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Parasite competition hidden by correlated coinfection: using surveys and experiments to understand parasite interactions

Pieter T. J. Johnson[1] and Ian D. Buller

Ecology and Evolutionary Biology, University of Colorado, Boulder, Colorado 80309 USA

Abstract. Within most free-living species exists a cryptic community of interacting parasites. By combining multiscale field data with manipulative experiments, we evaluated patterns of parasite coinfection in amphibian hosts and their underlying mechanisms. Surveys of 86 wetlands and 1273 hosts revealed positive correlations between two pathogenic trematodes (*Ribeiroia ondatrae* and *Echinostoma trivolvis*) both between wetlands and within individual hosts. In infection and coinfection experiments, *Ribeiroia* caused greater pathology than *Echinostoma*, including high host mortality (24%) and severe limb malformations (75%). No interactive effects were noted for host pathology, but both parasites decreased the per capita persistence of one another by 17–36%. Thus, in spite of consistently positive associations from field data, these parasites negatively affected the persistence of one another, likely via cross immunity (apparent competition). These findings underscore the danger of inferring parasite interactions from coinfection patterns and emphasize the potential disconnect between within-host processes (e.g., competition) and between-host processes (e.g., exposure and transmission). Here, correlated coinfections likely resulted from similarities in the parasites' host requirements and heterogeneity in host susceptibility or exposure. Understanding complex interactions among parasites depends critically on the scale under consideration, highlighting the importance of combining coinfection field studies with mechanistic experiments.

Key words: amphibian decline; amphibian malformations; coinfection; concomitant infection; disease ecology; infectious disease; polyparasitism.

Introduction

When many ecologists think of species communities, they perhaps imagine interactions among plants, herbivore consumers, and top-level predators. But within each of these easily-observed groups exists another community of interacting parasite species. Although most studies focus on single-host–single-pathogen interactions, growing evidence suggests that interactions among co-occurring parasites can influence host pathology, parasite transmission, and the evolution of virulence (Cox 2001, Pedersen and Fenton 2007, Lello et al. 2008, Telfer et al. 2008, Lively 2009). A promising frontier in understanding parasite interactions within hosts involves applying the principles of community ecology to within-host communities (Pedersen and Fenton 2007, Poulin 2007, Graham 2008). Community ecology provides a mechanistic framework for understanding parasite interactions, which can be direct, such as competition for attachment sites, competition for host resources, and predation upon one another (intraguild predation; e.g., Lello et al. 2004, Mideo 2009). Interactions can also be indirect or "host-mediated," often involving changes in immunity such as cross-immunity (apparent competition) and immune suppression (apparent facilitation) (Cattadori et al. 2007, Jolles et al. 2008). Based on a review of parasite interactions, Graham (2008) suggested that the outcome of intra-host interactions will depend on the types of resources used by each parasite (bottom-up controls) and which host defenses are activated (top-down controls; Pedersen and Fenton 2007).

Despite growing interest in parasite coinfections, surprisingly few studies have determined the factors responsible for observed correlations among different pathogens. Associations between parasites can be influenced by host behavior, ecology, exposure history, and pathology (Poulin 2007, Behnke 2008, Telfer et al.

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[1] E-mail: pieter.johnson@colorado.edu

2008), obscuring both the form of the interaction and whether it affects within-host (e.g., immunity) or between-host (e.g., transmission) processes (Hawley and Altizer 2010). For example, morbidity induced by one parasite can increase exposure to a second, even if within-host interactions are antagonistic (e.g., Karvonen et al. 2009). Mortality induced by one pathogen can also eliminate the availability of hosts for other parasites (Jolles et al. 2008). Collectively, such examples illustrate how parasite interactions vary as a function of scale, ranging from within individual hosts (infracommunity) to between host populations across the landscape (component community; Karvonen et al. 2007, Pedersen and Fenton 2007), underscoring the importance of combining field studies of parasite coinfection with controlled experiments to understand their interactions.

Communities of larval helminths within amphibian hosts provide a tractable study system to explore parasite interactions and how they vary with scale. In aquatic environments, amphibian hosts become infected by a diverse assemblage of helminth parasites, many of which have complex life cycles involving sequential transmission among multiple host species (Prudhoe and Bray 1982, Sutherland 2005). Unlike many microparasite infections, however, larval helminths do not reproduce within amphibian hosts, such that each parasite represents an independent and quantifiable infection event. Parasites within individual frogs and frogs within individual wetlands also provide well-delineated boundaries for exploring spatially nested communities. Finally, because amphibians acquire most larval helminths during the aquatic phase (i.e., tadpole), recently metamorphosed frogs provide a consistent and relatively standardized stage for comparison, reducing the challenges associated with parasites that accumulate with age (e.g., Cattadori et al. 2007, 2008, Telfer et al. 2008).

We combined large-scale field surveys with mechanistic experiments to determine patterns of parasite coinfection within amphibian hosts and identify the mechanisms responsible. We focused on the trematodes *Ribeiroia* and *Echinostoma*, each of which form encysted metacercariae in larval amphibians that can cause significant pathology in their hosts (see Plate 1). Using field data from 86 wetlands and 1273 frog hosts, we evaluated patterns of parasite co-occurrence and infection abundance at both the local (i.e., within host) and landscape (i.e., among wetlands) scales. To evaluate the underyling mechanisms of parasite interaction, we conducted a complementary laboratory experiment in which we exposed larval amphibians to realistic numbers of each parasite individually and in combination. Our goals were to compare the effects of each parasite and examine how their interactions influenced infection success and host pathology. By combining field and experimental approaches, we further aimed to incorporate the influence of multiple scales into understanding parasite interactions.

Methods

Field studies.—To compare patterns of infection and coinfection under natural conditions, we used data on *Ribeiroia* and echinostomes from 86 wetlands in the East Bay region of California, USA (Alameda, Contra Costa, and Santa Clara counties; see Plate 1). We focused on wetlands that supported Pacific chorus frogs (*Pseudacris regilla*) and rams horn snails (*Helisoma* spp.), each of which are suitable intermediate hosts for *Ribeiroia* and echinostome infections. We selected wetlands at random from among publicly accessible ponds and conducted preliminary visits in May 2009 to select sites with the requisite hosts. We returned to each pond in mid June to late July 2009 to collect metamorphosing (Gosner [1960] stages 44–46) *P. regilla* ($n \geq 10$ per site) for parasite quantification (see Sutherland 2005 and Johnson and Hartson 2009). Our focus was on *Ribeiroia* and parasites in the "echinostome" group, which include species in the genera *Echinostoma* and *Echinoparaphyrium*. We conducted analyses at three levels: first, we used contingency analysis to compare the frequency of wetland co-occurrence between the two parasites. Second, among wetlands that supported both parasites, we compared the average infection abundance of each using linear regression. Finally, we assessed the relationship between the presence and abundance of *Ribeiroia* and echinostomes within each frog (nested within a wetland) using mixed effects models (Zuur et al. 2009). Among wetlands that supported both parasites, we used generalized linear mixed models with a binomial distribution to compare whether frogs infected with one parasite were more or less likely to support the second parasite (recoding infection as infected vs. uninfected). Wetland was treated as a random effect using the R package lme4 and the Laplace approximation method (R Development Core Team 2008). Finally, to compare the abundances of *Ribeiroia* and echinostomes at the within-host scale, we used linear mixed effects models with snout–vent length ($\log_{10}$-transformed) as a covariate and wetland as a random effect in the R package nlme. Akaike's information criterion (AIC) was used to select among models with a random intercept alone or with both a random intercept and a random slope (see Zuur et al. 2009).

Laboratory experiment.—We collected egg masses of *Pseudacris regilla* from Lake Penhollow, Oregon, in June 2009 and transferred them to 1.5-L plastic containers after hatching. Tadpoles were fed a 1:1 mixture of Tetramin (Spectrum Brands, Madison, Wisconsin, USA) and *Spirulina* while water and containers were replaced twice per week (see Johnson and Hartson 2009). We obtained snails (*Helisoma* spp.) from field sites and determined their infection status by individually isolating them and examining the water for free-swimming cercariae. Cercariae were examined under a compound microscope to differentiate between *Ribeiroia* and echinosome parasites (see Johnson and McKenzie 2008, Szuroczki and Richardson 2009).

Although our echinostomes are likely *Echinostoma trivolvis*, we did not conduct molecular analyses to verify this and refer to them here as "echinostomes."

We conducted two experiments to evaluate the effects of *Ribeiroia* and echinostomes as a function of the timing and dosage of exposure. In experiment 1, which evaluated the effects of a pulse exposure on the survival of early-stage tadpoles, we randomly assigned 20 tadpoles (Gosner [1960] stage 26) to each of the following treatments: control (no parasite exposure), *Ribeiroia* (single exposure to 10 cercariae), echinostome (exposure to 10 cercariae), and *Ribeiroia* + echinostome (exposure to 10 cercariae of each parasite). Using a stereo-dissecting microscope, we isolated cercariae within four hours of release and pipetted them directly into each tadpole's container. Tadpoles in the control treatment were sham-exposed to water from uninfected snails. Ten days following the parasite exposures we euthanized all tadpoles and compared the incidence of mortality among treatments.

In experiment 2, we exposed tadpoles to each parasite later in development (Gosner stage 28), across a wider range of exposure levels, and raised animals to metamorphosis to evaluate host growth and malformations. We conducted a 2×3 factorial experiment in which tadpoles (Gosner stage 28) were exposed to *Ribeiroia* (none vs. 40 cercariae) and echinostome cercariae (none, 40 [light], or 160 [heavy] cercariae). We replicated each treatment as follows: control = 50; echinostome-light = 35; echinostome-heavy = 35; *Ribeiroia* only = 45; *Ribeiroia* + echinostome-light = 45; *Ribeiroia* + echinostome-heavy = 50 (total $n = 260$). Exposures were conducted as in experiment 1 with two exceptions. First, we administered the total parasite dosage in four exposure events distributed over a 10-day period to more closely mirror the continuous exposures encountered in nature. Second, we raised tadpoles to metamorphosis, recording time to metamorphosis (d), snout–vent length (SVL, mm), and mass (g). We examined metamorphosing frogs for abnormalities and necropsied ~20 from each treatment to quantify metacercariae. Tadpoles that died prior to metamorphosis were also necropsied.

Analysis.—We analyzed data on host survival (number of days alive) using parametric survival analysis, with individuals surviving for either 10 days (experiment 1) or until metamorphosis (experiment 2) classified as "censored." In experiment 2, malformation data (yes or no) were analyzed using generalized linear models with Firth's correction for separation (Firth 1993). We evaluated the effects of treatment on z-scores of host size, mass, and time-to-metamorphosis using two-way ANOVA. Days to metamorphosis was initially included as a covariate in analyses involving frog length and mass. Because no differences were observed between low (40 cercariae) and high (160 cercariae) echinostome exposures, we combined these into a single category for analyses. To analyze parasite recovery, we used either

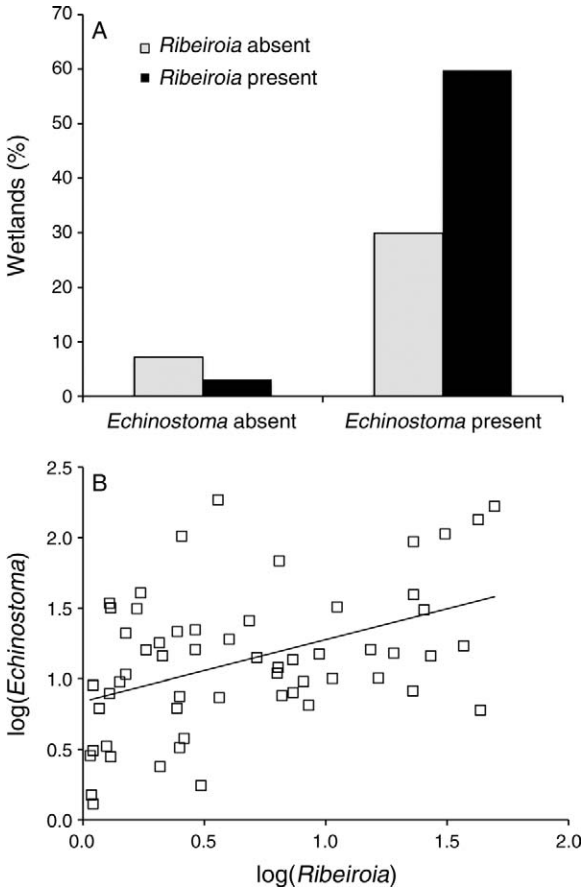

FIG. 1. Patterns of parasite co-occurrence and coinfection in California wetlands. (A) Percentage of wetlands that supported trematodes *Ribeiroia*, *Echinostoma*, or both parasites in amphibian hosts (*Pseudacris regilla*). (B) Average infection abundance of *Ribeiroia* and *Echinostoma* (each $\log_{10}$-transformed) among wetlands that supported both parasites.

(1) generalized linear models with a Poisson distribution for counts of metacercariae or (2) ANOVAs on the proportion of parasites recovered (arcsine-square-root transformed).

RESULTS

Field data.—*Ribeiroia* and echinostomes were significantly more likely to co-occur in a wetland than expected by chance (Fig. 1A; Pearson $\chi^2 = 5.39$, $P = 0.02$, $n = 86$ observations). Among sites where the parasites co-occurred ($n = 52$ sites), their mean abundances correlated positively at the wetland scale (Fig. 1B; $r = 0.45$, $P < 0.0001$). However, echinostome infection was consistently greater than that of *Ribeiroia* (paired t test, $t = 5.63$, df = 51, $P < 0.0001$), with overall mean (± SE) of 9.12 ± 1.72 for *Ribeiroia* and 26.9 ± 5.74 for echinostomes ($n = 52$). At the within-host scale, the presence of one parasite within a frog positively predicted co-occurrence by the second (generalized linear mixed models: echinostomes on *Ribeiroia*, $t = 1.97$, $P < 0.05$; *Ribeiroia* on echinostomes, $t = 2.69$, $P < 0.01$). After accounting for the effect of wetland, the $\log_{10}$-transformed abundances

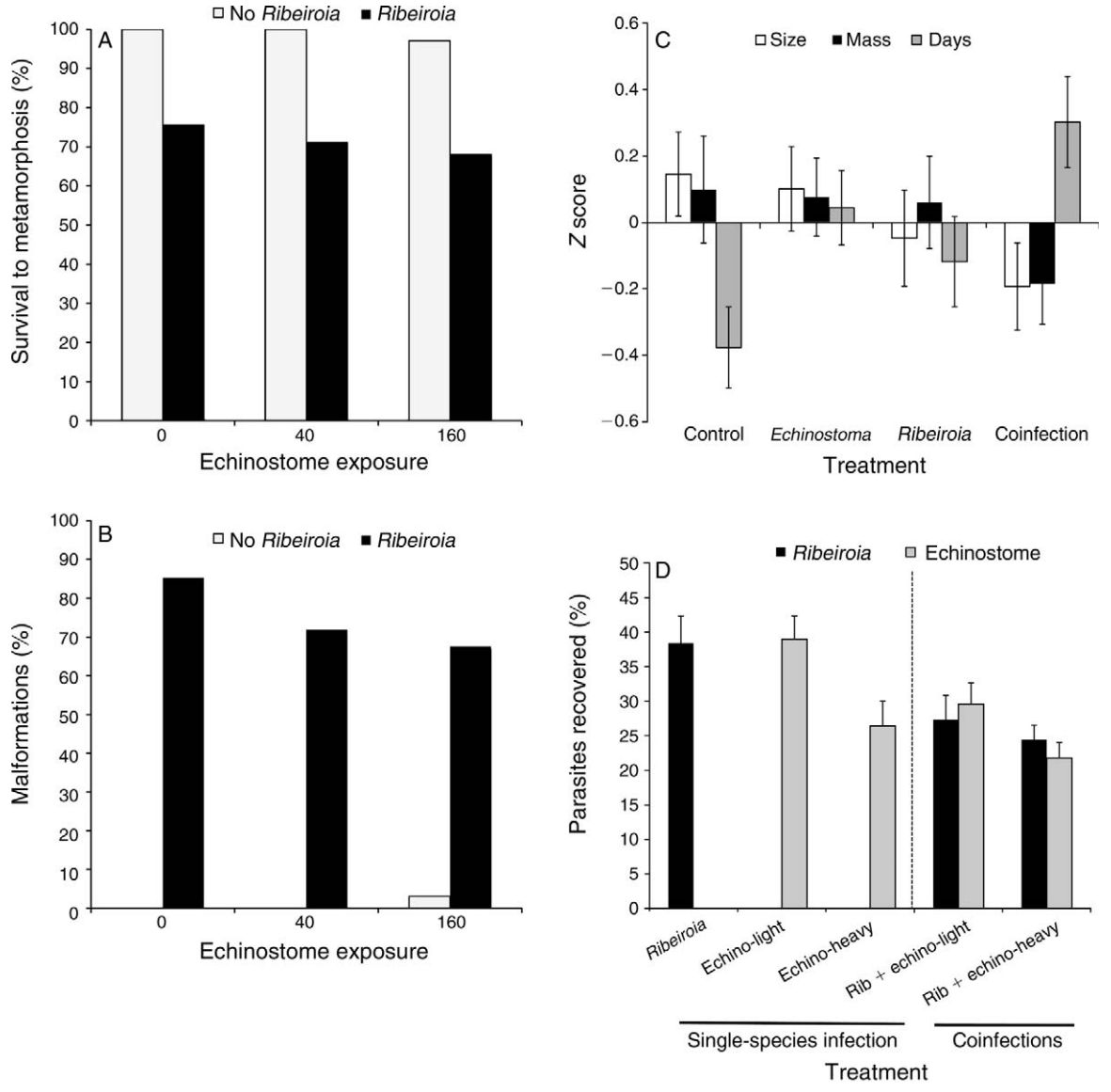

FIG. 2. Experimental infections with *Ribeiroia* and *Echinostoma*. (A) Effects of infection and coinfection on survival of larval *P. regilla* to metamorphosis. *Ribeiroia* exposures involved 0 or 40 cercariae while *Echinostoma* exposure involved 0, 40, or 160 cercariae. (B) Frequency (%) of malformations in metamorphosing *P. regilla* as a function of parasite treatment. (C) Effects of parasite infection and coinfection on the size, mass, and time to metamorphosis of *P. regilla* (all responses changed to *z* scores). (D) Effects of experimental condition on the recovery (%) of trematode metacercariae in metamorphosing frogs. *Ribeiroia* treatments included either 0 or 40 cercariae, whereas echinostome treatments included 0, 40, or 160 cercariae.

of each parasite also related positively to the other (linear mixed-effects model with random intercept and random slope: echinostomes on *Ribeiroia*, $t = 2.10$, $P < 0.05$; *Riberioia* on echinostomes, $t = 2.95$, $P < 0.01$; $n = 52$ wetlands and 854 frogs). Snout–vent length was a negative predictor only for echinostome abundance (log[SVL], $t = -2.11$, $P < 0.05$).

Experiment 1: Stage 26 exposure.—Among tadpoles exposed to parasites at Gosner stage 26, *Ribeiroia* reduced 10-day survival whereas there was no effect of echinostomes or their interaction (survival analysis, *Ribeiroia* $\chi^2 = 51.71$, $P < 0.0001$). Whereas no larvae from the control (unexposed) or echinostome-only treatments died, 44% of tadpoles exposed to *Ribeiroia* alone and 75% of tadpoles exposed to both parasites died, most within 7 days of exposure. On average, 60.8% of the administered *Ribeiroia* cercariae were recovered from experimental animals, but this number varied by treatment. Echinostome coinfection increased the number of *Ribeiroia* recovered whereas days post-exposure reduced recovery (generalized linear model, echinostome $\chi^2 = 6.97$, $P = 0.0083$; days post-exposure $\chi^2 = 262.41$, $P < 0.0001$). We recovered an average of 43% of administered echinostomes in animals from the coinfec-

tion treatment. Because all hosts in the echinostome-only treatment survived, we could not analyze the effects of treatment and days post-exposure on parasite recovery.

Experiment 2.—Among tadpoles exposed to *Ribeiroia*, 75.6% survived to metamorphosis relative to 100% survival in the control treatment (Fig. 2A; Survival analysis, *Ribeiroia* $\chi^2 = 43.24$, $P < 0.0001$). No larvae exposed to 40 echinostome cercariae and only one (2.9%) exposed to 160 cercariae died, which was not significantly different from the control (Fig. 2A; echinostome $\chi^2 = 2.39$, df = 1, $P = 0.12$). Among animals exposed to *Ribeiroia* that survived to metamorphosis ($n = 100$), with or without echinostome coinfection, 75% exhibited hind limb malformations (Fig. 2B; generalized linear model, *Ribeiroia* $\chi^2 = 162.13$, $P < 0.0001$), including cutaneous fusion (31.4%), bony triangles (26.9%), extra limbs or limb elements (28.9%), and missing limbs or digits (8.9%) ($n = 156$ abnormalities). Neither mortality nor malformation risk was significantly affected by concurrent exposure to echinostomes (survival, echinostome $\chi^2 = 2.85$, $P = 0.09$, interaction $\chi^2 = 2.01$, $P = 0.16$; malformations, echinostome $\chi^2 = 0.787$, $P = 0.37$, interaction $\chi^2 = 2.88$, $P = 0.09$; Fig. 2). *Ribeiroia* exposure decreased host size at metamorphosis (ANOVA, *Ribeiroia* $F_{1,211} = 4.112$, $P = 0.04$) and both parasites tended to delay time to metamorphosis (Fig. 2C), with a significant effect for echinostomes ($F_{1,215} = 9.46$, $P = 0.002$) and a marginal effect for *Ribeiroia* ($F_{1,215} = 3.40$, $P = 0.06$). Neither parasite influenced host mass at metamorphosis (ANOVA $F_{3,214} = 1.103$, $P = 0.349$).

Parasite recovery and interaction.—For both parasites, days to metamorphosis and parasite recovery were inversely related, such that fewer parasites were recovered with more time post-exposure (generalized linear model, days post-exposure on *Ribeiroia* recovery $\chi^2 = 33.16$, df = 1, $P = 0.0001$; days post-exposure on *Echinostoma* recovery = 15.62, df = 1, $P < 0.0001$). This effect was greater for *Ribeiroia* than for *Echinostoma*, with parameter estimates of −0.0626 and −0.0292, respectively. *Ribeiroia* and echinostomes also negatively affected metacercarial recovery of each another, such that their proportional recovery was lowest in coinfection treatments (Fig. 2D). *Ribeiroia* recovery decreased monotonically with increasing echinostome exposure (Fig. 2D; ANOVA, echinostome exposure level $F_{2,48} = 3.73$, $P = 0.031$; covariate, days post-exposure $F_{1,48} = 12.894$, $P = 0.001$). The same pattern was evident for echinostome recovery, although days to metamorphosis was no longer significant. Interestingly, however, echinostome recovery also decreased with higher echinostome exposure (Fig. 2D; ANOVA, echinostome exposure $F_{1,52} = 10.386$, $P = 0.002$; *Ribeiroia* exposure $F_{1,52} = 4.924$, $P = 0.031$). There was no significant interaction between echinostome exposure and *Ribeiroia* in predicting the number of echinostome metacercariae recovered ($F_{1,52} = 0.573$, $P = 0.45$).

Discussion

In nature, animal hosts are exposed to a "cocktail" of different parasites that ultimately form a dynamic community within the host (Pedersen and Fenton 2007). Interactions among co-occurring parasites can have significant effects both on each other and the host, suggesting that a broader understanding of parasite interactions has applied importance for medical-, veterinary-, and conservation-related disciplines (Fenton 2008, Graham 2008, Lello et al. 2008). Results of the current study reinforce the importance of interactions between parasites in determining infection but also highlight the significance of scale in affecting the outcome of such interactions. Using a combination of multiscale field surveys and manipulative experiments, we found strong patterns of association between the pathogens *Ribeiroia* and *Echinostoma* for each spatial scale examined; however, the direction and magnitude of these patterns differed between the field- and experimental approaches.

Based on infection data from 1273 frog hosts and 86 California wetlands, the trematodes *Ribeiroia* and echinostomes exhibited strongly positive associations at the landscape scale, where they were significantly more likely to co-occur than expected by chance and, when present together, correlated positively within wetlands. This pattern likely resulted from the similarity of the parasites' life cycles: both *Ribeiroia* and echinostomes depend on planorbid snails as first intermediate hosts, amphibians as second intermediate hosts, and birds or mammals as definitive hosts (Johnson and McKenzie 2008, Szuroczki and Richardson 2009). Wetlands conducive to definitive host activity (i.e., visits by frog-eating birds), for example, are more likely to support high abundances of both parasites. Echinostome infections were consistently higher than co-occurring *Ribeiroia* infections, perhaps owing to the lower host specificity of echinostomes, or to the higher pathology associated with *Ribeiroia* infection, which might limit observed infections via parasite-induced mortality. After accounting for wetland-level variation, both the presence and abundance of *Ribeiroia* and echinostome infections also correlated positively at the within-host scale. The positive association between these parasites within field-collected hosts likely reflects heterogeneity in host resistance and host exposure (Karvonen et al. 2009). In many systems, hosts vary in susceptibility, creating heterogeneity in infections even following comparable exposures (Karvonen et al. 2007, Cattadori et al. 2008). Naturally occurring hosts also differ in parasite exposure as a function of microhabitat use, behavior, or development time (Poulin 2007, Telfer et al. 2008), all of which could enhance infection heterogeneity and exposure to both parasites. Although not tested here, heterogeneities in parasite exposure could be influenced by prior or concurrent exposure to another parasite (e.g., Cattadori et al. 2008).

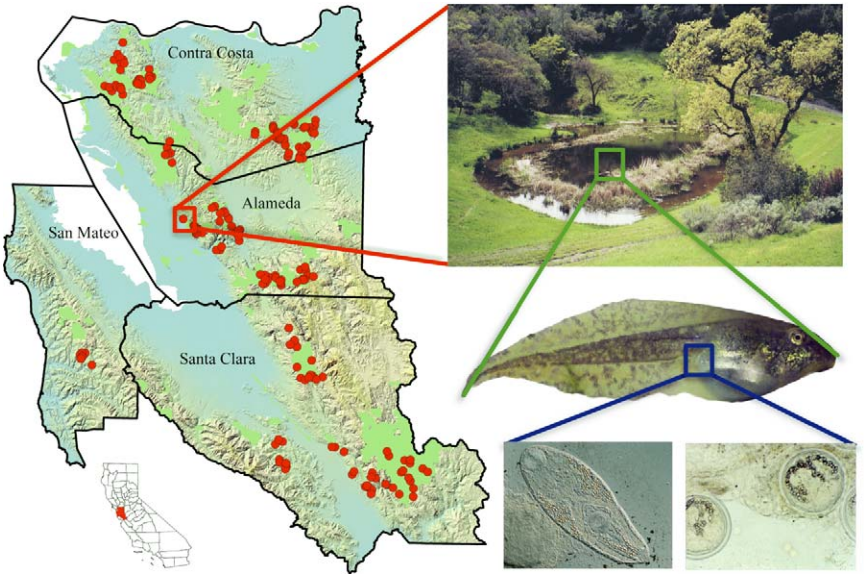

PLATE 1. Multiple, hierarchical scales of interaction between parasites: within individual hosts, between hosts within a pond, and among ponds across the landscape. Depicted is the four county region of East Bay, California, USA, in which surveys were conducted (study wetlands shown in red). Trematode parasites were isolated from chorus frogs at each site with a focus on *Ribeiroia ondatrae* (left) and *Echinostoma trivolvis* (right). Photo credits: P. T. J. Johnson, except tadpole photo by Jason Hoverman. The map was created by Katherine Dosch.

Despite the consistently positive associations between these parasites among wetlands and within frogs, however, our experimental results provided evidence of antagonistic parasite interactions within amphibian hosts. For both *Ribeiroia* and echinostomes, the presence of the second parasite strongly reduced the proportion of parasites recovered at metamorphosis, likely with importance consequences for transmission to definitive hosts. The addition of echinostome cercariae, for example, reduced *Ribeiroia* recovery in frogs by 28.8–36.4%, while *Ribeiroia* reduced echinostome recovery by 17.4–24%. Increases in echinostome exposure also reduced the proportion of successful echinostome metacercariae by 32.2%, but the per-parasite effects of intraspecific competition (0.21–0.26% reduction in recovery per parasite) were not as strong as those of *Ribeiroia* (0.43–0.60%). Given that *Ribeiroia* and echinostomes inhabit different portions of their frog hosts, with *Ribeiroia* in the epithelial tissue and echinostomes in the kidneys, these competitive effects are likely mediated through host immunity. These findings are consistent with the predictions of Graham (2008), who suggested that when resources are not limiting, top-down forces (i.e., immune function) will control interactions between co-occurring parasites, especially for ecologically similar parasites that are subjected to the same elements of the immune systems (Pedersen and Fenton 2007). Observed competitive effects likely owe to decreases in parasite persistence (i.e., increases in host clearance rate) after infection rather than changes in host susceptibility, given that we saw an increase in *Ribeiroia* recovery with coinfection when hosts were necropsied within 10 days (Telfer et al. 2008).

Interestingly, while echinostome addition reduced the recovery of *Ribeiroia*, we did not observe a decrease in host pathology in coinfected hosts. This suggests that the pathogenic effects of infection occur quickly enough following exposure so as not to be altered by subsequent decreases in infection due to parasite competition, even if such declines reduce transmission to the next host in the life cycle. However, the two parasites did cause sharp differences in amphibian host pathology. Only *Ribeiroia* caused substantial mortality and severe malformations, even at much lower exposures than used for echinostomes. Within *Ribeiroia* treatments (with or without echinostomes), ~25% of exposed hosts died following exposure, and 75% of surviving individuals exhibited malformations, relative to <3% mortality or malformations in echinostome-only treatments. The contrast in pathology between the two parasites likely resulted from differences in both the size of cercariae and their respective modes of entry (Rohr et al. 2009). *Ribeiroia* cercariae are larger than those of echinostomes and penetrate tadpole hosts directly, frequently causing a wound in the process, whereas echinostome cercariae enter hosts through the cloaca and travel to the kidneys (Holland et al. 2007). Nevertheless, even while less pathogenic, echinostome infections were both more widespread and consistently greater than those of *Ribeiroia*, with many frogs supporting >100 metacercariae and some with >1000, suggesting the cumulative effects of echinostome infection could be substantial in natural systems.

These results highlight the scale dependency of parasite interactions and the challenges inherent to quantifying the net effect of such interactions for host pathology and parasite transmission. While our exper-

iments provided evidence of negative interactions between parasites at the per capita (within-host) level, we cannot rule out the possibility that, in nature, these parasites interact positively at the population (between-host) level. For instance, both parasites delayed host time to metamorphosis, which would serve to increase a host's exposure to trematode cercariae in natural wetlands. Even if the per capita success of each parasite decreased with coinfections, the overall increase in exposure could be enough to elevate the infrapopulation abundance of each parasite and the resulting host pathology. This effect could be exacerbated if host exposure caused a reduction in behavioral avoidance, for example by reducing swimming behavior (see Taylor et al. 2004). Collectively, these findings illustrate the complexity inherent to understanding the consequences of parasite interactions, which are the product of processes occurring not only within individual hosts but also across space and over time, and the importance of combining field studies of parasite coinfection with mechanistic experiments. Continued application of community ecology principles to understand parasite communities has the potential to explain patterns of transmission and pathology in human as well as wildlife disease systems.

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