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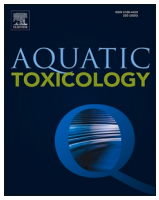

Temperature affects the toxicity of pesticides to cercariae of the trematode *Echinostoma trivolvis*

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ABSTRACT

Global climate change is predicted to have significant impacts on ecological interactions such as host-parasite relationships. Increased temperatures may also interact with other anthropogenic stressors, such as chemical contaminants, to exacerbate or reduce parasite transmission. However, studies on the effects of pesticides on non-target species are typically conducted at one standard temperature, despite the toxicity of many agrochemicals being temperature-dependent. Furthermore, most studies assessing the effects of temperature on pesticide toxicity have been conducted on host organisms, limiting our understanding of how temperature affects the toxicity of pesticides to free-living parasite stages as they move through the environment in search of a host. Using the free-swimming cercariae stage of the trematode *Echinostoma trivolvis*, we examined how the toxicities of three different pesticides (a carbamate insecticide, strobilurin fungicide, and triazine herbicide) vary by temperature by monitoring cercarial swimming activity over time. Our three main findings were: 1) a strong main effect of temperature across all pesticide trials – higher temperatures caused cercariae to cease swimming activity earlier, likely due to an increased rate of energy expenditure, 2) atrazine, azoxystrobin, and carbaryl were directly toxic to cercariae to some degree, but not in a predictable dose-dependent manner, and 3) the temperature at which pesticide exposure occurs could affect its toxicity to cercariae. The interaction between pesticide and temperature was most evident in the azoxystrobin exposure; azoxystrobin caused cercariae to cease swimming activity earlier at 30 °C but caused cercariae to maintain swimming activity longer at 18 °C relative to their respective pesticide-free control treatments. These findings highlight the importance of conducting toxicity assays at multiple temperatures and suggest that the combined effects of pesticides and temperature on host-parasite interactions may be difficult to generalize.

1. Introduction

Over 400 different pesticides are applied annually in the United States totaling more than 500 million kilograms applied across the landscape (Baker and Stone, 2015; Atwood and Paisley-Jones, 2017). As a result, organisms in many ecosystems are regularly exposed to pesticides. Pesticide exposure can cause a variety of effects at the level of the individual organism, including mortality, reduced growth, slowed development, and immunosuppression (Hayes et al., 2010; Egea--Serrano et al., 2012; Köhler and Triebskorn 2013). These individual-level effects, in turn, have the potential to alter the interactions between species dramatically. For example, pesticide exposure can make hosts more susceptible to parasitic infections and make animals more vulnerable to predation (Rohr et al., 2008; Relyea and Edwards, 2010). Pesticide use is predicted to increase significantly over the next 30 years as the global population's food demands grow (Rohr et al., 2019), punctuating the need for studies that examine their effects on ecological systems.

A significant challenge to assessing pesticide-mediated effects on natural systems is that exposures occur against the backdrop of temperature variation. Despite temperature variation being ubiquitous in nature, most studies that assess toxicity of pesticides are conducted at one standardized temperature (typically 20 – 23 °C; ASTM International, 2014). However, pesticide exposure can happen at a range of temperatures depending on local climatic conditions, timing of pesticide application, and occurrence of rain events that can carry pesticides into surface water. For example, Miles et al. (2017) found that water concentrations of the neonicotinoids peaked 5 to 7 weeks post-planting,

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despite typically being applied only once as a seed coating. Similarly, Rackliffe and Hoverman (2020) found that pesticide concentrations in streams do not necessarily peak at the time of pesticide application, instead spiking several times later in the season following rain events that flush them into water bodies. Because temperature can significantly modify key factors such as rates of chemical uptake, biotransformation, and detoxification (Laetz et al., 2014; Lau et al., 2015), trials at a single temperature may over- or underestimate pesticide toxicity. As incremental climate change progresses and pulse warming events become more common (Coumou and Rahmstorf 2012), many organisms are likely to be exposed to pesticides at warmer temperatures than previously experienced (Noyes and Lema 2015). Therefore, it is necessary to conduct toxicity assays across a range of temperatures to improve our understanding and ability to predict temperature-mediated effects on pesticide toxicity in nature.

Parasites play a key role in structuring communities and are estimated to account for over 40% of biodiversity in many ecosystems (Price 1980; Fenton and Brockhurst 2007; Dobson et al., 2008; Wood and Johnson 2015). Yet, most studies that assess the toxicity of pesticides on wildlife have focused on host organisms. Pesticides, however, are also toxic to parasites, especially free-living stages that actively move through the environment seeking hosts (Pietrock and Marcogliese 2003; Koprivnikar et al., 2006; Blanar et al., 2009; Hua et al., 2016; Sures et al., 2017). Thus, toxic effects during this stage can influence parasite survival and ability to locate hosts. However, predicting the effects of pesticide exposure on parasite stages is complicated by aspects of their biology. Free-swimming parasite life stages such as trematode cercariae are lecithotrophic, depending on a limited glycogen reserve and not feeding during their short (generally $<$ 48 hr) lifespan (Lawson and Wilson 1980). The rate at which this limited energy is expended increases as a function of temperature – as temperatures increase, swimming speeds increase, causing life span (but not necessarily net transmission efficiency) to decrease (Evans 1985; Fried and Pechenik 1995). Thus, although many pesticides have a positive temperature coefficient (i.e., toxicity increases with temperature; Boina et al., 2009), the shortened lifespan of cercariae at higher temperatures resulting from an increased rate of energy expenditure may reduce their total time exposed to a pesticide, reducing its effect on their survival. However, it is unclear how the toxicity of pesticides to free-swimming parasite stages changes according to temperature.

In this study, we used the cercariae of the trematode *Echinostoma trivolvis* to explore how the toxicities of three pesticides differ across three temperatures (18, 24, and 30 °C). To assess toxicities of the pesticides to cercariae, we monitored time-to-knockdown, which is a measure of how long cercariae display continuous swimming activity and thus how long they are able to seek out and infect a host. The three temperature treatments are all above the minimum emergence temperature threshold for *E. trivolvis* ($\leq$ 12 °C; Schmidt and Fried 1996; Morley and Lewis 2013) and below the upper bounds of water temperatures that cercariae are likely to experience pesticide exposure in North America. We chose pesticides of three different classes for exposures – a carbamate insecticide (carbaryl), a strobilurin fungicide (azoxystrobin), and a triazine herbicide (atrazine). In previous investigations, carbaryl and atrazine have had negative effects (carbaryl- Hua et al., 2016; Atrazine- Koprivnikar et al., 2006; Rohr et al., 2008) or no effects (carbaryl- Rohr et al., 2008) on the survival of cercariae depending on exposure concentration and experimental conditions. All three of these pesticides have documented temperature-dependent toxicity to at least one non-target taxa (Boone and Bridges 1999; Gaunt and Barker 2000; Boina et al., 2009; Velki and Ečimović 2015). Our predictions are 1) that cercariae would be knocked down earlier at higher temperatures due to an increased rate of energy expenditure (Evans 1985; Fried and Pechenik 1995), 2) each pesticide would be toxic to the cercariae and increase the rate of knockdown of cercariae relative to the control treatment, and 3) the pesticides would have a weaker effect on cercariae at higher temperatures because cercariae would rapidly expend their limited energy and cease swimming before toxic effects of the chemicals can significantly impact swimming behavior.

2. Material and methods

2.1. Study system

This study focused on the North American trematode species *Echinostoma trivolvis*. Fish and amphibian-eating avian and mammalian definitive hosts harbor the adult stage of *E. trivolvis* in the intestine. Eggs of the worm move into the environment in the feces, where miracidia hatch from the eggs and infect planorbid snails. Miracidia then transform into sporocysts, which in turn produce rediae. Germinal cells within the rediae undergo asexual reproduction, forming cercariae that emerge from the snail as a free-swimming larval stage. Cercariae then seek out and encyst in a secondary host, such as amphibian larvae, mollusks, or turtles as metacercariae (Kanev et al., 1995; Toledo et al., 2009). Our study focused on the cercarial stage of the life cycle because the external environment strongly influences its success in locating hosts (Pietrock and Marcogliese 2003). *E. trivolvis* is a widely distributed species (Johnson and McKenzie 2008), making it likely that it experiences pesticide exposure across a range of temperatures.

2.2. Parasite collection and identification, and husbandry

In June 2019, we collected 50 adult *Helisoma (Planorbella) trivolvis* snails (Burch 1982) from a pond (40.45511, −87.056093) at the Purdue Wildlife Area in West Lafayette, IN, USA. To screen each snail for echinostome infection, we placed them individually in 50-mL centrifuge tubes containing 25 mL of spring water and under a 100-W light for 1 h to induce shedding (Cohen et al., 1980). Using light microscopy, we identified cercaria belonging to the echinostomatid trematode family following the key in Schell (1985).

We used molecular methods to identify snails as being infected with *E. trivolvis*. We first isolated one cercaria from each echinostome-infected snail in a 1.5 mL microcentrifuge tube filled with 95% ethanol. For each isolated cercaria, we extracted DNA using DNeasy Blood and Tissue Kits (Qiagen). We stored eluted DNA at −20 °C. We used quantitative polymerase chain reaction (qPCR) to determine whether each sample was *E. trivolvis* using a primer set described in Fujino et al. (1997). These primers amplify a ~330 bp fragment of the *E. trivolvis* genome (GenBank Accession Number U42048). For each reaction, we added 10 μl SYBR Green, 0.5 μl of forward primer (5′-GTGAAATGTATGCTAATGA-3′; Fujino et al., 1997), 0.5 μl of reverse primer (5`-GTTGCGTTGCTATGTGGT-3′; Fujino et al., 1997), 7 μl of reverse osmosis water, and 2 μl of template. A CFX Connect (Bio-Rad Laboratories) was used to conduct qPCR. Each plate contained one negative control containing RO water as template and three positive controls containing template DNA from samples determined to be *E. trivolvis* in a previous study (>99% identity to *E. trivolvis* ITS-1 gene in Genbank [GQ463127.1], see online Supplementary Information for multiple sequence alignment; Billet et al., 2020). The qPCR protocol consisted of 3 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 55 °C. All positive controls successfully amplified. All unknown samples that peaked before cycle 40 were considered positive. Based on these results, we selected five *E. trivolvis*-infected snails as parasite sources for our experiments. *E. trivolvis* DNA extracted from the three positive controls and cercariae from the five source snails was deposited in the Yale Peabody Museum of Natural History (catalog numbers: YPM IZ 108300 to YPM IZ 108307).

To reduce shedding prior to the experiment, we individually isolated infected snails in 2-L cups filled with 1.5 L UV-irradiated well water and stored them at 7 °C until the start of experiments ($<$ 21d). We fed the snails a mixture of rabbit chow and spirulina powder ad libitum. Two days prior to the experiment, the snails were slowly acclimated to 23 °C.

2.3. Pesticide background

We chose technical-grade carbaryl, atrazine, and azoxystrobin (dissolved in 100% ethanol) for the experiments (ChemService, West Chester, PA, USA). All three pesticides can be applied multiple times throughout the growing season (e.g., Williams et al., 2010; Blandino et al., 2012), increasing the potential that cercariae will be exposed across a range of temperatures, especially in agricultural ponds where intermediate snail hosts are common (Johnson et al., 2007). For carbaryl, a 10 g/L stock was diluted in spring water to reach experimental concentrations of 600, 800, 1000, 1200, and 1400 μg/L. For azoxystrobin, a 0.5 g/L stock was diluted in spring water to reach experimental concentrations of 1, 5, 10, 20, 30 μg/L. For atrazine, 1 g/L stock was diluted in spring water to reach experimental concentrations of 10, 20, 50, 100, and 200 μg/L. These ranges reflect concentrations at or above those found in surface waters (Norris et al., 1983; Gilliom et al., 2007; Rodrigues et al., 2013). We added ethanol to each experimental concentration to match the ethanol concentration in the highest concentration reached in each experiment (carbaryl experiment – 0.014% ethanol; azoxystrobin experiment – 0.006% ethanol; atrazine experiment – 0.02% ethanol). Each experiment included a pesticide-free ethanol-vehicle control treatment, which acted as the control treatment, as ethanol at a low concentration has not been found to affect cercaria survival at these concentrations (Griggs and Belden 2008; Rohr et al., 2008; Jones et al., 2020).

2.4. Experimental design

We explored the effects of the three pesticides as a function of temperature on the swimming activity of *E. trivolvis* cercariae using time-to-knockdown assays. We used knockdown rather than mortality as the endpoint because knockdown represents when cercariae are functionally dead (i.e., no longer swimming and not seeking a second intermediate host). We defined normal swimming activity as consistent swimming and movement through the water column. A cercaria was considered knocked down when it was observed crawling on the bottom of the well and not swimming (Robinson et al., 2019). Knockdown can be treated as a discrete event because *E. trivolvis* cercariae are continuous swimmers and do not stop swimming during host-seeking. We did not observe any cercariae that displayed active swimming behavior after being marked as knocked down. We conducted a separate experiment for each pesticide.

We conducted the experiments at the Purdue University Aquaculture Research Lab in a temperature-controlled room, where temperature in the room was set to 18 °C for the experiments. Twelve temperature-controlled boxes made from 48 qt Styrofoam™ coolers containing seed germination heating mats (Seedfactor MET Certified Heating Mat) were constructed. These seed germination mats are equipped with a digital probe that allows them to self-regulate by monitoring the temperature of the heated substrate. Four boxes each were assigned to either the 18, 24, or 30 °C temperature regime, then assigned to one of three vertical shelves in a randomized block design.

We used covered 24-well tissue culture plates (127.8 mm x 81 mm; Corning Inc., Corning, NY, USA) as the experimental units within the temperature boxes. We placed six 24-well plates directly on top of the heating mat in each box and assigned each 24-well plate to one of the pre-determined pesticide concentrations. Within each box, each 24-well plate contained five wells of a single row assigned to one pesticide concentration, with the other 19 wells remaining empty. This setup was replicated across all 12 temperature boxes, resulting in 24 tissue culture wells for each pesticide concentration × temperature combination (N = 360 total cercariae/experiment). A new row of wells was used for each new experiment to prevent cross-contamination of pesticides.

We pipetted 500 μL of the assigned pesticide solution to each experimental well two hours before adding cercariae. In addition, a small dish with 5 mL of spring water was placed in the center of each box directly on the heating mat. The heating mat's temperature probe was placed inside the dish to monitor and maintain the temperature inside the box, with temperature recorded every two hours before the boxes were opened for the cercariae knockdown check. For each experiment, the temperature within the boxes was consistent, with a mean temperature within 1 °C of the nominal temperature ([*Mean* °C ± SD]: Atrazine trial – 18 °C treatment:17.3 ± 0.2, 24 °C treatment: 23.1 ± 0.9, 30 °C treatment: 29.3 ± 0.3; Carbaryl trial – 18 °C treatment:17.6 ± 0.5, 24 °C treatment: 23.3 ± 0.3, 30 °C treatment: 29.2 ± 0.4; Azoxystrobin trial – 18 °C treatment:17.4 ± 0.2; 24 °C treatment:23.2 ± 0.2, 30 °C treatment: 29.0 ± 0.1).

2.5. Cercariae addition

To isolate cercariae, five snails confirmed to be infected with *E. trivolvis* were individually placed in 50-mL tubes containing approximately 25 mL of spring water and illuminated with a 100 W light for 1 h to induce cercarial shedding. Water from each tube was poured into a single 150 mL cup to aggregate and mix the shed cercariae (Cohen et al., 1980). We then added 25 mL of spring water back to the tubes and placed the snails back under the light to continue shedding. Next, a glass pipette was used to collect and add a single cercaria to each experimental well. To prevent cross-contamination across pesticide concentrations, we used a different glass pipette for each pesticide concentration. We added cercariae to all of the wells in a single box before moving to the next box. Once all of the boxes on a shelf contained cercariae, we recorded the time.

To document swimming status of cercariae, each well plate was inspected under a dissecting microscope every two hours. Each plate was taken out of its temperature-controlled box for a maximum of two minutes for each check and immediately returned. Cercariae activity checks continued until all cercariae were knocked down. No cercariae displayed normal swimming behavior when observed following observation of knockdown. All three experiments ended at 28 h. Four replicates of one treatment (5 μg/L azoxystrobin, 24 °C) were spilled during an experiment and excluded from analysis.

2.6. Pesticide testing

A 10 mL sample of each pesticide concentration was collected the day of each experiment and stored at −20 °C prior to analysis. Briefly, each pesticide sample was spiked with a known concentration of internal standard, extracted by pouring through a 3 cc Oasis SPE cartridges (Waters INC, Milford, MA, USA), and then eluting with 3 mL of acetonitrile, which was evaporated in a speedvac concentrator (as in Rackliffe and Hoverman 2020). Concentrations were determined via LC/MS at the Purdue Bindley Bioscience Center (West Lafayette, IN, USA). The detected concentrations are as follows (from lowest to highest nominal concentration): carbaryl – 497, 513, 667, 688, and 778 μg/L (65.4% of nominal on average); azoxystrobin – 1, 5, 26, 33, and 44 μg/L (141% of nominal on average); atrazine – 21, 30, 71, 178, and 258 μg/L (161% of nominal on average). Because the detected concentrations deviate from the nominal concentrations, we refer to the detected concentrations hereafter and use the detected concentrations for analysis (Table 1).

2.7. Statistical analysis

We performed all statistical analyses using R version 4.0.3 (R Developmental Core Team 2020). We used three separate mixed-effects Cox proportional hazards models (*coxme* function, *Survival* package; Therneau 2021), one for each pesticide experiment, to examine the effects of pesticide concentration and temperature on the viability of cercariae. For each model, our response variable was time-to-knockdown (hrs). Each model contained pesticide concentration, temperature, and the interaction between pesticide concentration and temperature as predictors. We treated both pesticide concentration

Table 1
Summary of pesticide concentrations (detected concentrations and percent of nominal concentrations) for the carbaryl, atrazine, and azoxystrobin exposures.

Pesticide	Nominal Concentration (µg/L)	Detected Concentration (µg/L)	% Nominal
Carbaryl	0	0	–
	600	497	82.8
	800	513	64.1
	1000	667	66.7
	1200	688	57.3
	1400	778	55.6
Atrazine	0	0	–
	10	21	210.0
	20	30	150.0
	50	71	142.0
	100	178	178.0
	200	258	129.0
Azoxystrobin	0	0	–
	1	1	100.0
	5	5	100.0
	10	26	260.0
	20	33	165.0
	30	44	146.7

(6 levels) and temperature (3 levels) as categorical variables, with the pesticide-free control (0.0 mg/L) and the lowest temperature (18 °C) set as the reference categories. To control for potential non-independence of individuals housed in the same spatial block, box, and 24-well plate, we included a nested random effect structure (1|block/box/plate). We ran posthoc comparisons to explore interactions between temperature and pesticide concentration (*emmeans* function, *emmeans* package; Lenth 2020). Figures were generated using ggplot2 (Wickham 2016).

3. Results

In total, 1076 *E. trivolvis* cercariae were used to assess the interactive effects of temperature and pesticides on cercarial swimming activity. Across all three experiments, the median survival time in the pesticide-free control treatment at 18 °C, 24 °C, and 30 °C was 24 h, 16 h, and 11 h, respectively. 100% of cercariae were knocked down by 28 h. Survival curves generated from the time-to-knockdown assays are presented in Fig. 1.

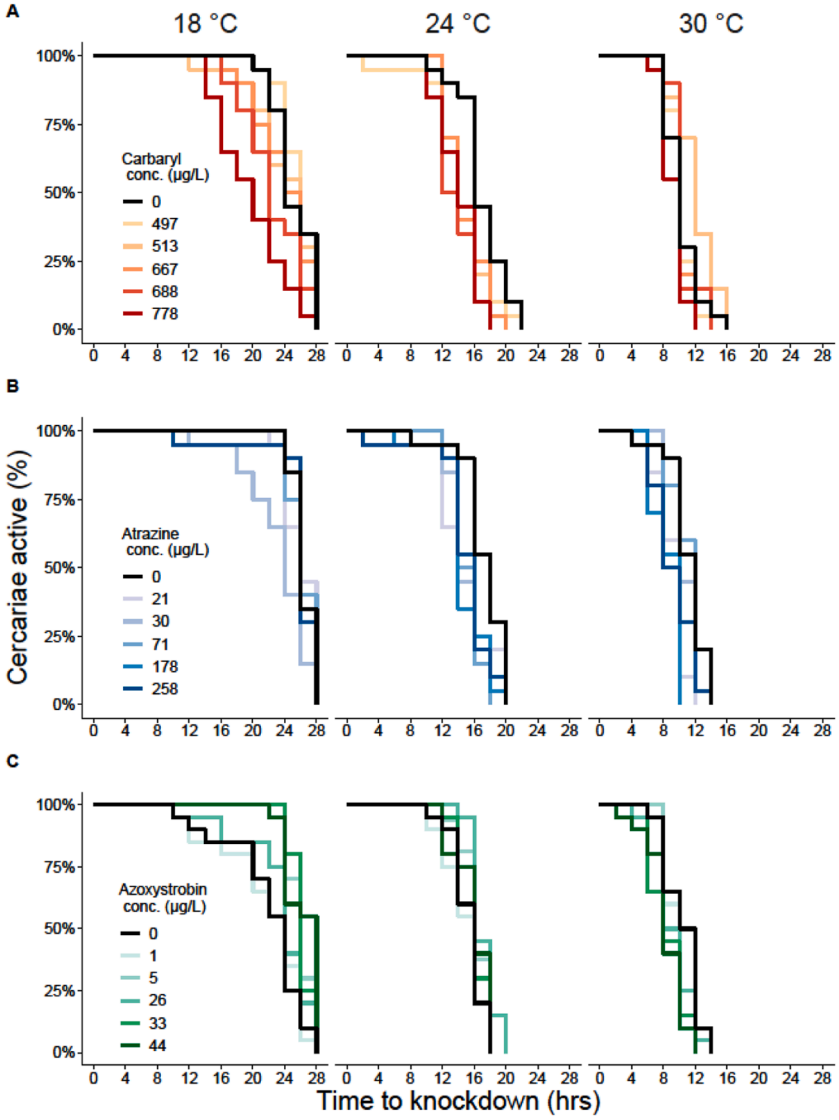

Fig. 1. Survival curves representing time to knockdown for *Echinostoma trivolvis* cercariae exposed to a range of concentrations of A) carbaryl, B) atrazine, and C) azoxystrobin at three different temperatures (18, 24, and 30 °C). Each curve represents the percentage of cercariae surviving every 2 h throughout the exposure period (maximum 28 h).

3.1. Carbaryl experiment

There was a significant main effect of temperature (temperature: $\chi^2 = 283.7$, df = 2, $P < 0.001$) and carbaryl concentration (carbaryl concentration: $\chi^2 = 32.7$, df = 5, $P < 0.001$), and an interaction between temperature and carbaryl concentration (temperature × carbaryl concentration: $\chi^2 = 18.3$, df = 10, $P = 0.049$; Fig. 2A) on rate of cercarial knockdown. Relative to the 18 °C treatment, the rate of knockdown was increased by the 24 °C ($\beta = 2.52 \pm 0.43$, $z = 5.86$, $P < 0.001$) and 30 °C ($\beta = 5.19 \pm 0.46$, $z = 11.2$, $P < 0.001$) treatments. Overall, the rate of cercarial knockdown was significantly increased by the highest carbaryl concentration, 778 µg/L ($\beta = 1.22 \pm 0.39$, $z = 3.1$, $P = 0.002$; Fig 3A). We also found that the effect of the 513 µg/L carbaryl treatment significantly differed across temperatures regimes. At 30 °C, the cercariae in the 513 µg/L carbaryl treatment had a reduced rate of knockdown relative to the pesticide-free control, whereas at 24 °C, the cercariae exposed to the 513 µg/L carbaryl treatment had an increased rate of knockdown relative to the pesticide-free control (Fig. 2B).

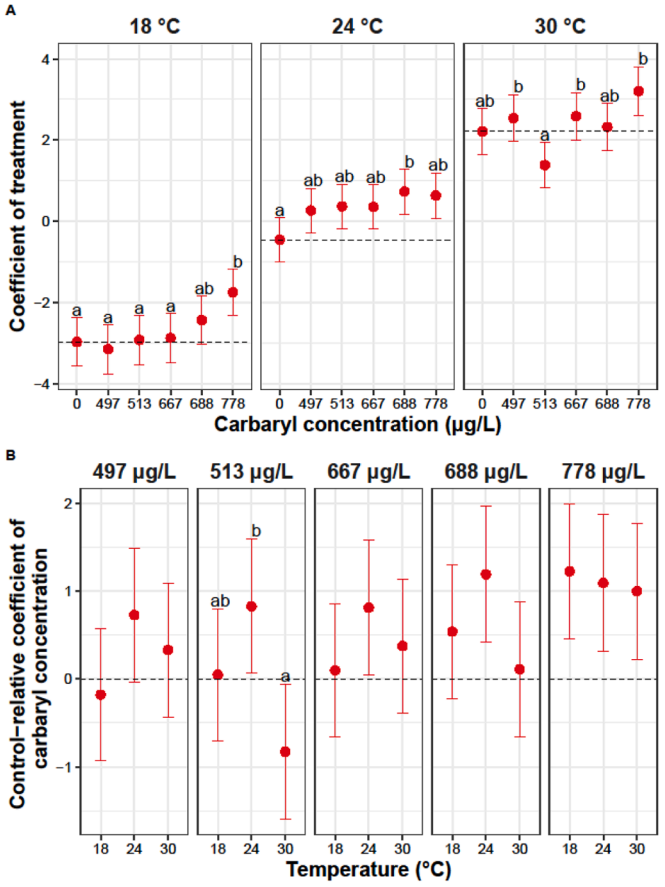

Fig. 2. Plot of coefficients ± 95% CI from mixed effects Cox proportional hazards regression model comparing time active (time-to-knockdown) of *Echinostoma trivolvis* cercariae across six carbaryl concentrations at three temperatures. Panel A represents the effects of each carbaryl treatment relative to the effect of temperature alone (i.e., 0 µg/L treatment). Coefficients are mean-centered and faceted by temperature treatment. Carbaryl treatments within each temperature facet sharing lower case letters are not significantly different from each other ($P < 0.05$). Panel B represents the interaction between temperature and carbaryl treatment and shows the coefficient of each carbaryl concentration relative to the 0 µg/L treatment at each temperature (i.e., horizontal dashed lines in Panel A). Coefficients within each carbaryl treatment facet sharing lower case letters are not significantly different from each other ($P < 0.05$).

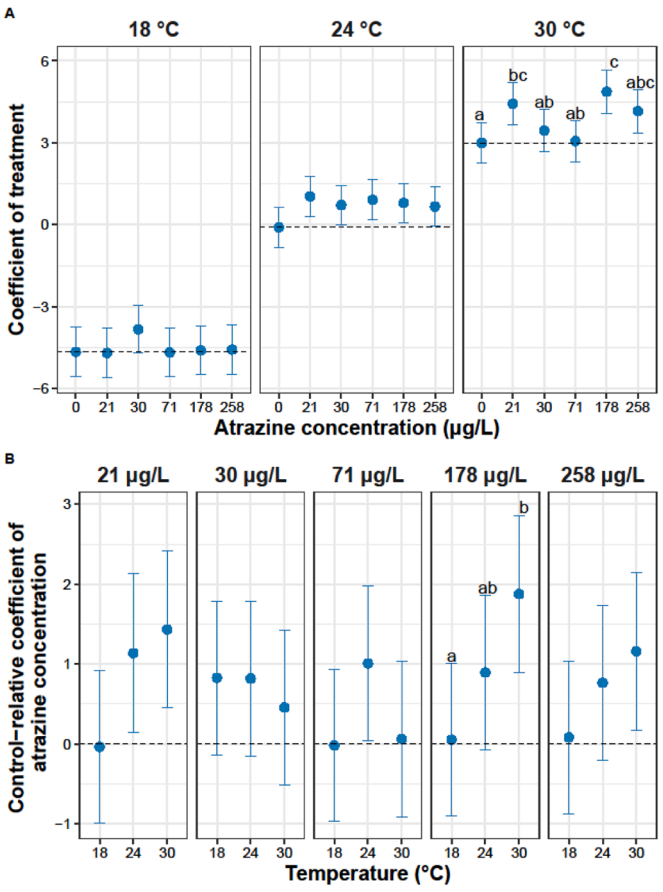

Fig. 3. Plot of coefficients ± 95% CI from mixed effects Cox proportional hazards regression model comparing time active (time-to-knockdown) of *Echinostoma trivolvis* cercariae across six atrazine concentrations at three temperatures. Panel A represents the effects of each atrazine treatment relative to the effect of temperature alone (i.e., 0 µg/L treatment). Coefficients are mean-centered and faceted by temperature treatment. Atrazine treatments within each temperature facet sharing lower case letters are not significantly different from each other ($P < 0.05$). Panel B represents the interaction between temperature and atrazine treatment and shows the coefficient of each atrazine concentration relative to the 0 µg/L treatment at each temperature (i.e., horizontal dashed lines in Panel A). Coefficients within each atrazine treatment facet sharing lower case letters are not significantly different from each other ($P < 0.05$).

3.2. Atrazine experiment

There was a significant main effect of temperature ($\chi^2 = 287.2$, df = 2, $P < 0.001$), atrazine concentration ($\chi^2 = 14.8$, df = 5, $P = 0.011$), and an interaction between temperature and atrazine concentration ($\chi^2 = 20.2$, df = 10, $P = 0.027$) on rate of cercarial knockdown. Relative to the 18 °C treatment, the rate of knockdown was increased by the 24 °C ($\beta = 4.55 \pm 0.65$, $z = 7.01$, $P < 0.001$) and 30 °C ($\beta = 7.64 \pm 0.69$, $z = 11.1$, $P < 0.001$) treatments. Rate of cercarial knockdown was significantly increased by the 21 µg/L ($\beta = 1.43 \pm 0.50$, $z = 3.3$, $P = 0.005$) and 178 µg/L ($\beta = 1.88 \pm 0.50$, $z = 3.7$, $P < 0.001$) atrazine treatments relative to the pesticide-free control, but only at 30 °C (Fig 3A). We also found that the effect of the 178 µg/L atrazine treatment significantly differed across temperatures regimes. The cercariae exposed to 178 µg/L atrazine at 30 °C had a significantly higher rate of knockdown than cercariae exposed to the same atrazine concentrations at 18 °C relative to their respective pesticide-free controls (Fig 3B).

3.3. Azoxystrobin experiment

There was a significant main effect of temperature ($\chi^2 = 253.9$, df = 2, $P < 0.001$) and an interaction between temperature and azoxystrobin concentration ($\chi^2 = 43.3$, df = 10, $P < 0.001$), but not a main effect of azoxystrobin concentration ($\chi^2 = 6.4$, df = 5, $P = 0.269$) on rate of cercarial knockdown. Relative to the 18 °C treatment, the rate of knockdown was increased by the 24 °C ($\beta = 3.46 \pm 0.47$, $z = 7.34$, $P < 0.001$) and 30 °C ($\beta = 5.79 \pm 0.51$, $z = 11.3$, $P < 0.001$) treatments. The effect of four azoxystrobin concentrations, 44 µg/L, 33 µg/L, and 26 µg/L, and 5 µg/L, significantly differed across temperatures regimes (Fig. 4B). Specifically, at these four concentrations, the pesticide-free control-relative rate of knockdown was significantly higher at 30 °C compared to cercariae in the same pesticide concentration at 18 °C. This finding was driven by two contrasting effects – whereas azoxystrobin treatments at 30 °C tended to increase the rate of knockdown, the same azoxystrobin treatments at 18 °C tended to decrease the rate of knockdown (Fig 4A). To explore the interaction between azoxystrobin concentration and temperature further, we assessed the interaction while treating concentration as a continuous variable rather than a categorical factor. This analysis yielded a qualitatively similar result – a negative relationship between azoxystrobin concentration and rate of knockdown at 18 °C ($\beta = -0.233$, 95% CI = −0.355 to −0.112) and a positive relationship between azoxystrobin concentration and rate of knockdown at 30 °C ($\beta = 0.194$, 95% CI = 0.076 to 0.312).

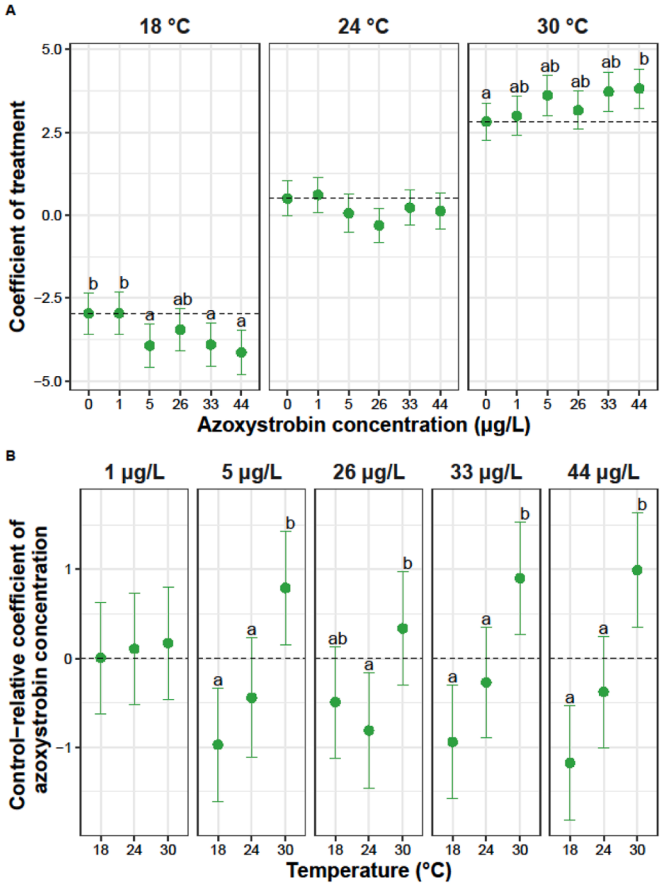

Fig. 4. Plot of coefficients ± 95% CI from mixed effects Cox proportional hazards regression model comparing time active (time-to-knockdown) of *Echinostoma trivolvis* cercariae across six azoxystrobin concentrations at three temperatures. Panel A represents the effects of each azoxystrobin treatment relative to the effect of temperature alone (i.e., 0 µg/L treatment). Coefficients are mean-centered and faceted by temperature treatment. Azoxystrobin treatments within each temperature facet sharing lower case letters are not significantly different from each other ($P < 0.05$). Panel B represents the interaction between temperature and azoxystrobin treatment and shows the coefficient of each azoxystrobin concentration relative to the 0 µg/L treatment at each temperature (i.e., horizontal dashed lines in Panel A). Coefficients within each azoxystrobin treatment facet sharing lower case letters are not significantly different from each other ($P < 0.05$).

4. Discussion

The effects of pesticide exposure on wildlife depend upon environmental context (Relyea and Hoverman 2006). However, ecotoxicological assays typically occur under standardized laboratory conditions, making predicting the effects of pesticides across a range of ecologically relevant conditions difficult. Here, we assessed how temperature affects the toxicity of three pesticides to the free-swimming cercarial stage of *Echinostoma trivolvis*. As expected, higher temperatures caused cercariae to cease swimming activity earlier, likely due to an increased rate of energy expenditure. Atrazine, azoxystrobin, and carbaryl were directly toxic to cercariae and increased the rate of knockdown in at least one concentration, but not necessarily in a predictable dose-dependent manner. Finally, the temperature at which pesticide exposure occurred affected its toxicity to cercariae. This interaction between temperature and pesticide exposure was most apparent in the azoxystrobin exposures, where azoxystrobin tended to increase the rate of knockdown at 30 °C but decrease the rate of knockdown at 18 °C relative to their respective control treatments.

Cercariae exposed to azoxystrobin at 18 °C tended to remain active longer relative to the pesticide-free control. In contrast, cercariae exposed to azoxystrobin at 30 °C tended to remain active for a shorter time relative to the pesticide-free control. This finding demonstrates that the effect of some pesticides can be difficult to predict from toxicity trials at a standardized temperature, as the magnitude and direction of the effect of a pesticide can shift across a range of biologically relevant temperatures. Strobilurin fungicides, which act on the respiration pathway in the mitochondrial membrane of fungi and prevent ATP formation, are highly toxic to many organisms, including aquatic invertebrates like *Daphnia* spp. and copepods (Warming et al., 2009; Wijngaarden et al., 2014). Warming et al. (2009) found azoxystrobin exposure affects respiration in *Daphnia magna*, affecting energy metabolism. Cercariae also rely on aerobic respiration to convert stored glucose to ATP (Bruce et al., 1971); therefore, a disruption to respiration is likely to affect cercarial swimming activity significantly. One potential explanation for the contrasting effects of azoxystrobin on cercariae at different temperatures is that at low temperatures (when energy demand is relatively low per unit time), the degree to which azoxystrobin reduces the efficiency of respiration is not strongly limiting and results in a slightly reduced rate of energy expenditure that reduces swimming speeds, extending the time spent swimming. At high temperatures, however (when energy demand per unit time is high), the disruptive effect of azoxystrobin on respiration may significantly increase the rate of anaerobic respiration, causing a build-up of lactic acid that reduces the lifespan of the cercariae (Rodrigues et al., 2013). The reduced solubility of oxygen at higher water temperatures may exacerbate this effect. Our findings suggest that increasing temperatures that will occur with anticipated climate changes will increase the negative effects of strobilurin fungicides on free-swimming parasite stages and highlight the importance of testing the toxicity of pesticides at temperatures that reflect a realistic range of conditions.

Previous studies at room temperature (20 – 24 °C) have demonstrated toxicity of atrazine to cercariae at 200 µg/L (Koprivnikar et al., 2006; Rohr et al., 2008) but not 20 µg/L (Koprivnikar et al., 2006). In the present study, the toxicity of atrazine to cercariae was temperature-dependent. Although all of the atrazine concentrations tended to increase the rate of knockdown in the treatment closest to room temperature (24 °C), none of them significantly differed from the control treatment. At 30 °C, however, the effects of the atrazine treatments on cercariae rate of knockdown were more variable, with both the

21 and 178 μg/L atrazine treatments increasing the rate of knockdown. Additionally, in the 178 μg/L atrazine treatment, the cercariae exposed at 30 °C tended to cease swimming activity faster than those exposed at 18 °C, relative to the control treatment at each temperature. This result was driven mainly by the increased negative effect of the atrazine treatment on cercariae at 30 °C. These findings suggests that atrazine may be more toxic to cercariae at higher temperatures and that toxicity tests at room temperature may underestimate its effects on cercariae, especially in agricultural regions at lower latitudes (i.e., warmer climates). Interestingly, the cercariae did not exhibit clear dose-dependent responses to atrazine at any temperature. Hua et al. (2016) also found that echinostome cercariae did not exhibit a dose-dependent response to pesticides, and the response tended to plateau as concentrations increased. Non-monotonic dose-response relationships are frequently reported for endocrine-disrupting compounds such as atrazine with hypothesized mechanisms including cytotoxicity, negative feedback, receptor down-regulation, selectivity, or competition (Vandenberg et al., 2012; Lagarde et al., 2015). Since most of this work has focused on free-living species, future work examining the mechanism underlying this effect in parasites would be valuable. Additionally, given that some pesticides, such as atrazine (Koprivnikar et al., 2006), show interspecific differences in their effects on cercariae, it will be important to test how its toxicity interacts with temperature for other trematode species.

Responses of cercariae to carbaryl most closely resembled a dose-dependent response, with cercariae tending to display sensitivity at the highest tested concentrations across all three temperature treatments. Given that it is rare for carbaryl concentrations in surface water to reach the concentrations used in the present study, it is unlikely that carbaryl causes significant mortality to echinostome cercariae in natural settings. This result agrees with previous studies that have demonstrated that acetylcholinesterase inhibiting insecticides typically have minimal effects on the cercariae of echinostomes and other species except for at very high concentrations (Rohr et al., 2008; Hua et al., 2016; Jones et al., 2020). We also found that the effect of the 513 μg/L carbaryl treatment differed across temperature treatments, slightly increasing the rate of knockdown at 24 °C and slightly decreasing the rate of knockdown at 30 °C. It is worth noting that there is a qualitatively similar pattern of elevated toxicity of carbaryl at 24 °C in the 497, 667, and 668 μg/L carbaryl treatments. This suggests that there may be a unimodal response of cercariae to carbaryl exposure across a realistic range of temperatures, but a larger sample size is needed to tease apart these effects.

5. Conclusions

Studies on how pesticides impact hosts and parasites across a range of ecologically relevant conditions are necessary to understand how pesticide contamination affects patterns of infectious disease. We demonstrated that temperature can significantly influence the effects of pesticides on free-swimming parasite stages. The effect of azoxystrobin on cercariae shifted directionally across temperatures, increasing the time to knockdown at 18 °C and decreasing the time to knockdown at 30 °C. Although pesticide exposure has been found to increase the life span of cercariae in other studies, these findings were the result of pesticide-induced paralysis, making cercariae "functionally dead" since they could no longer seek out a host (Jones et al., 2020). Future studies should integrate measures of swimming speed (e.g., Selbach and Poulin 2018) to better understand better how exposure to azoxystrobin and other pesticides influences host-searching behavior and swimming speeds. Additionally, future studies that assess the net effects of pesticide exposure on patterns of host infection across a range of temperatures will help inform how host-parasite interactions will change with the warming global climate.

CRediT authorship contribution statement

Logan S. Billet: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Alice Belskis:** Conceptualization, Methodology, Investigation, Formal analysis. **Jason T. Hoverman:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Statement

Data associated with this paper are deposited in the Purdue University Research Repository: https://purr.purdue.edu/publications/3933/1 (Hoverman and Billet, 2022). DNA vouchers are deposited in the Yale Peabody Museum of Natural History (catalog numbers: YPM IZ 108300 to YPM IZ 108307).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106102.

Literature Cited

ASTM International, 2014. Guide For Conducting Acute Toxicity Tests On Test Materials With Fishes, Macroinvertebrates, and Amphibians. ASTM International.
Atwood, D., Paisley-Jones, C., 2017. Pesticides Industry Sales and Usage: 2008-2012 Market Estimates. Page Pesticides Industry Sales and Usage.
Baker, N.T., and W.W. Stone. 2015. Estimated annual agricultural pesticide use for counties of the conterminous United States, 2008–12. Pages 1–9.
Billet, L.S., Wuerthner, V.P., Hua, J., Relyea, R.A., Hoverman, J.T., 2020. Timing and order of exposure to two echinostome species affect patterns of infection in larval amphibians. Parasitology 147, 1515–1523.
Blanar, C.A., Munkittrick, K.R., Houlahan, J., Maclatchy, D.L., Marcogliese, D.J., 2009. Pollution and parasitism in aquatic animals: a meta-analysis of effect size. Aquatic Toxicol. 93, 18–28.
Blandino, M., Galeazzi, M., Savoia, W., Reyneri, A., 2012. Timing of azoxystrobin + propiconazole application on maize to control northern corn leaf blight and maximize grain yield. Field Crops Res. 139, 20–29.
Boina, D.R., Onagbola, E.O., Salyani, M., Stelinski, L.L., 2009. Influence of Posttreatment Temperature on the Toxicity of Insecticides Against *Diaphorina citri* (Hemiptera: psyllidae). J. Econ. Entomol. 102, 685–691.
Boone, M.D., Bridges, C.M., 1999. The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*rana clamitans*). Environ. Toxicol. Chem. 18, 1482–1482.
Bruce, J.I., Ruff, M.D., Hasegawa, H., 1971. *Schistosoma mansoni*: endogenous and exogenous glucose and respiration of cercariae. Exp. Parasitol. 29, 86–93.
Burch, J.B., 1982. North American freshwater snails: identification keys, generic synonymy, supplemental notes, glossary, references. index. Walkerana 1, 217–365.
Cohen, L.M., Neimark, H., Eveland, L.K., 1980. *Schistosoma mansoni*: response of Cercariae to a Thermal Gradient. J. Parasitol. 66, 362–364.
Coumou, D., Rahmstorf, S., 2012. A decade of weather extremes. Nat. Clim. Chang. 2, 491–496.

Dobson, A., Lafferty, K.D., Kuris, A.M., Hechinger, R.F., Jetz, W., 2008. Homage to Linnaeus: how many parasites? How many hosts? Proc. Nat. Acad. Sci. 105, 11482–11489.
Egea-Serrano, A., Relyea, R.A., Tejedo, M., Torralva, M., 2012. Understanding of the impact of chemicals on amphibians: a meta-analytic review. Ecol. Evol. 2, 1382–1397.
Evans, N.A., 1985. The influence of environmental temperature upon transmission of the cercariae of *Echinostoma liei* (Digenea: echinostomatidae). Parasitology 90, 269–275.
Fenton, A., Brockhurst, M.A., 2007. The role of specialist parasites in structuring host communities. Ecol. Res. 23, 795–804.
Fried, B., Pechenik, J., 1995. Effect of temperature on survival and infectivity of *Echinostoma trivolvis* cercariae: a test of the energy limitation hypothesis. Parasitology 111, 373–378.
Fujino, T., Zhiliang, W., Nagano, I., Takahashi, Y., Fried, B., 1997. Specific primers for the detection of genomic DNA of *Echinostoma trivolvis* and *E. caproni* (Trematoda: echinostomatidae). Mol. Cell. Probes 11, 77–80.
Gaunt, P., Barker, S.A., 2000. Matrix solid phase dispersion extraction of triazines from catfish tissues; examination of the effects of temperature and dissolved oxygen on the toxicity of atrazine. Int. J. Environ. Pollut. 13, 284–312.
Gilliom, R.J., Barbash, J.E., Crawford, C.G., Hamilton, P.A., Martin, J.D., Nakagaki, N., Nowell, L.H., Scott, J.C., Stackelberg, P.E., Thelin, G.P., Wolock, D.M., 2007. The quality of our nation's waters: pesticides in the nation's streams and ground water, 1992–2001. U.S. Geological Survey Circular 1291, 172 p-172 p.
Griggs, J.L., Belden, L.K., 2008. Effects of atrazine and metolachlor on the survivorship and infectivity of *Echinostoma trivolvis* trematode cercariae. Arch. Environ. Contam. Toxicol. 54, 195–202.
Hayes, T.B., Khoury, V., Narayan, A., Nazir, M., Parka, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T., Gallipeau, S., 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). Proc. Natl. Acad. Sci. U.S.A. 107, 4612–4617.
Hua, J., Buss, N., Kim, J., Orlofske, S.A., Hoverman, J.T., 2016. Population-specific toxicity of six insecticides to the trematode *Echinoparyphium sp*. Parasitology 143, 542–550.
Johnson, P.T.J., Chase, J.M., Dosch, K.L., Hartson, R.B., Gross, J.A., Larson, D.J., Sutherland, D.R., Carpenter, S.R., 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proc. Natl. Acad. Sci. 104, 15781–15786.
Pages Johnson, P.T.J., McKenzie, V.J., 2008. Effects of environmental change on helminth infections in amphibians: exploring the emergence of *Ribeiroia* and *Echinostoma* infections in North America, editors. In: Toledo, R., Fried, B. (Eds.), The Biology of Echinostomes: From the Molecule to the Community. Springer, New York, pp. 249–280.
Jones, D.K., Davila, D.D., Nguyen, K.H., Rohr, J.R., 2020. Effect of Agrochemical Exposure on *Schistosoma mansoni* Cercariae Survival and Activity. Environ. Toxicol. Chem. 39, 1421–1428.
Kanev, I., Fried, B., Dimitrov, V., Radev, V., 1995. Redescription of *Echinostoma trivolvis* (Cort, 1914) (Trematoda: echinostomatidae) with a discussion on its identity. Syst. Parasitol. 32, 61–70.
Köhler, H.R., Triebskorn, R., 2013. Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond? Science 341, 759–765.
Koprivnikar, J., Forbes, M.R., Baker, R.L., 2006. Effects of atrazine on cercarial longevity, activity, and infectivity. J. Parasitol. 92, 306–311.
Laetz, C.A., Baldwin, D.H., Hebert, V.R., Stark, J.D., Scholz, N.L., 2014. Elevated temperatures increase the toxicity of pesticide mixtures to juvenile coho salmon. Aquat. Toxicol. 146, 38–44.
Lagarde, F., Beausoleil, C., Belcher, S.M., Belzunces, L.P., Emond, C., Guerbet, M., Rousselle, C., 2015. Non-monotonic dose-response relationships and endocrine disruptors: a qualitative method of assessment. Environ. Health 14, 13.
Lau, E.T.C., Karraker, N.E., Leung, K.M.Y., 2015. Temperature-dependent acute toxicity of methomyl pesticide on larvae of 3 Asian amphibian species. Environ. Toxicol. Chem. 34, 2322–2327.
Lawson, J.R., Wilson, R.A., 1980. The survival of the cercariae of *Schistosoma mansoni* in relation to water temperature and glycogen utilization. Parasitology 81, 337–348.
Lenth, R.V. 2020. emmeans: estimated Marginal Means, aka Least-Squares Means.
Miles, J.C., Hua, J., Sepulveda, M.S., Krupke, C.H., Hoverman, J.T., 2017. Effects of clothianidin on aquatic communities: evaluating the impacts of lethal and sublethal exposure to neonicotinoids. PLoS One 12 e0174171–e0174171.
Morley, N.J., Lewis, J.W., 2013. Thermodynamics of cercarial development and emergence in trematodes. Parasitology 140, 1211–1224.
Norris, L.A., Lorz, H.W., Gregory, S.Z., 1983. Influence of forest and range land management on anadromous fish habitat in western North America: forest chemicals. Pages 96–96 General Technical Report PNW-149.
Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Curr Zool 61, 669–689.
Pietrock, M., Marcogliese, D.J., 2003. Free-living endohelminth stages: at the mercy of environmental conditions. Trends Parasitol. 19, 293–299.
Price, P.W., 1980. Evolutionary Biology of Parasites. (MPB-15), Volume 15. Princeton University Press.
Rackliffe, D.R., Hoverman, J.T., 2020. Population-level variation in neonicotinoid tolerance in nymphs of the Heptageniidae. Environ. Pollut. 265, 114803–114803.
Relyea, R.A., Edwards, K., 2010. What Doesn't Kill You Makes You Sluggish: how Sublethal Pesticides Alter Predator–Prey Interactions. Copeia 2010, 558–567.
Relyea, R., Hoverman, J., 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecol. Lett. 9, 1157–1171.
Robinson, S.A., Richardson, S.D., Dalton, R.L., Maisonneuve, F., Bartlett, A.J., Solla, S.R., 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.
Rodrigues, E.T., Lopes, I., Pardal, M.Â., 2013. Occurrence, fate and effects of azoxystrobin in aquatic ecosystems: a review. Environ. Int. 53, 18–28.
Rohr, J.R., Barrett, C.B., Civitello, D.J., Craft, M.E., Delius, B., DeLeo, G.A., Hudson, P.J., Jouanard, N., Nguyen, K.H., Ostfeld, R.S., Remais, J.V., Riveau, G., Sokolow, S.H., Tilman, D., 2019. Emerging human infectious diseases and the links to global food production. Nature Sustain. 2, 445–456.
Rohr, J.R., Raffel, T.R., Sessions, S.K., Hudson, P.J., 2008. Understanding the net effects of pesticides on amphibian trematode infections. Ecol. Appl. 18, 1743–1753.
Schell, S.C., 1985. Handbook of Trematodes of North America North of Mexico. University Press of Idaho, Moscow, Idaho, USA, pp. 1–263.
Schmidt, K.A., Fried, B., 1996. Emergence of Cercariae of *Echinostoma trivolvis* from *Helisoma trivolvis* under Different Conditions. J. Parasitol. 82, 674–676.
Selbach, C., Poulin, R., 2018. Parasites in space and time: a novel method to assess and illustrate host-searching behaviour of trematode cercariae. Parasitology 1–6.
Sures, B., Nachev, M., Selbach, C., Marcogliese, D.J., 2017. Parasite responses to pollution: what we know and where we go in 'Environmental Parasitology'. Parasites and Vectors 10, 1–19.
Therneau, T.M. 2021. A Package for Survival Analysis in R.
Toledo, R., Esteban, J.G., Fried, B., 2009. Recent Advances in the Biology of Echinostomes. Page 204 Advances in Parasitology, 1st Ed. Elsevier Ltd.
Vandenberg, L.N., Colborn, T., Hayes, T.B., Heindel, J.J., Jacobs, D.R., Lee, D.H., Shioda, T., Soto, A.M., vom Saal, F.S., Welshons, W.V., Zoeller, R.T., Myers, J.P., 2012. Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. Endocr. Rev. 33, 378–455.
Velki, M., Ečimović, S., 2015. Changes in exposure temperature lead to changes in pesticide toxicity to earthworms: a preliminary study. Environ. Toxicol. Pharmacol. 40, 774–784.
Warming, T.P., Mulderij, G., Christoffersen, K.S., 2009. Clonal variation in physiological responses of *Daphnia magna* to the strobilurin fungicide azoxystrobin. Environ. Toxicol. Chem. 28, 374–380.
Wickham, H., 2016. ggplot2: Elegant Graphics For Data Analysis. Springer-Verlag, New York.
Wijngaarden, R.P.A., van, D.J.M., Belgers, M.I., Zafar Matser, A.M., Boerwinkel, M.-C., Arts, G.H.P., 2014. Chronic aquatic effect assessment for the fungicide azoxystrobin. Environ. Toxicol. Chem. 33, 2775–2785.
Williams, M.M., Boerboom, C.M., Rabaey, T.L., 2010. Significance of Atrazine in Sweet Corn Weed Management Systems. Weed Technol. 24, 139–142.
Wood, C.L., Johnson, P.T.J., 2015. A world without parasites: exploring the hidden ecology of infection. Front. Ecol. Environ. 13, 425–434.