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Timing and order of exposure to two echinostome species affect patterns of infection in larval amphibians

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Abstract

The study of priority effects with respect to coinfections is still in its infancy. Moreover, existing coinfection studies typically focus on infection outcomes associated with exposure to distinct sets of parasite species, despite that functionally and morphologically similar parasite species commonly coexist in nature. Therefore, it is important to understand how interactions between similar parasites influence infection outcomes. Surveys at seven ponds in northwest Pennsylvania found that multiple species of echinostomes commonly co-occur. Using a larval anuran host (*Rana pipiens*) and the two most commonly identified echinostome species from our field surveys (*Echinostoma trivolvis* and *Echinoparyphium* lineage 3), we examined how species composition and timing of exposure affect patterns of infection. When tadpoles were exposed to both parasites simultaneously, infection loads were higher than when exposed to *Echinoparyphium* alone but similar to being exposed to *Echinostoma* alone. When tadpoles were sequentially exposed to the parasite species, tadpoles first exposed to *Echinoparyphium* had 23% lower infection loads than tadpoles first exposed to *Echinostoma*. These findings demonstrate that exposure timing and order, even with similar parasites, can influence coinfection outcomes, and emphasize the importance of using molecular methods to identify parasites for ecological studies.

Introduction

Aquatic environments often contain a diverse community of macroparasite species that make up a significant portion of free-living biomass, play an important role in food webs and serve as a source of infectious disease (Hechinger and Lafferty, 2005; Lafferty *et al.*, 2008; Johnson and Hoverman, 2012; Preston *et al.*, 2013). Indeed, this parasite biodiversity has led to a recognition of the importance of research focusing on how multi-parasite interactions influence infection outcomes within a host (Johnson and Hoverman, 2012; Hoverman *et al.*, 2013; Wuerthner *et al.*, 2017). This research has shown that when a host is challenged by two parasites simultaneously, the coinfection can increase, decrease or have no effect on the competitive ability of each parasite, depending on factors such as competition for resources and cross-reactive immunity (Pedersen and Fenton, 2007). Moreover, hosts are often challenged by different parasite species sequentially due to spatial, diel and seasonal variations in prevalence and emergence (Fingerut *et al.*, 2003, Studer and Poulin, 2012, Marino *et al.*, 2017). Thus, priority effects driven by both exposure timing and order can also strongly influence infection dynamics and result in different outcomes compared to simultaneous infection. For example, Hoverman *et al.* (2013) found that infection of larval amphibians with echinostome trematodes prior to exposure to the trematode species *Ribeiroia ondatrae* reduced *R. ondatrae* infection success. Because multiple parasite species commonly co-occur and the prevalence of different parasite species is variable through space and time, studies that elucidate the consequences of coinfection on infection outcomes are important for a broader understanding of disease dynamics in natural populations (Telfer *et al.*, 2008, 2010; Ezenwa *et al.*, 2010; Knowles, 2011).

Echinostomes (Echinostomatidae) are a diverse and common group of trematode parasites, with molecular evidence suggesting that over ten echinostome species exist in the United States (Detwiler *et al.*, 2010). Echinostomes are frequently used as a model parasite in ecological studies, particularly in amphibian systems due to their ability to cause intensity-dependent pathology and disease (Fried *et al.*, 1997; Holland *et al.*, 2007). Much of the research on echinostome-driven pathology in amphibians has focused on one species, *Echinostoma trivolvis* (Szuroczki and Richardson, 2009). However, despite slight morphological variations between some echinostome species, echinostomes can be difficult to differentiate without molecular tools (Kostadinova *et al.*, 2003; Detwiler *et al.*, 2010). Because of this, many experimental studies use field-collected echinostomes with identification limited to family, and some field surveys have likely underestimated the true macroparasite biodiversity in a system (Koprivnikar *et al.*, 2007; Hoverman *et al.*, 2012, 2013; Buss and Hua, 2018). The frequent

co-occurrence of multiple echinostome species suggests that some experimental studies may have used two or more species (Detwiler *et al.*, 2010, 2012), with the potential for interactions within the host that may affect infection success and pathology. However, the interactions between different echinostome species in second intermediate hosts have not been well explored (but see Leung and Poulin, 2011).

Amphibians present an excellent model system for assessing the influence of timing and order of exposure to multiple echinostome species on infection outcomes. Amphibians are a second intermediate host for a variety of trematode species and frequently co-occur with echinostomes (Hoverman *et al.*, 2012). Echinostomes and other trematodes typically use freshwater snails as a first intermediate host. The rediae in the first intermediate host then release free-swimming cercariae into the water column, which enter tadpoles *via* the cloacal opening and encyst in the kidney as metacercariae (Szuroczki and Richardson, 2009). Echinostome cercariae typically encyst preferentially in the right kidney, which has been viewed as an evolutionary adaptation to reduce parasitic impairment of both organ structures and thus lessen the impact of infection on host and parasite fitness (Thiemann and Wassersug, 2000; Johnson *et al.*, 2014). Because there is a considerable number of field and experimental studies of the echinostome-amphibian system (Johnson and McKenzie, 2008), it is of particular interest to explore how different echinostome species vary in infection success and how coinfection with two or more co-occurring echinostome species may influence infection outcomes.

Here, we used observational field data of echinostome prevalence across three months in seven ponds to inform laboratory experiments that explore how coinfection with two commonly co-occurring echinostome species influences infection outcomes in larval leopard frogs (*Rana pipiens*). Specifically, we examined how the timing of host exposure to two common echinostomes (*E. trivolvis* and *Echinoparyphium* lineage 3), presented separately and in combination, influences patterns of infection. When using single parasite exposures, we predicted that direct interspecific interactions would not strongly influence infection loads and that infection loads would be similar when the parasite species are present separately and in combination. When using two sequential parasite exposures, we predicted tadpoles exposed to the two parasite species sequentially would have higher infection loads than tadpoles exposed to the same parasite species sequentially, due to negative intraspecific interactions that would prime the immune system (Cox, 2001).

Material and methods

Field surveys

As part of a broader project to collect *Helisoma trivolvis* snails infected with *E. trivolvis*, we surveyed seven ponds in northwestern Pennsylvania from May to July 2018. We surveyed each pond once a month and collected *H. trivolvis*, which is a first intermediate host for echinostomes. We collected snails using unstandardized dipnet sweeps through submerged vegetation with the goal of collecting a minimum of 50 snails per site, keeping only snails >5 mm; snails below this threshold tend to be immature and rarely support infections (Richgels *et al.*, 2013). The number of collected snails varied by site and month [Month: mean (range); May: 143.7 (32–330); June: 235.7 (125–300); July: 175 (100–200)].

To screen collected snails for echinostome infection, we isolated individual snails in 50 mL tubes filled with 35 mL of UV-irradiated well water and placed them 10 cm below a light source for 1 h to induce cercarial shedding (Szuroczki and Richardson, 2009). We identified echinostome-infected snails by placing cercariae on slides under a compound scope following Schell (1985). We then isolated a single cercaria from each echinostome-infected snail and preserved it in 95% ethanol. Although it is possible that some snails were infected with multiple echinostome species, the overall prevalence of double infections is typically low (~2.5%; Sousa, 1993; Lafferty *et al.*, 1994). Moreover, experimental work has shown that double infection with echinostomatids is particularly unstable; one species is typically eliminated by the other (Dönges, 1972). This makes it possible, but unlikely, that any individual snail was double infected and shedding cercariae from two echinostome species.

Trematode identification

We extracted the genomic DNA from each trematode sample using a Qiagen DNeasy extraction kit. The internal transcribed spacer region (ITS-1) of ribosomal DNA was amplified by polymerase chain reaction (PCR), using forward (BD1, 5′ – GTC GTA ACA AGG TTT CCG TA – 3′, Bowles and McManus, 1993) and reverse (4S, 5′ – TCT AGA TGC GTT CGA A(G/A) T GTC GAT G – 3′, Bowles and McManus 1993) primers. The cycling parameters for the PCR were 95 °C for 2 min followed by 35 cycles of 94°C for 1 min, 50 °C for 45 s and 72 °C for 1 min, finishing with an elongation at 72 °C for 7 min. We then cleaned the PCR products with QIAquick PCR Purification Kit (Qiagen). Sequencing was conducted by the Purdue Genomics Core Facility in the forward and reverse directions using a BigDye terminator kit (Applied Biosystems) and an ABI 3730XL sequencer.

A total of 89 ITS1 sequences were generated. We condensed these 89 sequences into 14 unique haplotypes using DnasSP v6 prior to the phylogenetic analysis (Rozas *et al.*, 2017). We aligned one sample representing each of the 14 haplotypes with 23 previously published sequences of echinostomatids (Detwiler *et al.*, 2010) from GenBank automatically using the MUSCLE alignment in the program MEGA version X (Kumar *et al.*, 2018) and then rechecked manually by eye. Phylogenetic analysis was conducted with a Bayesian inference approach (MrBayes 3.2.7a; Ronquist *et al.*, 2012). We used the General Time Reversible plus Invariant sites plus Gamma distributed model of nucleotide substitution for our analysis. Two simultaneous Bayesian runs were conducted [with the default Markov chain Monte Carlo (MCMC) settings], and run for a total of 5.0×10^6 generations per run. We sampled trees and parameters every 100 generations and the first 25% of each run was discarded as burn-in (Hua *et al.*, 2016). We rooted the tree with three outgroup taxa. We then used this tree to identify our novel sequences based on Bayesian support values.

Experimental animal collection and husbandry

Based on our field surveys, the two most abundant echinostomatids at our field sites were *Echinostoma trivolvis* (hereafter *Echinostoma*) and *Echinoparyphium* lineage 3 (hereafter *Echinoparyphium*). We maintained a subset of the ten snails each infected with *Echinostoma* (site name – number of snails; LOG – 3; MAP – 3; CRK – 2; RMD – 2) and *Echinoparyphium* (site name – number of snails; LOG – 2; MAP – 3; CRK – 3; RMD – 1; JER – 1) in the lab for the experiments. Snails were housed individually in 1 L cups filled with 0.8 L of UV-irradiated well water and held at 7 °C to reduce shedding prior to the start of experiments. One day prior to the start of the experiments, snails were slowly acclimated to 23 °C. Snails were fed a mixture of rabbit chow and spirulina powder *ad libitum*.

We collected ten leopard frog (*R. pipiens*) egg masses from a pond at the Purdue Wildlife Area (PWA) in West Lafayette, IN

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Table 1. Summary of the 12 treatments used in experiments 1 and 2

Experiment	Exposure regime	Day 0	Day 3	Total number of tadpoles assessed
1	Simultaneous	50 *Echinostoma*	No parasites	10
		50 *Echinoparyphium*	No parasites	10
		25 *Echinostoma* + 25 *Echinoparyphium*	No parasites	10
2	Sequential	25 *Echinostoma*	25 *Echinostoma*	10
		25 *Echinoparyphium*	25 *Echinoparyphium*	10
		25 *Echinostoma*	25 *Echinoparyphium*	10
		25 *Echinoparyphium*	25 *Echinostoma*	9
		25 *Echinostoma*	No parasites	9
		25 *Echinoparyphium*	No parasites	10
		No parasites	25 *Echinostoma*	9
		No parasites	25 *Echinoparyphium*	9
		No parasites	No parasites	10

In experiment 1, *Rana pipiens* tadpoles experienced a single exposure to 50 *Echinostoma trivolvis*, 50 *Echinoparyphium* lineage 3 or 25 cercariae of each species on day 0. In experiment 2, *R. pipiens* tadpoles were exposed to a factorial combination of 25 *E. trivolvis*, 25 *Echinoparyphium* lin. 3 or no parasites.

on 12 April 2018. We distributed the egg masses into 180 L outdoor culturing pools that had been filled with aged well water and covered with 70% shade cloth to prevent predator oviposition. After hatching, tadpoles were fed rabbit chow (Purina) *ad libitum* until the start of experiments. Tadpole health was checked daily. Two days prior to the start of experiments, we brought 150 haphazardly chosen leopard frog tadpoles [snout-vent length (SVL) = 10.9 ± 1.1 mm S.D., stage = 27.5 ± 0.7 S.D.; Gosner, 1960] into the lab to acclimate to indoor conditions (23 °C, 12:12 light cycle).

Experiment 1: simultaneous exposures

The first experiment examined whether infection loads differed between exposure to each echinostome species separately or combined. A single tadpole was assigned to each experimental unit. Experimental units were completely randomized across a shelving unit and assigned to one of three treatments: (1) exposure to 50 *Echinostoma* cercariae; (2) exposure to 50 *Echinoparyphium* cercariae; or (3) exposure to 25 *Echinostoma* cercariae and 25 *Echinoparyphium* cercariae simultaneously ($N = 10$ tadpoles per treatment). Initial parasite exposures took place in 130 mL cups filled with 75 mL UV-irradiated well water. After 12 h, the tadpoles were transferred to a 1 L cup filled with 800 mL of UV-irradiated well water, as cercariae efficacy is highest during the first 8 h after shedding and declines with time (Rohr *et al.*, 2008).

To obtain cercariae to add to the experimental units, we individually shed the echinostome-infected snails for 1 h, as described previously, and then homogenized the cercariae by species in 1 L cups. We collected and counted free-swimming cercariae with a glass pipette under a dissecting scope and transferred the cercariae directly into each experimental unit. While dosing experimental units, we renewed the source parasites in 1 L cups every hour by discarding the unused cercariae and adding newly shed cercariae so that all cercariae entered experimental units within 2 h of emergence from the snail, which is when they are most infective.

We checked for tadpole mortality daily for 5 days and fed each tadpole rabbit chow *ad libitum* during the experiment. We ended the experiment after 5 days, as this allows echinostome cercariae to successfully encyst in the kidneys and minimizes time for tadpoles to clear infection (Hoverman *et al.*, 2013). We euthanized (MS-222 overdose) and individually preserved tadpoles in 70% ethanol. Prior to necropsy, we weighed, staged and measured SVL and the total length of each tadpole. To quantify infection load, we first dissected the left and right kidney structures (primary kidney, nephric duct, pronephros; hereafter referred to as left and right kidneys) of each tadpole, placed them between two microscope slides, and counted the total number of metacercariae on each side under a compound microscope (Schotthoefer *et al.*, 2003). We also examined the rest of the body for metacercariae, but all cysts were found in the kidneys.

Experiment 2: sequential exposures

The second experiment examined how the timing and sequence of exposure of tadpoles to *Echinostoma* and *Echinoparyphium* influence total echinostome infection loads. Each experimental unit was randomly assigned to a factorial treatment combination of parasite exposure on day 0 (25 *Echinostoma*, 25 *Echinoparyphium* or no parasites) and parasite exposure on day 3 (25 *Echinostoma*, 25 *Echinoparyphium* or no parasites). The nine treatments are summarized in Table 1. The experimental setup and parasite exposure methods were identical to experiment 1. Tadpoles were exposed to parasites on two dates, day 0 and day 3. On each day, we added either 25 cercariae of the appropriate species or no cercariae to each experimental unit. We checked for tadpole mortality daily and fed each tadpole rabbit chow *ad libitum* during the experiment. The experiment was ended on day 5, 2 days after the second exposure. We euthanized (MS-222 overdose) and individually preserved tadpoles in 70% ethanol. Tadpoles were necropsied as described in experiment 1. Because experiment 1 and experiment 2 occurred simultaneously in the same randomized experimental array, we draw comparisons between the two experiments.

Statistical analyses

Because our field surveys were unstandardized and limited in scope, we restricted our analysis of the field data to summary statistics and qualitative descriptions to inform our experimental design.

Our main response variable for the experimental studies was overall infection load (count of metacercariae recovered). Although there are slight size variations between the two echinostome species (Fried *et al.*, 1998), this is not a reliable metric to differentiate a large quantity of metacercarial cysts, and molecular identification of so many parasites is not feasible. We used a negative binomial generalized linear model (nbGLM) for these analyses as our count data were overdispersed. To examine whether

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infection loads differed among single exposures to *Echinostoma* cercariae, *Echinoparyphium* cercariae or a combination of both parasites in experiment 1, we conducted an nbGLM with treatment as the main predictor and final SVL as a covariate, as tadpole size can influence susceptibility (Marino *et al.*, 2017). To examine how the timing and sequence of exposure of tadpoles to *Echinostoma* and *Echinoparyphium* cercariae affected total echinostome infection loads in experiment 2, we conducted an nbGLM with treatment as the main predictor and final SVL as a covariate. We also conducted a second nbGLM with day 0 treatment and day 3 treatment as the main predictors and final SVL as a covariate to determine if differences in infection loads differed based on the species used for the initial or secondary exposure.

To better determine if intraspecific or interspecific interactions influenced total echinostome infection loads in experiments 1 and 2, we compared the observed metacercarial load of each treatment to an expected value (Hoverman *et al.*, 2013). The expected parasite infection load was calculated by adding together the average number of parasites recovered from treatments exposed to only 25 cercariae on either day 0 or day 3 (Table 1); this value represents the infection load that would be expected if infection was additive (i.e. no antagonistic or synergistic interactions). This value was then compared to the observed parasite infection load for the treatments exposed to 50 total cercariae (Table 1). For example, to determine if interspecific interactions influenced infection load in the treatment exposed to 25 *Echinostoma* + 25 *Echinoparyphium* simultaneously on day 0, we first added the average number of parasites recovered from the treatment exposed to 25 *Echinostoma* on day 0 (mean = 9.1 metacercariae) with the average number of parasites recovered from the treatment exposed to 25 *Echinoparyphium* on day 0 (mean = 8.9 metacercariae). We then used a single sample *t*-test to determine whether the observed infection load differed from the expected infection load for each of the treatments receiving a total of 50 cercariae. We also conducted a series of repeated *G*-tests of goodness-of-fit to compare the observed distribution of metacercariae recovered from each kidney (left and right) with those expected assuming completely random, equal distribution on the left and right side.

We performed all statistical analyses using R version 3.5.1 (R Core Team, 2018). We determined that our metacercarial cyst load count data for was overdispersed and that using a Poisson distribution was inappropriate using the dispersiontest() function in the 'AER' package (dispersion = 2.45, $Z = 5.24$, $P < 0.001$; Kleiber and Zeileis, 2008). Negative binomial GLMs were conducted using the glm.nb() function in the 'MASS' package (Venables and Ripley, 2002). We used the Anova() function in the 'car' package to estimate *P* values (Fox and Weisberg, 2011). Estimated marginal means were calculated with the 'emmeans' package (Lenth *et al.*, 2019), and Tukey post hoc tests were used to determine where significant differences among the treatments occurred with the cld() function in the 'multcomp' package (Hothorn *et al.*, 2008). We used the *t*.test() function to compare observed and expected metacercarial loads. Figures were made using 'ggplot2' (Wickham, 2016). We did not log-transform SVL, as it was normally distributed, and transformation did not improve normality. We excluded four individual tadpoles (but no more than one in any treatment) from analyses as their kidneys were degraded prior to cyst counting.

Research ethic approval for infection of tadpoles

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. The authors have involved the minimum number of animals to produce statistically reproducible results. All methods for the infection of tadpoles were approved by the Purdue University IACUC (protocol 1701001530). Animals were collected under the Indiana Department of Natural Resources permit 18-066.

Results

Echinostome identification and prevalence in natural ponds

Using the sequenced ITS region from individual cercariae, each obtained from a different *H. trivolvis* snail host, we identified 89 echinostome samples consisting of 14 distinct haplotypes in the field survey. By comparing these 14 haplotypes to previously published sequences of echinostomatids (Detwiler *et al.*, 2010; Fig. 1), we identified our samples as belonging to three species: *E. trivolvis* (haplotypes 10–13, $N = 37$), *Echinostoma revolutum* (haplotype 14, $N = 1$) and *Echinoparyphium* lineage 3 (haplotypes 1–9, $N = 51$) (Table 2). *E. trivolvis* and *Echinoparyphium* lineage 3 were identified from snails at all seven sites (Fig. 2). Both species were identified at 13 out of 21 samplings and both species co-occurred in 7/21 samplings at five different sites. The single *E. revolutum* sample was identified from a June sampling at one pond. Prevalence in snail hosts by month and site ranged from 0 to 6% for *E. trivolvis* and 0–12.5% for *Echinoparyphium* lineage 3. Our field surveys provided substantial evidence that *E. trivolvis* and *Echinoparyphium* lineage 3 commonly co-occur in our study region.

Experiment 1: simultaneous exposures

For tadpoles that experienced a single exposure to separate or combined echinostomatid species, we found that infection loads significantly differed between the three treatments ($\chi^2 = 6.4$, D.F. = 2, $P = 0.041$; Fig. 3A). The mean metacercarial load of individuals exposed to a mixed cohort of *Echinostoma* and *Echinoparyphium* was 58.2% higher than the mean metacercarial load of individuals exposed to *Echinoparyphium* alone ($Z = 2.353$, $P = 0.048$). Although individuals exposed to only *Echinostoma* tended to have higher infection loads than individuals exposed only to *Echinoparyphium*, this difference was not statistically significant ($Z = -1.969$, $P = 0.120$). Mean metacercarial load of individuals exposed to a mixed cohort of *Echinostoma* and *Echinoparyphium* did not differ from the mean metacercarial load of individuals exposed to only *Echinostoma* ($Z = -0.313$, $P = 0.947$). We did not find that infection loads in any treatment differed significantly from the expected value ($t \leqslant 2.0$, D.F. = 9, $P \geqslant 0.076$; Fig. 3A). Only one individual died during this experiment, so we did not assess the effects of treatment on mortality.

Experiment 2: sequential exposures

For the hosts in the four treatments that experienced two exposures to either *Echinostoma* or *Echinoparyphium* on day 0 and day 3 (D–G, Table 2), we found evidence that mean metacercarial load was significantly different between treatments ($\chi^2 = 12.8$, D.F. = 1, $P = 0.005$; Fig. 3B). Mean metacercarial load of individuals exposed to *Echinoparyphium* on day 0 and *Echinostoma* on day 3 was ~36.8% lower than either treatment with exposure to *Echinostoma* on day 0 ($Z \geqslant 3.029$, $P \leqslant 0.013$). The mean metacercarial load of individuals exposed to *Echinoparyphium* on day 0 and *Echinoparyphium* on day 3 did not significantly differ from the other three treatments (Fig. 3B). We also found evidence that mean metacercarial load was significantly influenced by the parasite species used at exposure on day 0 ($\chi^2 = 6.4$, D.F. = 1, $P = 0.012$). Following both exposures, mean metacercarial load of individuals exposed to *Echinostoma* on day 0 was 30% higher than mean

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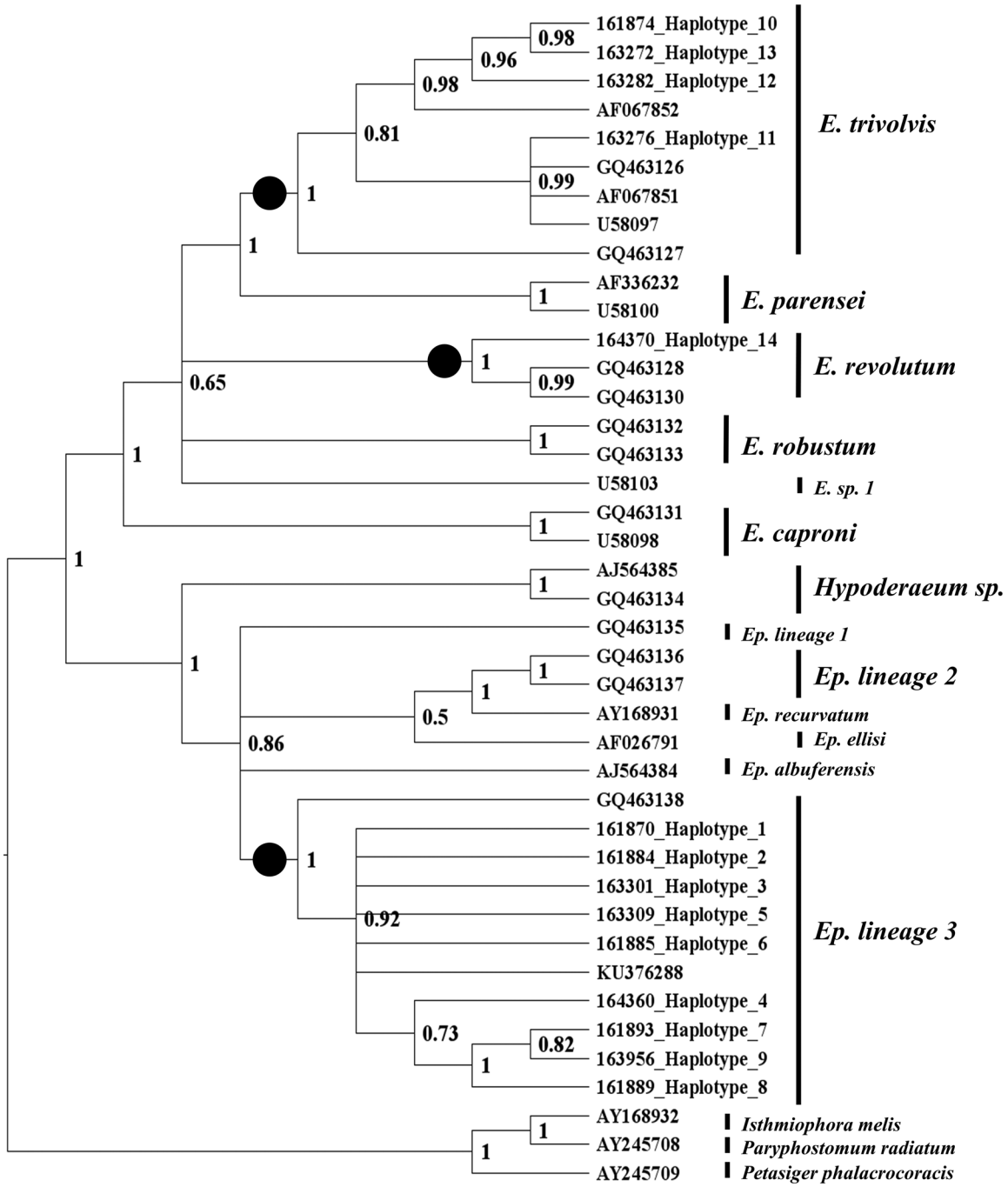

Fig. 1. Phylogenetic estimate of relationships within Echinostomatidae based on the ITS1 gene inferred from Bayesian support values and rooted with three outgroup species. Support values are shown near the nodes. Nodes supported by ⩾95% posterior probability are considered highly supported. The circles denote the species or lineages which were detected in our sampling.

metacercarial load of individuals exposed to *Echinoparyphium* on day 0, regardless of day 3 treatment. When comparing expected infection loads with observed infection loads by treatment, mean metacercarial load of individuals exposed to *Echinoparyphium* on day 0 and *Echinostoma* on day 3 was 32% lower than the expected value ($t = -3.98$, D.F. = 9, $P = 0.003$; Fig. 3B). Observed infection loads did not differ significantly from the expected value in the other three treatments (all $P > 0.05$; Fig. 3B). We found no significant differences in mean metacercarial load between any of the treatments receiving only 25 total cercariae that were used to calculate expected infection values ($\chi^2 = 2.7$, D.F. = 3, $P = 0.436$). Given that only four tadpoles died during the experiment, we did not assess the effects of treatment on tadpole mortality.

Kidney encystment bias

Of the 4375 cercariae used across all treatments, 1816 successfully encysted (41.5%). Of those that successfully encysted, 715 encysted in the left kidney (39.4%) and 1101 encysted in the right kidney (60.6%), and a repeated *G*-test of goodness-of-fit found that the bias toward the right kidney was statistically significant ($G = 82.7$, D.F. = 1, $P < 0.001$). The bias of metacercariae toward the right kidney was consistent within all seven treatments that received 50 cercariae ($P < 0.05$).

Discussion

In natural systems, hosts are typically challenged by a wide array of co-occurring parasites (Rynkiewicz *et al.*, 2015). Accordingly,

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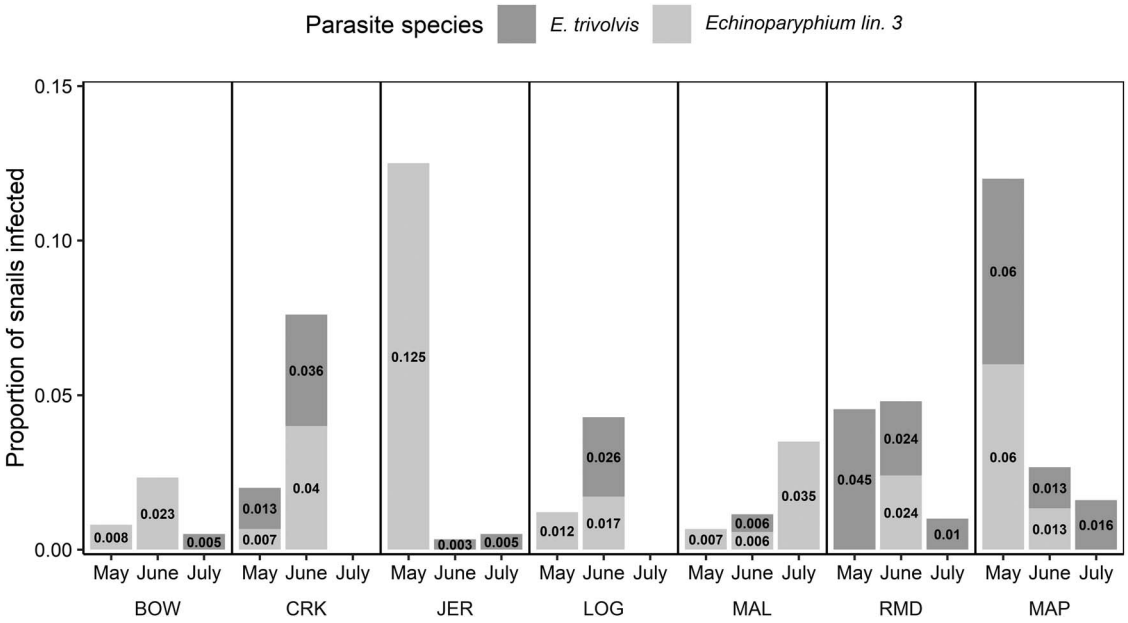

Fig. 2. The prevalence of *Echinoparyphium* lineage 3 and *Echinostoma trivolvis* in seven ponds in northwest Pennsylvania over a three-month survey in 2018. Each bar depicts the proportion of infected *Helisoma trivolvis* snails, separated by trematode species.

Table 2. Haplotype frequencies of the 89 Echinostomatidae ITS1 sequences obtained from cercariae for this study.

Haplotype	Species	*N* (% of total)
1	*Echinoparyphium* lineage 3	43 (48.3%)
2	*Echinoparyphium* lineage 3	1 (1.1%)
3	*Echinoparyphium* lineage 3	1 (1.1%)
4	*Echinoparyphium* lineage 3	1 (1.1%)
5	*Echinoparyphium* lineage 3	1 (1.1%)
6	*Echinoparyphium* lineage 3	1 (1.1%)
7	*Echinoparyphium* lineage 3	1 (1.1%)
8	*Echinoparyphium* lineage 3	1 (1.1%)
9	*Echinoparyphium* lineage 3	1 (1.1%)
10	*Echinostoma trivolvis*	21 (23.6%)
11	*E. trivolvis*	14 (15.7%)
12	*E. trivolvis*	1 (1.1%)
13	*E. trivolvis*	1 (1.1%)
14	*Echinostoma revolutum*	1 (1.1%)

disease ecologists have shifted focus from studies of single parasite infection outcomes to an assemblage of two or more parasites to better understand the influence of co-infection in disease outcomes, within-host parasite communities, and community disease dynamics (Telfer *et al.*, 2010; Hoverman *et al.*, 2013; Ezenwa, 2016; Wuerthner *et al.*, 2017). While this prior research has demonstrated that coinfection with two functionally different parasites can have profound impacts on infection outcomes, there has been little focus on infection outcomes associated with coinfection by functionally and morphologically similar species (Miura *et al.*, 2005; Detwiler *et al.*, 2010, 2012). Using molecular methods, we found that two echinostome species, *E. trivolvis* and *Echinoparyphium* lineage 3, commonly co-occur in aquatic ecosystems. Using this qualitative field survey to inform our experimental design, we demonstrated that both single and sequential exposures to these two echinostome species can alter infection loads in a larval amphibian host.

In treatments that experienced a single exposure to two echinostome species – separately or combined – we found that mean metacercarial load of individuals exposed to a mixed cohort of *Echinostoma* and *Echinoparyphium* was 58% higher than the mean metacercarial load of individuals exposed to only *Echinoparyphium* but similar to the mean metacercarial load of individuals exposed to only *Echinostoma.* Individuals exposed to only *Echinostoma* had 49% higher infection loads than individuals exposed only to *Echinoparyphium*, but this difference was not statistically significant. Thus, co-occurring echinostome species can be highly variable in their ability to successfully infect larval amphibians and simultaneous infection with multiple species can cause infection loads equal to or higher than either species alone.

One potential mechanism for this observation is that in coinfection, one parasite facilitates increased infection success in the other. Because the two parasite species were present at the same time, it is unlikely that an immune response played a significant role in facilitation. Instead, infection by the more successful parasite may have increased the infection success of the other parasite by enhancing its ability to enter the host. For example, cellular damage caused by *Echinostoma* encystment may have increased the concentration of chemical cues released from the cloaca (e.g. amino acids; Haas *et al.*, 2000), thereby increasing the ability of *Echinoparyphium* to enter the host. Alternatively, hosts exposed to only *Echinoparyphium* on day 0 may have had lower infection loads due to density-dependent regulation resulting from intraspecific interference or a stronger behavioural response by the host (Ebert *et al.*, 2000; Karvonen *et al.*, 2003; Poulin, 2010). There may be an evolved mechanism for parasites to avoid penetration of previously infected hosts, although this has mainly been explored with the miracidia stage rather than the highly abundant cercaria stage (Haas *et al.*, 2000; Allan *et al.*, 2009; Vannatta *et al.*, 2020). Because the metacercariae of the two species used are morphologically indistinct, it remains uncertain how coinfection influenced the success of each species alone; however, the patterns in

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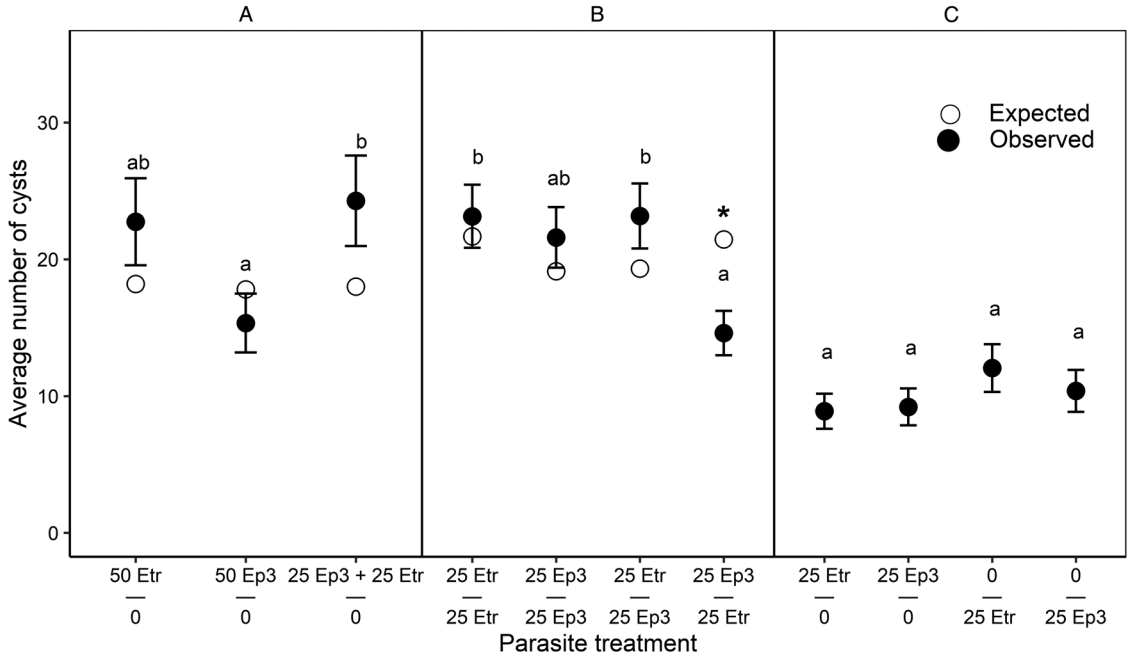

Fig. 3. Parasite infection loads (estimated marginal mean for number of metacercariae recovered ± s.e.) in *Rana pipiens* tadpoles after different combinations of exposure to two echinostome species at one or two time points. For each treatment, the day 0 exposure is shown above the day 3 exposure and separated by '—'. Panel A shows the treatments receiving 50 cercariae at once on day 0; Panel B shows the treatments receiving 25 cercariae on day 0 and 25 cercariae on day 3; Panel C shows the treatments receiving 25 cercariae on either day 0 or day 3. Within each panel, treatments sharing lower case letters are not significantly different from each other ($P > 0.05$). For each treatment in panel A and B, the expected total infection load calculated from mean infection loads in panel C is shown as an open circle. An asterisk denotes a significant difference between the expected and observed value ($P < 0.05$). Ep3 represents *Echinoparyphium* lineage 3 and Etr represents *Echinostoma trivolvis*.

this experiment suggest that infection success can differ between echinostome species but that simultaneous coinfection may increase the infection success of one or both species.

In treatments that experienced parasite exposures at two separate time points, we found that the mean metacercarial load of individuals exposed to *Echinoparyphium* on day 0 and *Echinostoma* on day 3 was 37% lower than either treatment with exposure to *Echinostoma* on day 0. We also found that individuals exposed to *Echinoparyphium* on day 0 had a 23% lower infection load relative to individuals exposed to *Echinostoma* on day 0. Together, these results demonstrate that priority effects can influence the overall infection success of two co-occurring echinostome species, but that the interaction may be asymmetric and dependent on exposure order. These results are unlikely to be explained by the energetic demands of the previously encysted parasites, as metacercariae have low resource demands (Smyth and Halton, 1983). Instead, the finding that initial exposure to *Echinoparyphium* resulted in overall lower infection loads than initial exposure to *Echinostoma* could indicate that either: (1) *Echinoparyphium* exposure induces a strong immune response that confers cross-immunity to a later echinostome infection; or (2) *Echinostoma* exposure results in helminth-induced immunosuppression that prevents cross-immunity to a later echinostome exposure from occurring. Helminth-induced immunosuppression is a well-documented strategy used by trematodes to increase their survival in the host (Maizels *et al.*, 2009; Taylor *et al.*, 2012), but it is unclear if this occurs in the metacercarial stage that infects amphibians. There is, however, evidence to suggest that early exposure to trematode cercariae can heighten resistance to a later challenge by a functionally different parasite (Hoverman *et al.*, 2013; Wuerthner *et al.*, 2017; Koprivnikar *et al.*, 2019). The fact that mean infection load of individuals exposed to *Echinoparyphium* on day 0 and *Echinostoma* on day 3 was significantly lower than the expected value supports the cross-immunity hypothesis and suggests that the *E. trivolvis* on day 3 had an infection success lower than what would be expected if the infection was additive (i.e. antagonism). Collectively, these results suggest that exposure timing and order can strongly influence coinfection outcomes, even with functionally similar parasites; however, more research is needed to reveal the mechanism underlying these priority effects.

A limitation of this study is that in coinfected tadpoles, we were unable to differentiate between the cysts of two echinostome species. Several studies of amphibian trematode infections have used fluorescing dye to label cercariae used for exposures on different days, a potentially useful method for future studies of infection dynamics with morphologically similar parasites (Leung and Poulin, 2011; Hoverman *et al.*, 2013; LaFonte and Johnson, 2013; Johnson *et al.*, 2014; LaFonte *et al.*, 2015; Koprivnikar *et al.*, 2019). Another limitation is that because our field surveys were limited in scope, we can draw few conclusions about how the prevalence of each species changes through the year in natural systems. For example, a temporal gap in peak abundance of *Echinostoma* and *Echinoparyphium* lineage 3 could lead to predictably staggered infections similar to those explored experimentally here. Alternatively, spatial separation of snails infected with different echinostomes within a pond could lead to temporal gaps in exposure based on host habitat choices. Further standardized surveys will be needed to determine if this is the case. Finally, because echinostome infection loads can significantly vary in natural situations, our narrow range of exposures likely does not capture the range of outcomes, such as increased pathology or mortality, that might occur with echinostome coinfections. Future studies should explore how coinfection with different echinostomes at a range of exposure loads alters disease outcomes and the success of each parasite.

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As the principles of community ecology become increasingly important to the field of disease ecology, clarifying the dynamics of within-host parasite interactions has become necessary (Pedersen and Fenton, 2007; Johnson *et al.*, 2015). Moreover, molecular studies continue to reveal genetic distinctions between morphologically similar species. Thus, it is important to explore whether there are non-additive interactions between functionally and morphologically similar macroparasite species or whether the effects of parasite exposure can be generalized by morphotype. Our study demonstrates that echinostome infection success in larval anurans can differ significantly based on the species makeup, density and timing of exposure. We also found evidence for priority effects based on exposure order; individuals exposed to *Echinoparyphium* on day 0 tended to have lower final infection loads than individuals exposed to *Echinostoma* on day 0. This finding adds to the existing literature demonstrating priority effects during coinfection and emphasizes that priority effects can occur even between functionally similar species (Hoverman *et al.*, 2013; Devevey *et al.*, 2015; Wuerthner *et al.*, 2017). Given these findings, we recommend that workers using field-collected echinostomes as a model parasite for disease studies use molecular methods to confirm which species will be used. While the cost of sequencing can be prohibitive, techniques such as qPCR or PCR with species-specific primers (e.g. Fujino *et al.*, 1997) may provide a more affordable way to rapidly identify the presence or absence of DNA from a specific species. Studies should continue to focus on how cryptic parasite diversity in natural systems influences disease outcomes, and how these functionally similar organisms interact and compete for within-host resources.

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Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. The authors have involved the minimum number of animals to produce statistically reproducible results. All methods for the infection of tadpoles were approved by the Purdue University IACUC (protocol 1701001530). Animals were collected under Indiana Department of Natural Resources permit 18-066.

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