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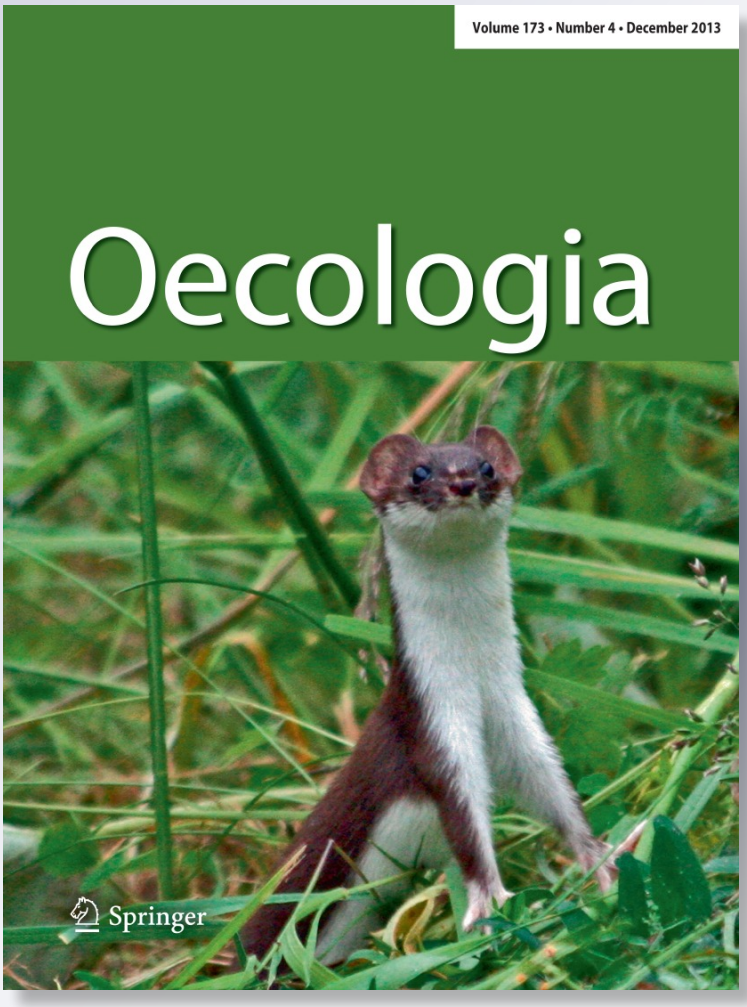

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COMMUNITY ECOLOGY - ORIGINAL RESEARCH

Does timing matter? How priority effects influence the outcome of parasite interactions within hosts

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Abstract In nature, hosts are exposed to an assemblage of parasite species that collectively form a complex community within the host. To date, however, our understanding of how within-host–parasite communities assemble and interact remains limited. Using a larval amphibian host (Pacific chorus frog, *Pseudacris regilla*) and two common trematode parasites (*Ribeiroia ondatrae* and *Echinostoma trivolvis*), we experimentally examined how the sequence of host exposure influenced parasite interactions within hosts. While there was no evidence that the parasites interacted when hosts were exposed to both parasites simultaneously, we detected evidence of both intraspecific and interspecific competition when exposures were temporally staggered. However, the strength and outcome of these priority effects depended on the sequence of addition, even after accounting for the fact that parasites added early in host development were more likely to encyst compared to parasites added later. *Ribeiroia* infection success was reduced by 14 % when *Echinostoma* was added prior to *Ribeiroia*, whereas no such effect was noted for *Echinostoma* when *Ribeiroia* was added first. Using a novel fluorescent-labeling technique that allowed us to track *Ribeiroia* infections from different exposure events, we also discovered that, similar to the interspecific interactions, early encysting parasites reduced the encystment success of later arriving parasites by 41 %, which could be mediated by host immune responses and/or competition for space. These results suggest that parasite identity interacts with host immune responses to mediate parasite interactions within the host, such that priority effects may play an important role in structuring parasite communities within hosts. This knowledge can be used to assess host–parasite interactions within natural communities in which environmental conditions can lead to heterogeneity in the timing and composition of host exposure to parasites.

Keywords Amphibian · Coinfection · Developmental windows · Immune priming · Indirect competition

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Introduction

In natural populations, a diverse array of macroparasites, microparasites, and mutualists form a complex community within each individual (Thrall et al. 2007). Because of the recent emergence of infectious diseases, there has been an increasing focus on understanding how parasite communities assemble and interact within their hosts (Pedersen and Fenton 2007; Telfer et al. 2010). To understand how parasite communities assemble within hosts, there is a need to integrate fundamental ecological concepts developed for free-living species into host–parasite systems (Pedersen and Fenton 2007; Poulin 2007; Graham 2008). For example, interactions between parasites may be direct, involving competition for host resources or attachment sites within the host (Cattadori et al. 2007; Mideo 2009), or indirect, via the host's immune system (Graham 2008; Fenton and Perkins

2010; Telfer et al. 2010; Hawley and Altizer 2011). Depending on the characteristics of the specific parasites involved, indirect interactions can be either positive (i.e. immune suppression) or negative (i.e. cross-reactive immune responses) (Pedersen and Fenton 2007; Cobey and Lipsitch 2013). Thus, analogous to free-living species, parasite communities within hosts can be structured by both bottom–up (e.g., resource competition) and top–down (e.g., activity of the host immune system) processes (Pedersen and Fenton 2007; Fenton 2008; Graham 2008; Johnson and Buller 2011). Because the outcome of parasitic interactions within the host will ultimately affect host pathology, parasite transmission, and virulence evolution (Cox 2001; Pedersen and Fenton 2007; Lello et al. 2008; Telfer et al. 2008; Lively 2009), studies that explore the outcome of parasite interactions within hosts are especially important (Adamson et al. 1992; Adamson and Noble 1993; Sousa 1994).

Interactions between parasites are likely to be influenced by the order in which the host encounters each parasite. For free-living species, it is well established that community assembly is strongly influenced by the historical sequence of species addition into the community. Species arriving early can inhibit or facilitate the establishment of later arriving species (Connell and Slatyer 1977; Alford and Wilbur 1985; Menge and Sutherland 1987). While such 'priority effects' have been identified across a multitude of systems, less is known regarding priority effects in intra-host parasite communities (Jackson et al. 2006; Karvonen et al. 2009; Leung and Poulin 2011). Priority effects are likely to play an important role in structuring intra-host parasite communities for a number of reasons. Parasite transmission in natural populations is a dynamic process, influenced by spatial and temporal heterogeneity in the abundance of both hosts and parasites (Mideo et al. 2008; Hawley and Altizer 2011). In addition, the ability of hosts to resist or tolerate infection can vary temporally, such as between seasons (Hawley and Altizer 2011) or through development (Holland et al. 2007; Kelly et al. 2010; Johnson et al. 2011). These factors may modulate potential direct and indirect interactions among parasites within hosts, suggesting that priority effects could be important for structuring intra-host parasite communities (Jackson et al. 2006; Karvonen et al. 2009). Integrating priority effects into host–parasite systems has the potential to generate novel insights into the factors that affect parasite community structure and assembly.

Amphibians and their digenetic trematodes (flukes) provide a useful study system to explore the influence of priority effects on within-host interactions and parasite community assembly (Koprivnikar et al. 2012). Amphibians are intermediate or definitive hosts for a diversity of trematode species (Schell 1985; Sutherland 2005), many of which use freshwater snails as first intermediate hosts. Amphibians are, therefore, exposed to a wide range of trematode infections during their aquatic larval stage. These parasites generally encyst as metacercariae, which do not reproduce within the amphibian (Smyth and Halton 1983). Across the landscape, it is common to find multiple trematode species co-infecting amphibians (Johnson and Buller 2011). Recently, *Ribeiroia ondatrae* and *Echinostoma* spp. in particular have gained notoriety for their capacity to induce severe pathology and limb deformities in amphibians (Johnson 2008). However, studies have only recently started to examine the within-host interactions of trematodes. Laboratory experiments that have followed simultaneously coinfected amphibian hosts through metamorphosis have demonstrated reduced persistence of both parasite species compared to hosts that were exposed to a single parasite species (Johnson and Buller 2011; Johnson and Hoverman 2012). Because these parasites encyst in different locations within amphibians (*R. ondatrae* encysts around the limb buds while *E. trivolvis* encysts in the kidneys), it has been suggested that these negative interactions are indirect, potentially mediated by host immune responses (Pedersen and Fenton 2007).

Here, we extend these studies by examining the role of priority effects in interactions between functionally similar and taxonomically related parasites (larval trematodes). We focus on a system consisting of two common trematode species (*R. ondatrae* and *E. trivolvis*) (Johnson and Buller 2011) and their most abundant host (*Pseudacris regilla*) (Johnson et al. 2013). Using laboratory experiments, we manipulated the timing of host exposure to each of the parasites separately and in combination with the other parasite to examine intraspecific and interspecific interactions and priority effects on infection success. Using a novel fluorescent-labeling technique, we also tracked the in situ infection success and persistence of *Ribeiroia* infections from each of the exposure events. Different fluorescent labels allowed us to identify parasites by exposure event, even for multiple exposures of the same parasite species, and thereby further investigate intraspecific priority effects within living hosts. Based on previous work demonstrating negative interactions between the parasites, we predicted that early arriving parasites would reduce the infection success of late arriving parasites and that interspecific competition would likely be stronger than intraspecific competition.

Materials and methods

Species collection and husbandry

We collected adult rams horn snails (*Helisoma trivolvis*) from field sites in California and Oregon to obtain *R.*

ondatrae and *E. trivolvis* (hereafter referred to as *Ribeiroia* and *Echinostoma*, respectively) for the experiments. The snails were screened for infection by isolating individuals in 50-mL centrifuge tubes containing 40 mL of treated tap water for 24 h. The snails were then divided into sources for each parasite species by identifying free-swimming cercariae that had emerged into the water (following Schell 1985). *Ribeiroia* cercariae were identified by the presence of an esophageal diverticulum. *Echinostoma* cercariae were identified by the presence of collar spines. At our collection sites, molecular data support the predominance of *E. trivolvis* (S. Orlofske et al., unpublished data); however, given their morphological similarity with other echinostomes (e.g., *E. revolutum*, *Echinoparyphrium* spp.), we cannot rule out the possibility that our species represented a closely related echinostome (Johnson 2008). The two groups of infected snails were maintained separately in 20-L tubs containing treated tap water until used in the experiment.

Egg masses ($n \sim 100$) of the Pacific chorus frog (*P. regilla*) were collected from Lake Penhollow (experiments 1 and 2) or Obsidian Lake East (43.70N, 121.23W; experiment 3), Oregon, and shipped overnight to the University of Colorado. After hatching, the tadpoles were randomly assigned to 50-L tubs containing treated tap water and held at 22 °C in a temperature-controlled room. Tadpoles were fed a 1:1 diet of ground TetraMin and TetraVeggie fish flakes (Tetra, Blacksburg, VA, USA) ad libitum until used in an experiment.

Experiment 1

The first experiment examined how simultaneous exposure to *Ribeiroia* and *Echinostoma* (i.e. coinfection) affected the infection success of each parasite species. The experiment was a completely randomized design consisting of three treatments: (1) exposure to 10 *Ribeiroia* cercariae; (2) exposure to 10 *Echinostoma* cercariae; and (3) exposure to 10 *Ribeiroia* cercariae and 10 *Echinostoma* cercariae ($n = 8$ tadpoles per treatment). Our experimental units were 2-L tubs containing 1 L of treated tap water. The experiment was conducted at 22 °C and a 12:12 day:night photoperiod. A single tadpole (Gosner 1960, stage 26) was randomly assigned to each container and fed a 1:1 diet of ground TetraMin and TetraVeggie fish flakes (Tetra) every other day during the experiment. To obtain cercariae for the experiment, snails were isolated as above for up to 3 h, and the free-swimming cercariae were collected using a stereo dissection scope and glass pipette. The cercariae from different snails were pooled together before use. Within 4 h of emergence, all cercariae were isolated, counted, and transferred into the experimental units. Because tadpoles are known to clear parasitic infections over time (Johnson et al. 2011), we terminated the experiment after 6 days to focus on the initial infection success of the parasites. Tadpole survival was 100 % in all treatments and no pathology (e.g., malformations) was detected in the experiment. All tadpoles were euthanized in a 0.5 % solution of MS-222 and preserved in 10 % neutral-buffered formalin. Tadpoles were systematically necropsied to determine infection abundance of each parasite (number of encysted metacercariae). During the necropsies, the entire tadpole was examined including the epithelial tissue, tail muscle, gills, intestines, and kidneys (following Johnson and Hartson 2009).

Experiment 2

The second experiment examined how the sequence of exposure to *Ribeiroia* and *Echinostoma* affected the infection success of each parasite species (i.e. priority effects). The experiment was a completely randomized factorial combination of parasite exposure at time 1 (no parasites, *Ribeiroia* or *Echinostoma*) crossed with parasite exposure at time 2 (no parasites, *Ribeiroia* or *Echinostoma*) with 10 replicate tadpoles per treatment (see Table 1). Experimental units and setup were identical to experiment 1. Parasites were obtained as described above and tadpoles were exposed on two dates (exposure 1 = day 0, and exposure 2 = day 3). On the first exposure date, we added 15 cercariae of the appropriate parasite species to the tubs. On the second exposure date, we added 20 cercariae of the appropriate parasite species. The difference in the number of parasites used for each exposure was determined by parasite availability. Importantly, previous research has demonstrated that parasite dosage (i.e. number of parasites in the exposure) does not influence the percentage of parasites that successfully encyst (Johnson and Hoverman

Table 1 Summary of the nine experimental treatments ($n = 10$ per treatment) used in experiment 2

Treatment	Time 1	Time 2	Total parasites
1	No parasites	No parasites	0
2	No parasites	*Echinostoma*	20
3	No parasites	*Ribeiroia*	20
4	*Echinostoma*	No parasites	15
5	*Ribeiroia*	No parasites	15
6	*Ribeiroia*	*Ribeiroia*	35
7	*Echinostoma*	*Echinostoma*	35
8	*Ribeiroia*	*Echinostoma*	35
9	*Echinostoma*	*Ribeiroia*	35

Pseudacris regilla tadpoles were exposed to a factorial combination of no parasites, *Echinostoma trivolvis*, or *Ribeiroia ondatrae* (time 1 = day 0, and time 2 = day 3). For each parasite, exposures at time 1 consisted of 15 cercariae while exposures at time 2 consisted of 20 cercariae

2012). Tadpoles were fed a 1:1 diet of ground TetraMin and TetraVeggie fish flakes (Tetra) every other day during the experiment. The experiment was terminated 2 days after exposure 2 (day 5). Tadpole survival was 100 % in all treatments and no pathology (e.g., malformations) was detected in the experiment. Tadpoles were euthanized in a 0.5 % solution of MS-222 and preserved in 10 % neutral-buffered formalin. Tadpoles were necropsied as described above. On average, tadpoles had reached Gosner stage 30 by the end of the experiment and stage did not differ among treatments (ANOVA $F_{8,80} = 1.9$, $P = 0.061$).

Experiment 3

The third experiment used a novel parasite-labeling technique to examine the infection success and persistence of *Ribeiroia* from each of the sequential exposure events, with parasites labeled a specific color for each exposure event. This allowed us to differentially track the fate of parasites from early versus late exposures within living hosts. The experiment was a completely randomized design consisting of three treatments ($n = 14$ per treatment) that manipulated host exposure to *Ribeiroia* cercariae at two time points (days 0 and 7). The three treatments were: (1) exposure on day 0 only, (2) exposure on day 7 only, and (3) exposure on days 0 and 7 (Table 2). The exposures on day 0 consisted of a low dose (5 cercariae) while the exposures on day 7 consisted of a high dose (30 cercariae). The dosage used at day 0 (i.e. 5 cercariae) was chosen to minimize competition for space and focus on hypothesized interactions mediated by the host's immune system. The experiment was conducted at 22 °C and a 14:10 day:night photoperiod. Forty-two tadpoles, at Gosner stage 29, were randomly assigned to a treatment group, and each placed in an individual 1-L tub containing 750 mL of treated tap water. Tadpoles were fed daily and water changed every 5 days to maintain water quality throughout the experiment. Parasites were obtained as described above. Cercariae were fluorescently labeled using the fatty acid analog probes BODIPY 558/568 C12 (red dye; parasites added on day 0) or BODIPY FL C12 (green dye; parasites added on day 7) from Molecular Probes following Keeney et al. (2008) and LaFonte and Johnson (submitted). From the purchased dyes, 100 mM working solutions were created by dissolving the dyes in dimethyl sulfoxide. These stocks were added to 50-mL centrifuge tubes containing the free-swimming cercariae to achieve a 100-nM concentration, and incubated for 45 min at 25 °C. Cercariae were then rinsed twice in treated tap water before being counted and transferred to the experimental tubs (within 5 h of emergence).

Table 2 Summary of the three experimental treatments ($n = 14$ per treatment) used in experiment 3

Treatment	Time 1	Time 2	Total parasites
1	5 *Ribeiroia* cercariae	30 *Ribeiroia* cercariae	35
2	5 *Ribeiroia* cercariae	No parasites	5
3	No parasites	30 *Ribeiroia* cercariae	30

P. regilla tadpoles were exposed to a sequential combination of fluorescently-labeled *R. ondatrae* cercariae (time 1 = day 0, and time 2 = day 7)

Half of the tadpoles from each treatment were removed from the experiment at 36 h after the second exposure to examine infection success and the remaining half at 21 days to examine parasite persistence ($n = 7$ per treatment). All tadpoles were euthanized, and systematically necropsied to determine the abundance of parasites from each of the exposure events (number of encysted metacercariae) using a Leica MZ FLIII fluorescence stereomicroscope equipped with TXR and GFP2 fluorescent filter sets suitable for BODIPY 558/568 C12 (red; parasites added on day 0) and BODIPY FL C12 (green; parasites added on day 7), respectively. One individual died during the experiment (day 0 exposure only), but there was no other mortality or pathology.

Statistical analyses

We used analysis of variance (ANOVA) to examine the effects of coinfection on the infection success of each parasite species in experiment 1. A separate analysis was conducted for each parasite species using the presence or absence of the other parasite species as the main factor. We calculated the proportion of parasites recovered out of the total administered for each parasite species and proportions were arcsine-square root transformed prior to analysis.

For the second experiment, we conducted multiple analyses with the dataset to assess treatment effects on total parasite infection success and the infection success of each parasite species. In the first analysis, we used a two-way ANOVA to examine the effects of parasite species (*Ribeiroia* or *Echinostoma*) and exposure time (time 1 or time 2) on parasite infection success. For this analysis, we focused on treatments with single exposures to the parasites (treatments 2–5 in Table 1). This analysis allowed us to assess whether infection success differed between the parasite species and between exposure times. Due to differences in the number of parasites added to the treatments, we calculated the proportion of parasites recovered out of the total administered for each parasite species and proportions were arcsine square root transformed prior to analysis.

We also conducted analyses to determine if intra- and interspecific interactions influenced parasite infection success. Because infection success differed between parasite species and exposure times and different numbers of

parasites were added at the two time points (see "Results"), we used the four treatments described above to calculate the expected parasite infection success in the intra- and interspecific interaction treatments. This value was calculated as the average number of parasites recovered for parasite species X at time 1 plus the average number of parasites recovered for parasite species X at time 2 divided by the total exposure level (i.e. 35 parasites). For instance, the expected total parasite infection success if tadpoles were exposed to *Ribeiroia* at time 1 then *Echinostoma* at time 2 was 38 % (11.5 *Ribeiroia* + 1.8 *Echinostoma*/35 total parasites). We used the single sample *t* test to determine whether our observed parasite infection success differed from the expected values for each of the intra- and interspecific interaction treatments (treatments 6–9 in Table 1).

For the third experiment, we used a Generalized Linear Model with negative binomial distribution using the ''glm.nb'' function in the ''MASS'' package of R statistical software (Rohr et al. 2010; R Development Core Team 2010) to examine the effects of exposure treatment, sampling time, and their interaction on parasite detection at each exposure. We then examined the effect of infection treatment on parasite detection at each time point, to examine whether the presence of parasites from prior exposure influenced infection success (36 h) or parasite persistence (21 days).

Results

Experiment 1

In short-term trials, there was no evidence that simultaneous coinfection altered the ability of either parasite to infect the host (Fig. 1). Infection success did not differ for *Echinostoma* in treatments with and without coinfecting *Ribeiroia* ($F_{1,14} = 0.4$, $P = 0.553$). Although there was a trend for fewer *Ribeiroia* in treatments with coinfecting *Echinostoma*, this trend was not significant ($F_{1,14} = 0.2$, $P = 0.663$). On average, the infection success of *Ribeiroia* was twice the infection success of *Echinostoma*.

Experiment 2

For hosts receiving a single parasite exposure, parasite infection success was strongly influenced by parasite species and the timing of the exposure (Fig. 2). On average, infection success was 31 % higher in hosts infected with *Ribeiroia* (mean infection success = 59 %) than *Echinostoma* (mean infection success = 28 %) ($F_{1,36} = 18.1$, $P < 0.001$). Additionally, infection success was 36 % higher in hosts exposed at time 1 compared to time 2

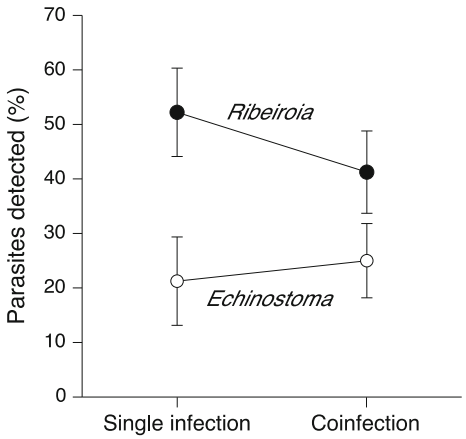

Fig. 1 Infection success (mean % of metacercariae detected ±1SE) of *Ribeiroia ondatrae* (*filled circles*) and *Echinostoma trivolvis* (*open circles*) in *Pseudacris regilla* tadpoles exposed to either parasite in isolation (single infection) or both parasites simultaneously (coinfection)

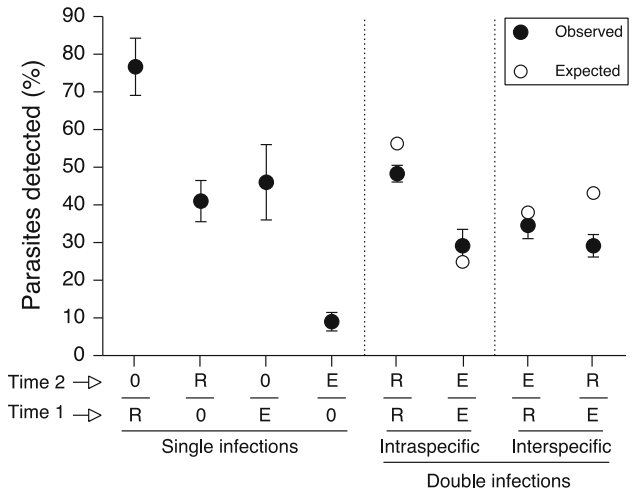

Fig. 2 Parasite infection success (mean % of metacercariae detected ±1SE) observed within *P. regilla* tadpoles when tadpoles were exposed at two time points (time 1 = day 0 and time 2 = day 3) to a factorial combination of either 0 no parasites, E *Echinostoma*, or R *Ribeiroia*. For the intra- and interspecific infection treatments, the predicted total infection success (*open circles*) is also shown (see text for calculation)

($F_{1,36} = 23.5$, $P < 0.001$). However, there was no interaction between parasite species and exposure time ($F_{1,36} = 0.002$, $P = 0.968$).

We used the data from these single parasite exposures to compare expected to observed parasite infection success in the intraspecific and interspecific interaction treatments. In the intraspecific interaction treatment (same parasite at time 1 and time 2), the infection success of *Ribeiroia* was 8 % lower than expected ($t = -3.59$, $df = 9$, $P = 0.006$). However, the infection success of *Echinostoma* did not differ from the expected value ($t = 0.98$, df = 9, $P = 0.351$). In the interspecific interaction treatment (one

parasite at time 1, the other at time 2), total parasite success depended on the sequence of addition and was asymmetric. There was no evidence of a reduction in total infection success when *Ribeiroia* was added at time 1 and *Echinostoma* was added at time 2 ($t = -0.973$, $df = 9$, $P = 0.178$). However, total infection success was 14 % lower than expected when *Echinostoma* was added at time 1 and *Ribeiroia* was added at time 2 ($t = -2.98$, $df = 9$, $P = 0.016$). This effect was mainly driven by lower than expected success of *Ribeiroia* ($t = -2.45$, $df = 9$, $P = 0.037$) rather than *Echinostoma* ($t = -1.82$, $df = 9$, $P = 0.052$; electronic supplementary material). Combining the results from the intraspecific and interspecific treatments, we found that interspecific competition was 81 % stronger than intraspecific competition for *Ribeiroia*.

Experiment 3

Similar to the second experiment, the sequence of exposure played a significant role in *Ribeiroia* persistence. In this experiment, however, we were able to determine the persistence of parasites from individual exposure events through the use of the fluorescent biomarker. At 21 days post-exposure, the persistence of parasites that were added at the second exposure (day 7) was 41 % lower in tadpoles that had also been exposed to parasites at the first exposure (day 0) compared to those that had not been exposed at day 0 ($Z = -2.039$, $P = 0.042$; Fig. 3b). The persistence of parasites that established during the first exposure was not influenced by the second exposure event at 21 days post-exposure ($Z = -0.273$, $P = 0.785$; Fig. 3a). Initial infection success (the number of encysted parasites) from either the first or the second exposure did not differ between treatments at 36 h post-exposure (parasites from first exposure: $Z = 0.284$, $P = 0.777$; parasites from second exposure: $Z = -0.107$, $P = 0.915$; Fig. 3). Thus, these results expand on our understanding of intraspecific priority effects by demonstrating that parasites involved in the second exposure bear the brunt of the reduction in total infection success seen in intraspecific interaction treatments (both here and in experiment 2).

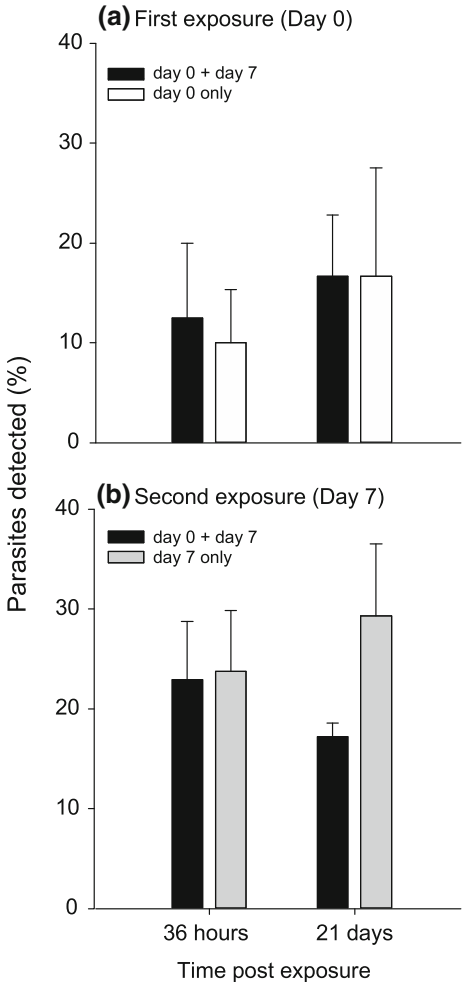

Fig. 3 Infection success and persistence of *R. ondatrae* (mean number of metacercariae detected ±1SE) in *P. regilla* tadpoles following sequential exposure to fluorescent-labeled cercariae. Parasites detected from **a** the first exposure on day 0 and **b** parasites detected from the second exposure on day 7. Tadpoles were either exposed only on day 0 (*white*), only on day 7 (*gray*) or at both time points (*black*). Infection success was assessed 36 h after the second exposure and persistence was assessed 21 days after the second exposure

Discussion

In disease ecology, there is an increasing focus on understanding the factors that influence the assembly of parasite communities within hosts (Pedersen and Fenton 2007; Poulin 2007; Graham 2008; Johnson and Hoverman 2012). Using principles from community ecology, research has addressed how direct and indirect interactions between parasites and the host's immune system can influence infection success (Cattadori et al. 2007; Mideo 2009; Hawley and Altizer 2010; Telfer et al. 2010). However, the influence of priority effects on the outcome of parasite interactions within hosts has received comparatively little attention (Jackson et al. 2006; Karvonen et al. 2009; Leung and Poulin 2011). By combining experiments that examined simultaneous and temporally staggered exposures to parasites, we demonstrate that priority effects can alter intra- and interspecific competition and, ultimately, infection success of potentially virulent parasites.

In treatments involving a single exposure to a single parasite species, we found a 36 % reduction in infection success for parasites administered in the second exposure event compared to those from the first exposure event for both *Ribeiroia* and *Echinostoma*, suggesting that the timing

of exposure strongly influenced parasite loads. This information provided critical baseline data that could be used to examine the influence of intra- and interspecific interactions on infection success. In general, increased resistance to infection as amphibians develop likely explains the reduced success of parasites added late in the experiments, as found in previous studies of amphibian–parasite interactions (Schotthoefer et al. 2003; Holland et al. 2007; Rohr et al. 2010; Johnson et al. 2011). Collectively, this research suggests that there are critical windows during host development that influence resistance and disease outcomes (Sollid et al. 2003; Cunningham et al. 2005; Villeneuve et al. 2005; Holland et al. 2007; Holland 2009; Kelly et al. 2010; Johnson et al. 2011).

Our experiments revealed evidence for interspecific competition between the two parasite species and these effects were dependent on the sequence of addition and the identity of the parasite. Notably, there was no evidence for interspecific competition when both parasite species were added simultaneously. However, when parasite addition was temporally staggered, we detected evidence for interspecific competition (i.e. priority effects) after accounting for the expected reduction in infection success between time points, based on single infections. Interestingly, these effects were asymmetric. While the infection success of *Echinostoma* added second was not influenced by prior exposure to *Ribeiroia* (and likewise, short-term persistence of *Ribeiroia* was not significantly influenced by subsequent exposure to *Echinostoma*), *Ribeiroia* infection success was reduced by 14 % when *Echinostoma* was added prior to *Ribeiroia*. Importantly, although *Ribeiroia* also showed reduced infection success in the intraspecific treatments, interspecific competition was 81 % stronger than intraspecific competition in Experiment 2.

Because *Echinostoma* and *Ribeiroia* encyst in different locations within amphibians (kidneys and limb buds, respectively) and encysted metacercariae generally have low resource demands (Smyth and Halton 1983), competitive interactions are likely mediated by the host's immune system (Johnson and Hoverman 2012). Additional evidence for the role of host's immune system comes from the contrasting outcomes between the simultaneous and temporally staggered exposures. Because there is generally a time lag between initial exposure to parasites and activation of the immune response, simultaneous exposure to different parasites is unlikely to provide sufficient time for the immune system to mediate interspecific interactions that reduce initial infection success. However, exposures that are staggered by at least 2 days appear to be adequate to prime immune function and mediate competitive interactions. Importantly, the strength of the interspecific priority effects assessed here may be underestimated. The short time scale of the second experiment may have reduced our ability to detect the effects of parasite clearance by the host. Johnson and Buller (2011) found negative interspecific interactions in hosts that had reached metamorphosis (having been exposed to both parasites simultaneously as larvae), in contrast to the positive associations between *Ribeiroia* and *Echinostoma* in coinfected individuals at 10 days post-exposure (see also Johnson and Hoverman 2012). This is consistent with the notion that time since exposure is a critical component of parasite persistence within the host.

The asymmetric nature of the competitive interaction between the parasites when exposures were staggered was unexpected. This could suggest that activation of the host's immune response was contingent on parasite identity rather than a general response to infection. If activation of the host's immune system was a general response to trematode infection (Karvonen et al. 2009), prior exposure to either parasite species should have reduced the recovery of both parasite species when added later in the experiment. The fact that prior exposure to *Echinostoma* reduced encystment of *Ribeiroia* at the second exposure but no reciprocal effect was observed suggests that host immune responses to *Echinostoma* may be cross-reactive for other trematodes, whereas any immune response to *Ribeiroia* may be more species-specific. Thus, parasite identity could interact with host immune responses to mediate parasite interactions within the host. Consistent with our findings, Johnson and Buller (2011) found reductions in the persistence of *Ribeiroia*, but not *Echinostoma*, in simultaneously coinfected hosts followed through metamorphosis. However, because disease outcomes associated with macroparasites are often density-dependent (Anderson and May 1978), there is a need for future studies that manipulate exposure dosage to determine if the observed competitive interactions are sensitive to the numbers of parasites interacting within the host.

We also found evidence for intraspecific competition and, by using a novel method to label parasites from particular exposure events with different colored dyes, we showed that this competition manifested through priority effects. In particular, we found reduced persistence of *Ribeiroia* following repeated exposures indicating the existence of intraspecific competition. The observed intraspecific interactions for *Ribeiroia* may have been mediated by both host immune responses (i.e. top–down effects) and/or competition for space (i.e. bottom–up effects). Because of the large size of *Ribeiroia* and the small size of the limb bud region of the tadpoles tested, competition for space within the tadpole could have reduced the ability for later arriving parasites to encyst. To minimize the effects of competition for space, our third experiment used just five cercariae for the initial exposures and parasites were marked using a novel

fluorescent-labeling technique to track their fate within the host. Despite an average of just one encysted parasite from the first exposure, we found a 41 % reduction in the persistence of late arriving parasites at 21 days compared to controls. By comparison, there was no reduction in the persistence of parasites from the initial exposure, suggesting that later arriving parasites do not displace early encysting parasites. Importantly, these patterns in persistence were not associated with initial differences in infection success; the number of encysted parasites at 36 h post-exposure was not different among treatment groups. Together, these results suggest that later arriving parasites are gradually cleared, potentially by the host's immune system, but only when the host had been previously exposed. Thus, in addition to competition for space, host immune priming (Hamilton et al. 2008) may play a role in mediating intraspecific competition. Such immune priming has been shown to induce heterogeneity in host susceptibility, with important ramifications for disease dynamics (Tate and Rudolf 2012).

In contrast, we did not find evidence of intraspecific competition for *Echinostoma*. Because of the relatively small size of *Echinostoma*, the low number of parasites added, and the lower infection success of *Echinostoma* relative to *Ribeiroia*, intraspecific competition for space within the relatively large amphibian kidney was likely low. Indeed, surveys of wild amphibians have frequently detected >1,000 metacercariae in the kidneys (Johnson 2008), suggesting that high *Echinostoma* loads are common. Thus, higher parasite exposure levels may be necessary to observe intraspecific competition for this parasite species. While few studies have examined intraspecific competition among amphibian parasites, our results suggest the strength of intraspecific interactions is mediated by a combination of parasite size, availability of area for encystment, and host immune responses.

The dynamics of within-host interactions among parasite species are increasingly recognized as important determinants of disease outcomes. Similar to free-living communities (Menge and Sutherland 1987; Robinson and Dickerson 1987; Lawler and Morin 1993; Werner and Peacor 2003), communities of parasites within hosts can be influenced by bottom–up and top–down processes as well as the historical sequence of parasite community assembly. Our demonstration of priority effects indicates that top–down processes, particularly cross-reactive host immune responses can influence parasite community assembly within the host. Additionally, stage-dependent susceptibility of the host (i.e. top–down processes) played a role in reducing parasite recovery over time. These findings are in line with studies of priority effects in invertebrates, suggesting parasite competition is largely host-mediated (Ulrich and Schmid-Hempel 2012). These results collectively suggest that the timing of host exposure to parasites is crucial for understanding parasite community structure (i.e. composition and abundance) within the host. Ultimately, this knowledge can be used to assess host–parasite interactions within natural communities. For instance, amphibian breeding phenology and parasite emergence from intermediate snail hosts are dependent on climate variables such as temperature and precipitation (Duellman and Trueb 1994; Poulin 2006). Consequently, differential host and parasite responses to environmental conditions can lead to heterogeneity in the timing of host exposure to parasites during development, which can affect host pathology (e.g., malformation frequency; Johnson and Hartson 2009; Johnson et al. 2011). Moreover, hosts are exposed to parasites on an ongoing basis, representing multiple exposure events. In order to fully understand the forces structuring parasite communities within free-living hosts, it will be necessary to understand how priority effects cascade through multiple repeated exposures. With the increasing focus on natural patterns of parasite abundance within populations, future studies that specifically incorporate temporal factors operating within sites will be essential for understanding host–parasite interactions.

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