed to pesticides including atrazine, carbaryl, malathion or glyphosate (concentrations of 201 μg/L, 33.5 μg/L, 9.6 μg/L and 3700 μg/L, respectively) for 1 week had increased susceptibility to infection by *E. trivolvis*. Similarly, larval *L. pipiens*, but not American toads (*Anaxyrus americanus* Holbrook, 1836) exposed to either 500 or 1000 μg/L of carbaryl for 1 week, had higher parasite loads than controls when challenged by the trematode *Echinoparyphium* Dietz, 1909 (Pochini and Hoverman 2017b). In addition, we found that exposure to clothianidin had minimal effects on the free-swimming infective stage (cercariae) of the *Echinostoma* sp.

Increased susceptibility of amphibians to parasites following exposure to pesticides is thought to be due to a variety of disruptions to immune functions. Different pesticides can affect amphibian immunity in distinct ways including reducing circulating eosinophils, T cell proliferation and antibody responses (Kiesecker 2002; Christin et al. 2003; Gilbertson et al. 2003). Atrazine, for example, is a known endocrine disruptor because it can mimic various vertebrate hormones (Rohr and McCoy 2009) which can result in modifications to immune functions. Carbaryl is another pesticide that has been shown to reduce larval amphibian activity levels (Bridges 1999) which would reduce behavioural immunity and thus ability to reduce parasite infection. Short-term exposure (1 week) to neonicotinoids has previously been found to decrease corticosterone production in wood frogs (*Lithobates sylvaticus*), while chronic exposure can elicit a moderate state of physiological stress (Gavel et al. 2019). These measures of stress are linked to immune function (Belden and Kiesecker 2005; Davis and Maerz 2009); however, it is possible that neonicotinoids require long exposures at high concentrations to affect immunity compared to other pesticide classes.

Despite this apparent lack of influence on host parasitism, Pochini and Hoverman (2017b) highlight the importance of looking at multiple host species when determining the effects of pesticides on non-target organisms because some amphibian species were more resilient to subsequent parasite challenge. In addition, while our results indicate that short-term exposures to clothianidin do not affect susceptibility to parasitism in *L. pipiens*, it is possible that larval amphibians are chronically exposed to neonicotinoids for longer periods in nature before facing challenge by trematodes such as *Echinostoma* sp. Adult *L. pipiens* will commonly breed and lay eggs in late April to early May, developing to metamorphosis in about 60 to 80 days (Harris et al. 1998; MacCulloch 2002). The larval development of *L. pipiens* likely coincides with peak concentrations of neonicotinoids entering agricultural waterways during rainfall and crop-planting events (Hladik et al. 2014). As neonicotinoids are still a relatively new insecticide, less is known about their concentrations in the environment, compared to other contaminants. However, Hladik et al. (2014) found that clothianidin concentrations peaked in streams after a rainfall event at a concentration of 0.257 μg/L. Clothianidin has a half-life in water of 40.3 days (Hladik et al. 2014), though studies have found that other neonicotinoids such as thiamethoxam can have negligible degradation at water depths greater than 8 cm (Lu et al. 2015). In comparison, first-generation neonicotinoids such as imidacloprid that have been used for longer periods have been found in the environment at concentrations up to 320 μg/L (Morrissey et al. 2015) and clothianidin has been noted to be persistent in the environment and accumulate (USEPA 2003). We believe our concentrations to be relevant. As trematode parasites become most plentiful when water temperatures start to warm in early summer (Szuroczki and Richardson 2009), it is likely that *L. pipiens* larvae will have been chronically exposed to neonicotinoids for weeks before most parasite challenge would occur. In addition, other amphibian species, such as the green frog (*L. clamitans*), have long larval periods where chronic exposure to both pesticides and trematode parasites can occur over 2 breeding seasons (see Skelly et al. 2006). This disparity between lab and field conditions, and life history differences between species, highlights the need for further research investigating the effects of chronic exposure to clothianidin (and other neonicotinoids) on the susceptibility to parasites for a variety of amphibians, before making inferences regarding the safety of these insecticides to these non-target organisms. Alongside susceptibility to parasitism, tolerance of infection is another important aspect to consider.

Our results indicate that sub-chronic exposure to clothianidin and trematodes did not affect *L. pipiens* growth. The impacts of chronic exposure to neonicotinoids on

L. sylvaticus LeConte, 1825 have previously been assessed by Robinson et al. (2017), where much like our results, the study found no effect of chronic exposure to commercially formulated imidacloprid or thiamethoxam at concentrations ranging between 1 and 100 μg/L on amphibian growth. Our results are further corroborated by a study conducted by Robinson et al. (2019) which found no effect of chronic exposure to commercially formulated thiamethoxam or clothianidin (at 2.5 or 250 μg/L) on *L. pipiens* growth. In nature, amphibians are commonly faced with trade-offs in terms of energy expenditure (Gervasi and Foufopoulos 2008). For example, amphibians that develop in desiccating pond environments may accelerate their time to metamorphosis but have a corresponding weaker immune system (Gervasi and Foufopoulos 2008). Decreases in amphibian growth in the presence of pesticides and parasites, i.e. reduced tolerance of infection, have been noted by other studies. For example, Koprivnikar (2010) found a significantly reduced growth in trematode-infected larval *L. pipiens* chronically exposed to 3 μg/L of atrazine relative to tadpoles only exposed to the pesticide stressor. As the larval amphibians in our present study showed no evidence of increased investment in immune function, this may have corresponded with no need for an energetic-trade off in terms of reduced growth. However, reduced infection tolerance may only manifest under chronic rather than sub-chronic contaminant exposure; thus, tadpole growth and condition should be assessed over longer periods than the 6-day post-infection here.

Alongside no effect of clothianidin exposure on tadpole infection, we found that exposure to clothianidin for 16 days at 1 and 100 μg/L had no significant impact on *L. pipiens* blood cell profiles, where N:L ratios in all treatments resemble baseline ratios for leopard frogs (Davis and Maerz 2011). Our results are supported by a study conducted by Gavel et al. (2019), which found that chronic exposure (~46 days) to commercially formulated clothianidin at 2.5 and 250 μg/L also had no impact on *L. sylvaticus* leukocyte profiles. Our results are also in accordance with a study conducted by Paetow et al. (2012), which found that 21-day exposure of juvenile *L. pipiens* to varying concentrations of atrazine and glyphosate (concentrations ranged from 1.7 to 4.28 μg/L and 7 to 100 μg/L, respectively) did not have an impact on leukocyte profiles. However, leukocyte responses in amphibians have previously been shown to be affected by exposure to pesticides (Kiesecker 2002). In a laboratory study conducted by Kiesecker (2002), *L. sylvaticus* tadpoles that were chronically exposed to a mixture of pesticides had altered proportions of circulating leukocytes and, in particular, had decreased numbers of circulating eosinophils. Similarly, adult *L. pipiens* acutely exposed to 21 μg/L of atrazine for 8 days, had suppressed leukocyte responses and decreased phagocytic activity (Brodkin et al. 2007). The lack of observed effect in circulating leukocytes in our study helps to explain the lack of differences seen in parasite susceptibility. In Kiesecker (2002), pesticide-induced alterations to circulating leukocytes were associated with an increased susceptibility to the trematode *Ribeiroia* sp. Our results indicate that short-term exposures to clothianidin likely do not affect leukocyte-mediated immune function in *L. pipiens* or alter their susceptibility to trematode parasites.

While our study focused on the immunometrics of susceptibility to parasites, the effects of neonicotinoids on amphibian susceptibility to infection can be multifaceted. In particular, behaviour can have a large impact on tadpole infections by trematode parasites, with tadpoles evading cercariae either through swimming away or changing their swimming patterns (Koprivnikar et al. 2006b; Budischak et al. 2009). While our data combined with Gavel et al. (2019) suggests that sub-chronic and chronic exposures to clothianidin do not affect tadpole immune function, a recent study found that chronic exposure to neonicotinoids can alter predator escape behaviour in post-metamorphic juvenile *L. sylvaticus* (Lee-Jenkins and Robinson 2018). Contaminant-induced reductions in tadpole anti-parasite behaviours can cause increased susceptibility to trematode parasitism, such as those reported for road salt exposure (Milotic et al. 2017). These results indicate that more research is required to ascertain whether chronic exposure to clothianidin can similarly affect tadpole susceptibility to parasites through behavioural mechanisms and the extent to which housing or experimental protocols allow such behaviours to be expressed.

It is important not only to quantify impacts of contaminants on hosts but also on motile parasite infectious stages if these can also be exposed in natural settings. In the present study, we found no impacts of clothianidin on *Echinostoma* sp. mortality, but we did find a slight effect on swimming behaviour. Specifically, cercariae exposed to 0.23 μg/L of clothianidin were less likely to continue normal swimming behaviour over time. We observed decreases in normal swimming behaviour at the 5-h mark (Fig. 2), where the cercariae had slowed their spiraling swimming pattern to resemble slow circling and started crawling on the bottom of the well. This indicates that exposure to clothianidin could potentially affect transmission by reduced cercariae ability to encounter hosts because *Echinostoma* sp. are typically most active and infective until 6–8 h after emergence (Fried et al. 1997). Previous studies have also noted that low concentrations of contaminants can affect parasite infectivity, while unusually high concentrations are required to have an effect on mortality (Pietrock and Marcogliese 2003). The reduction in *Echinostoma* sp. activity at lower concentrations of clothianidin could also be indicative of reduced infectivity as well (i.e. ability to establish within hosts), thus possibly affecting infection levels as well. However, as a 0.23-μg/L clothianidin concentration was not assessed in our tadpole experiments, further research is required to ascertain the impacts of low concentrations of

clothianidin on parasite-host interactions, particularly because non-monotonic responses can occur (e.g. Milotic et al. 2017).

Overall, our study indicates that sub-chronic exposure to clothianidin likely does not have an impact on amphibian-trematode relationships through alterations of host susceptibility or parasite survival and does not have synergistic, additive or antagonistic effects on infection tolerance. This is in contrast to previous studies with other contaminants, which have noted that parasites and pesticides can act synergistically to have negative impacts on amphibians (Kiesecker 2002; Christin et al. 2003). While our results suggest that changes in amphibian parasitism are unlikely to result from clothianidin-induced reductions in cercarial performance and/or tadpole immunosuppression, there are other components that we did not quantify in relation to neonicotinoids. For example, our study did not investigate the effects of clothianidin on the freshwater snail intermediate hosts of *Echinostoma* sp. necessary for cercariae development. Previous studies have found that freshwater snails have a very high tolerance to neonicotinoids (Prosser et al. 2016; Miles et al. 2017), so they are likely to be present and serve as intermediate hosts; however, whether pesticide exposure is harmful to infected snails, or reduces cercariae output (Koprivnikar and Walker 2011), should be examined.

Interestingly, the Miles et al. (2017) study found that exposure to high concentrations of clothianidin increased mortality of invertebrate snail predators. Furthermore, the study noted that water bugs (*Belostoma flumineum* Say, 1832) exposed to 100 μg/L of clothianidin decreased their consumption of snails by as much as 62% (Miles et al. 2017). These results could hold implications for amphibian exposure to trematode parasites. Previous studies have shown that the effects of anthropogenic contaminants on snail populations can have cascading effects for other trophic levels. For example, Johnson et al. (2007) illustrated that agricultural runoff contributed to eutrophic conditions, creating a habitat where snail populations thrived. This indirectly increased *R. ondatrae* infections in amphibians and subsequently increased amphibian mortality (Johnson et al. 2007). Similarly, Rohr et al. (2015) found that the presence of aquatic invertebrates that consume cercariae, or consume snails serving as first intermediate hosts, was associated with reduced trematode infections in larval amphibians. If neonicotinoids indirectly decrease snail mortality through their negative effects on their invertebrate predators, similar results could occur wherein amphibians face higher parasitism loads and greater mortality. Future research is required to ascertain the potential multitrophic level impacts of neonicotinoids on amphibian-parasite relationships.

In summary, our study indicated that sub-chronic exposure to 1 or 100 μg/L of clothianidin did not affect parasite infection levels, blood cell profiles or growth of *L. pipiens*, thus showing no evidence of a negative synergistic relationship between clothianidin and parasites during short-term exposures. Furthermore, our study illustrated that clothianidin exposure did not affect *Echinostoma* sp. infectious stage (cercariae) mortality, but there was a slight negative effect on swimming behaviour at 0.23 μg/L. However, many gaps remain in our understanding of the impacts of neonicotinoids on amphibian-parasite relationships. Most importantly, there is a need for an assessment of whether longer-term, chronic exposure to clothianidin or the other neonicotinoids can affect amphibian susceptibility or tolerance to parasites. Furthermore, the effects of neonicotinoids on multiple amphibian and trematode species, as well as other trophic levels, should be investigated. By undertaking a community ecology approach in assessing the impacts of neonicotinoids on non-target organisms, we can deduce more ecologically relevant conclusions required for effective management strategies in the conservation of amphibians.

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Data availability All data generated or analysed during this study are included in this published article and its supplementary information files.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Statement on the welfare of animals All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Environment and Climate Change Canada Wildlife Eastern Animal Care Committee, SR01-2016). This article does not contain any studies with human participants performed by any of the authors.

References

Anderson JC, Dubetz C, Palace VP (2015) Neonicotinoids in the Canadian aquatic environment: a literature review on current use products with a focus on fate, exposure, and biological effects. Sci Total Environ 505:409–422. https://doi.org/10.1016/j.scitotenv.2014.09.090

Ballabeni P, Ward PI (1993) Local adaptation of the trematode Diplostomum phoxini to the European minnow Phoxinus phoxinus, in its second intermediate host. Funct Ecol 7:84–90

Bates D, Maechler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. J Stat Softw 67:1–48. https://doi.org/10.18637/jss.v067.i01

Beaver PC (1937) Experimental studies on *Echinostoma revolutum* (Froelich), a fluke from birds and mammals. Ill Biol Monogr 15:1–96

Belden LK, Kiesecker JM (2005) Glucocorticosteroid hormone treatment of larval treefrogs increases infection by Alaria sp. trematode cercariae. J Parasitol 91:686–688. https://doi.org/10.1645/GE-397R

Beldomenico PM, Begon M (2010) Disease spread, susceptibility and infection intensity: vicious circles? Trends Ecol Evol 25:21–27

Blaustein AR, Han BA, Relyea RA, Johnson PTJ, Buck JC, Gervasi SS, Kats LB (2011) The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. Ann N Y Acad Sci 1223:108–119. https://doi.org/10.1111/j.1749-6632.2010.05909.x

Blaustein AR, Gervasi SS, Johnson PTJ, Hoverman JT, Belden LK, Bradley PW, Xie GY (2012) Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philos Trans R Soc B 367:1688–1707

Boone MD, Semlitsch RD, Little EE, Doyle MC (2007) Multiple stressors in amphibian communities: effects of chemical contamination, bullfrogs, and fish. Ecol Appl 17:291–301

Bridges CM (1999) Effects of a pesticide on tadpole activity and predator avoidance behavior. J Herpetol 33:303–306. https://doi.org/10.2307/1565728

Brodkin MA, Madhoun H, Rameswaran M, Vatnick I (2007) Atrazine is an immune disruptor in adult northern leopard frogs (Rana pipiens). Environ Toxicol Chem 26:80–84

Brunner FS, Eizaguirre C (2016) Review: Can environmental change affect host/parasite-mediated speciation? Zoology 119:384–394. https://doi.org/10.1016/j.zool.2016.04.001

Budischak SA, Belden LK, Hopkins WA (2009) Relative toxicity of malathion to trematode-infected and noninfected Rana palustris tadpoles. Arch Environ Contam Toxicol 56:123–128. https://doi.org/10.1007/s00244-008-9167-9

Bush AO, Lafferty KD, Lotz JM, Shostak AW (1997) Parasitology meets ecology on its own terms: Margolis et al. J Parasitol 83:575–583

Carey C (2000) Infectious disease and worldwide declines of amphibian populations, with comments on emerging diseases in coral reef organisms and in humans. Environ Health Perspect 108:143–150. https://doi.org/10.1289/ehp.00108s1143

Carey C, Bryant CJ (1995) Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ Health Perspect 103:13–17. https://doi.org/10.2307/3432406

Christin MS, Dendron AD, Brousseau P et al (2003) Effects of agricultural pesticides on the immune system of Rana pipiens and on its resistance to parasitic infection. Environ Toxicol Chem 22:1127–1133

Coors A, Meester LD (2008) Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in Daphnia magna. J Appl Ecol 45:1820–1828. https://doi.org/10.1111/j.1365-2664.2008.01566.x

Cortes-Gomez AM, Ruiz-Agudelo CA, Valencia-Aguilar A, Ladle RJ (2015) Ecological functions of neotropical amphibians and reptiles: a review. Univ Sci 20:229–245. https://doi.org/10.11144/Javeriana.SC20-2.efna

Davis AK, Maerz JC (2009) Effects of larval density on hematological stress indices in salamanders. J Exp Zool Part A 311:697–704. https://doi.org/10.1002/jes.557

Davis AK, Maerz JC (2011) Assessing stress levels of captive-reared amphibians with hematological data: implications for conservation initiatives. J Herpetol 45:40–44. https://doi.org/10.1670/10-180.1

Davis AK, Maney DL, Maerz JC (2008) The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct Ecol 22:760–772. https://doi.org/10.1111/j.1365-2435.2008.01467.x

Fournier DA, Skaug HJ, Ancheta J, Ianelli J, Magnusson A, Maunder MN, Nielsen A, Sibert J (2012) AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. Optim Methods Softw 27:233–249. https://doi.org/10.1080/10556788.2011.597854

Fox J, Weisberg S (2011) Cox proportional-hazards regression for survival data in R. In: An R companion to applied regression, second edi. Sage, Thousand Oaks, pp 1–20

Fried B, Pane PL, Reddy A (1997) Experimental infection of Rana pipiens tadpoles with Echinostoma trivolvis cercariae. Parasitol Res 83:666–669

Gavel MJ, Richardson SD, Dalton RL, Soos C, Ashby B, McPhee L, Forbes MR, Robinson SA (2019) Effects of two neonicotinoid insecticides on blood cell profiles and corticosterone concentrations of wood frogs (Lithobates sylvaticus). Environ Toxicol Chem 38:1273–1284

Gervasi SS, Foufopoulos J (2008) Costs of plasticity: responses to desiccation decrease post-metamorphic immune function in a pond-breeding amphibian. Funct Ecol 22:100–108. https://doi.org/10.1111/j.1365-2435.2007.01340.x

Gilbertson M, Haffner GD, Droullard KG, Albert A, Dixon B (2003) Immunosuppression of the northern leopard frog (*Rana pipiens*) induced by pesticide exposure. Environ Toxicol Chem 22(1):101–110

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190. https://doi.org/10.2307/3890061

Hadji-Azimi I, Coosemans V, Canicatti C (1987) Atlas of adult Xenopus laevis laevis hematology. Dev Comp Immunol 11:807–874. https://doi.org/10.1016/0145-305X(87)90068-1

Harris ML, Bishop CA, Struger J, Ripley B, Bogart JP (1998) The functional integrity of northern leopard frog (Rana pipiens) and green frog (Rana clamitans) populations in orchard wetlands. Effects of pesticides and eutrophic conditions on early life stage development. Environ Toxicol Chem 17:1351–1363. https://doi.org/10.1002/etc.5620170720

Hayes TB, Case P, Chui S, Chung D, Haeffele C, Haston K, Lee M, Mai VP, Marjuoa Y, Parker J, Tsui M (2006) Pesticide mixtures, endocrine disruption, and amphibian declines: are we underestimating the impact? Environ Health Perspect 114:40–50. https://doi.org/10.1289/ehp.8051

Hayes TB, Falso P, Gallipeau S, Stice M (2010) The cause of global amphibian declines: a developmental endocrinologist's perspective. J Exp Biol 213:921–933. https://doi.org/10.1242/jeb.040865

Hladik ML, Kolpin DW, Kuivila KM (2014) Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. Environ Pollut 193:189–196. https://doi.org/10.1016/j.envpol.2014.06.033

Hrynyk MA, Brunetti C, Kerr L, Metcalfe CD (2018) Effect of imidacloprid on the survival of *Xenopus* tadpoles challenged with wild type frog virus 3. Aquat Toxicol 194:152–158. https://doi.org/10.1016/j.aquatox.2017.11.005

Hua J, Buss J, Kim J et al (2016) Population-specific toxicity of six insecticides to the trematode Echinoparyphium sp. Parasitology 143:542–550. https://doi.org/10.1017/S0031182015001894

Johnson PTJ, Chase JM, Dosch KL, Hartson RB, Gross JA, Larson DJ, Sutherland DR, Carpenter SR (2007) Aquatic eutrophication promotes pathogenic infection in amphibians. Proc Natl Acad Sci 104:15781–15786. https://doi.org/10.1073/pnas.0707763104

Johnson PT, Rohr JR, Hoverman JT et al (2012) Living fast and dying of infection: host life history drives interspecific variation in infection and disease risk. Ecol Lett 15:235–242. https://doi.org/10.1111/j.1461-0248.2011.01730.x

Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, Daszak P (2008) Global trends in emerging infectious diseases. Nature 451:990–994. https://doi.org/10.1038/nature06536

Kiesecker JM (2002) Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? Proc Natl Acad Sci U S A 99:9900–9904. https://doi.org/10.1073/pnas.152098899

Koprivnikar J (2010) Interactions of environmental stressors impact survival and development of parasitized larval amphibians. Ecol Appl 20:2263–2272. https://doi.org/10.1890/09-1558.1

Koprivnikar J, Walker PA (2011) Effects of the herbicide atrazine's metabolites on host snail mortality and production of trematode cercariae. J Parasitol 97:822–827. https://doi.org/10.1645/GE-2814.1

Koprivnikar J, Forbes MR, Baker RL (2006a) On the efficacy of anti-parasite behaviour: a case study of tadpole susceptibility to cercariae of Echinostoma trivolvis. Can J Zool 84:1623–1629

Koprivnikar J, Forbes MR, Baker RL (2006b) Effects of atrazine on cercarial longevity, activity, and infectivity. J Parasitol 92:306–311. https://doi.org/10.1645/GE-624R.1

Koprivnikar J, Forbes MR, Baker RL (2007) Contaminant effects on host-parasite interactions: atrazine, frogs, and trematodes. Environ Toxicol Chem 26:2166–2170. https://doi.org/10.1897/07-220.1

Koprivnikar J, Marcogliese DJ, Rohr JR, Orlofske SA, Raffel TR, Johnson PTJ (2012) Macroparasite infections of amphibians: what can they tell us? Ecohealth 9:342–360. https://doi.org/10.1007/s10393-012-0785-3

Koprivnikar J, Hoye BJ, Urichuk TM, Johnson PT (2019) Endocrine and immune responses of larval amphibians to trematode exposure. Parasitol Res 118:275–288. https://doi.org/10.1007/s00436-018-6154-6

Kuznetsova A, Brockhoff PB, Haubo Bojesen R (2016) lmerTest: tests in linear mixed effects models. R package version 2.0–33

Lee-Jenkins SS, Robinson SA (2018) Effects of neonicotinoids on putative escape behaviour of juvenile wood frogs (Lithobates sylvaticus) chronically exposed as tadpoles. Environ Toxicol Chem 37:3115–3123. https://doi.org/10.1002/etc.4284

Lu Z, Challis JK, Wong CS (2015) Quantum yields for direct photolysis of neonicotinoid insecticides in water: implications for exposure to non-target aquatic organisms. Environ Sci Technol Lett 2:188–192. https://doi.org/10.1021/acs.estlett.5b00136

MacCulloch RD (2002) The ROM field guide to amphibians and reptiles of Ontario. McClelland & Stewart Ltd

Mann RM, Hyne RV, Choung CB, Wilson SP (2009) Amphibians and agricultural chemicals: review of the risks in a complex environment. Environ Pollut 157:2903–2927. https://doi.org/10.1016/j.envpol.2009.05.015

Marcogliese DJ, Pietrock M (2011) Combined effects of parasites and contaminants on animal health: parasites do matter. Trends Parasitol 27:123–130. https://doi.org/10.1016/j.pt.2010.11.002

Martin TR, Conn DB (1990) The pathogenicity, localization, and cyst structure of echinostomatid metacercariae (trematode) infecting the kidneys of the frogs Rana clamitans and Rana pipiens. J Parasitol 76:414–419

Mason R, Tennekes H, Sanchez-Bayo F, Jepsen PU (2013) Immune suppression by neonicotinoid insecticides at the root of global wildlife declines. J Environ Immunol Toxicol 1:3–12. https://doi.org/10.7178/jeit.1

Miles JC, Hua J, Sepulveda MS, Krupke CH, Hoverman JT (2017) Effects of clothianidin on aquatic communities: evaluating the impacts of lethal and sublethal exposure to neonicotinoids. PLoS One 12:e0174171. https://doi.org/10.4231/R7RX992T

Milotic D, Milotic M, Koprivnikar J (2017) Effects of road salt on larval amphibian susceptibility to parasitism through behavior and immunocompetence. Aquat Toxicol 189:42–49. https://doi.org/10.1016/j.aquatox.2017.05.015

Milotic M, Milotic D, Koprivnikar J (2018) Exposure to a cyanobacterial toxin increases larval amphibian susceptibility to parasitism. Parasitol Res 117:513–520

Morrissey CA, Mineau P, Devries JH, Sanchez-Bayo F, Liess M, Cavallaro MC, Liber K (2015) Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ Int 74:291–303

Nakagawa S, Cuthill I (2007) Effect size, confidence interval and statistical significance: a practical guide for biologists. Biol Rev 82:591–605. https://doi.org/10.1111/j.1469-185X.2007.00027.x

OECD (2009) Test no. 231: amphibian metamorphosis assay, OECD guidelines for testing of chemicals section 2: effects on biotic systems. 33

Orlofske SA, Belden LK, Hopkins WA (2013) Larval wood frog (Rana [= Lithobates] sylvatica) development and physiology following infection with the trematode parasite, Echinostoma trivolvis. Comp Biochem Physiol A Mol Integr Physiol 164:529–536. https://doi.org/10.1016/j.cbpa.2012.12.013

Orlofske SA, Belden LK, Hopkins WA (2017) Effects of Echinostoma trivolvis metacercariae infection during development and metamorphosis of the wood frog (Lithobates sylvaticus). Comp Biochem Physiol A Mol Integr Physiol 203:40–48. https://doi.org/10.1016/j.cbpa.2016.08.002

Paetow LJ, McLaughlin JD, Cue RI et al (2012) Effects of herbicides and the chytrid fungus Batrachochytrium dendrobatidis on the health of post-metamorphic northern leopard frogs (Lithobates pipiens). Ecotoxicol Environ Saf 80:372–380. https://doi.org/10.1016/j.ecoenv.2012.04.006

Pietrock M, Marcogliese DJ (2003) Free-living endohelminth stages: at the mercy of environmental conditions. Trends Parasitol 19:293–299. https://doi.org/10.1016/s1471-4922(03)00117-x

Pochini KM, Hoverman JT (2017a) Reciprocal effects of pesticides and pathogens on amphibian hosts: the importance of exposure order and timing. Environ Pollut 221:359–366. https://doi.org/10.1016/j.envpol.2016.11.086

Pochini KM, Hoverman JT (2017b) Immediate and lag effects of pesticide exposure on parasite resistance in larval amphibians. Parasitology 144:817–822. https://doi.org/10.1017/S0031182016002560

Poulin R (1999) The functional importance of parasites in animal communities: many roles at many levels? Int J Parasitol 29:903–914

Prosser RS, De Solla SR, Holman EAM et al (2016) Sensitivity of the early-life stages of freshwater mollusks to neonicotinoid and butenolide insecticides. Environ Pollut 218:428–435. https://doi.org/10.1016/j.envpol.2016.07.022

Puglis HJ, Boone MD (2011) Effects of technical-grade active ingredient vs. commercial formulation of seven pesticides in the presence or absence of UV radiation on survival of green frog tadpoles. Arch Environ Contam Toxicol 60:145–155. https://doi.org/10.1007/s00244-010-9528-z

R Core Team (2017) R: a language and environment for statistical computing, 3.4.0. Doc. Free. available internet http//www. r-project. org

Robinson SA, Richardson SD, Dalton RL, Maisonneuve F, Trudeau VL, Pauli BD, Lee-Jenkins SSY (2017) Sublethal effects on wood frogs chronically exposed to environmentally relevant concentrations of two neonicotinoid insecticides. Environ Toxicol Chem 36:1101–1109. https://doi.org/10.1002/etc.3739

Robinson SA, Richardson SD, Dalton RL, Maisonneuve F, Bartlett AJ, Solla SR, Trudeau V, Waltho N (2019) Assessment of sub-lethal effects of neonicotinoid insecticides on the life-history traits of two frog species. Environ Toxicol Chem. https://doi.org/10.1002/etc.4511

Rohr JR, McCoy KA (2009) A qualitative meta-analysis reveals consistent effects of atrazine on freshwater fish and amphibians. Environ Health Perspect 118:20–32. https://doi.org/10.1289/ehp.0901164

Rohr JR, Raffel TR, Sessions SK, Hudson PJ (2008) Understanding the net effects of pesticides on amphibian trematode infections. Ecol Appl 18:1743–1753

Rohr JR, Civitello DJ, Crumrine PW, Halstead NT, Miller AD, Schotthoefer AM, Stenoien C, Johnson LB, Beasley VR (2015) Predator diversity, intraguild predation, and indirect effects drive parasite transmission. Proc Natl Acad Sci 112:3008–3013. https://doi.org/10.1073/pnas.1415971112

Rohr JR, Brown J, Battaglin WA, McMahon TA (2017) A pesticide paradox: fungicides indirectly increase fungal infections. Ecol Appl 27:2290–2302. https://doi.org/10.1002/eap.1607

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Methods 9:671–675

Skaug HJ, Fournier DA, Bolker BM, et al (2016) Generalized linear mixed models using "AD Model Builder"

Skelly DK, Bolden SR, Holland MP et al (2006) Urbanization and disease in amphibians. In: Collinge SK, Ray C (eds) Disease ecology: community structure and pathogen dynamics. Oxford University Press, New York, pp 153–167

Spolyarich N, Hyne RV, Wilson SP, Palmer CG (2011) Morphological abnormalities in frogs from a rice-growing region in NSW, Australia, with investigations into pesticide exposure. Environ Monit Assess 173:397–407. https://doi.org/10.1007/s10661-010-1395-6

Starner K, Goh KS (2012) Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010-2011. Bull Environ Contam Toxicol 88: 316–321. https://doi.org/10.1007/s00128-011-0515-5

Sures B, Nachev M, Selbach C, Marcogliese DJ (2017) Parasite responses to pollution: what we know and where we go in "environmental parasitology.". Parasit Vectors 10:1–19. https://doi.org/10.1186/s13071-017-2001-3

Szuroczki D, Richardson JML (2009) The role of trematode parasites in larval anuran communities: an aquatic ecologist's guide to the major players. Oecologia 161:371–385. https://doi.org/10.1007/s00442-009-1388-8

Therneau T (2015) A package for survival analysis in S, version 2.38

Torchin ME, Byers JE, Huspeni TC (2005) Differential parasitism of native and introduced snails: placement of parasite fauna. Biol Invasions 7:885–894

Trudeau VL, Schueler FW, Navarro-Martin L, Hamilton CK, Bulaeva E, Bennett A, Fletcher W, Taylor L (2013) Efficient induction of spawning of northern leopard frogs (Lithobates pipiens) during and outside the natural breeding season. Reprod Biol Endocrinol 11:14. https://doi.org/10.1186/1477-7827-11-14

USEPA (2003) EFED risk assessment for the seed treatment of clothianidn 600FS on corn and canola. 91pp. Available online at: https://www3.epa.gov/pesticides/chemsearch/clearedreviews/csrPC-04430920-Feb-03_a.pdf Accessed 31 Jan 2019

Venables WN, Ripley BD (2002) Modern applied statistics with S., Fourth. Springer, New York

Vu M, Weiler B, Trudeau VL (2017) Time- and dose-related effects of a gonadotropin-releasing hormone agonist and dopamine antagonist on reproduction in the northern leopard frog (Lithobates pipiens). Gen Comp Endocrinol 254:86–96. https://doi.org/10.1016/j.ygcen.2017.09.023

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM (2009) Mixed effects models and extensions in ecology with R. Springer Science and Business Media, New York

Zuur AF, Ieno EN, Elphick CS (2010) A protocol for data exploration to avoid common statistical problems. Methods Ecol Evol 1:3–14. https://doi.org/10.1111/j.2041-210X.2009.00001.x

Zuur AF, Hilbe JM, Ieno EN (2015) A beginner's guide to GLM and GLMM with R. Highland Statistics Ltd., Newburgh