Parasite diversity and coinfection determine pathogen infection success and host fitness

Pieter T. J. Johnson[1] and Jason T. Hoverman[2]

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

While the importance of changes in host biodiversity for disease risk continues to gain empirical support, the influence of natural variation in parasite diversity on epidemiological outcomes remains largely overlooked. Here, we combined field infection data from 2,191 amphibian hosts representing 158 parasite assemblages with mechanistic experiments to evaluate the influence of parasite richness on both parasite transmission and host fitness. Using a guild of larval trematode parasites (six species) and an amphibian host, our experiments contrasted the effects of parasite richness vs. composition, observed vs. randomized assemblages, and additive vs. replacement designs. Consistent with the dilution effect hypothesis extended to intrahost diversity, increases in parasite richness reduced overall infection success, including infections by the most virulent parasite. However, the effects of parasite richness on host growth and survival were context dependent; pathology increased when parasites were administered additively, even when the presence of the most pathogenic species was held constant, but decreased when added species replaced or reduced virulent species, emphasizing the importance of community composition and assembly. These results were similar or stronger when community structures were weighted by their observed frequencies in nature. The field data also revealed the highly nested structure of parasite assemblages, with virulent species generally occupying basal positions, suggesting that increases in parasite richness and antagonism in nature will decrease virulent infections. Our findings emphasize the importance of parasite biodiversity and coinfection in affecting epidemiological responses and highlight the value of integrating research on biodiversity and community ecology for understanding infectious diseases.

microbiome | parasite competition | emerging infectious disease | ecosystem function | amphibian decline

Ecological research has focused increasingly on the importance of changes in biodiversity, thereby forming a fundamental link between community and ecosystem ecology (1–4). The loss or gain of species into a community, often in association with anthropogenic activities, can have remarkable effects on productivity, carbon storage, nutrient cycling, and species invasions (5–8). More recently, this line of inquiry has been extended to explore the role of biodiversity in affecting parasite transmission (i.e., the dilution effect; ref. 9). Building from historical research on agricultural plant communities (10), a series of recent studies has reported an inverse relationship between host diversity and the risk of disease in humans, plants, birds, amphibians, and corals (9). On the basis of experimental manipulations of host diversity, common mechanisms for this relationship involve changes in susceptible host density, such that higher diversity leads to a concomitant decline in susceptible hosts, or in encounter reduction, with added species interfering with parasite transmission (11, 12).

Despite a growing emphasis on how host diversity affects parasite transmission, few studies have examined the effects of parasite diversity on disease dynamics. Simultaneous infections by multiple parasite species are common in many systems (13, 14), yet how this cryptic component of diversity affects infectivity and host morbidity remains largely conjectural. Theory predicts that, unlike the many weak interactions that characterize free-living communities, parasites within a host have a high likelihood of interacting strongly (15, 16). Indeed, multipathogen interactions are believed to play an influential role in HIV and malaria in humans, colony collapse disorder in honey bees, and emerging infections in coral reefs (17–20). Given that many parasites cause limited pathology within their hosts (21), antagonism among coinfecting parasites could represent a novel mechanism of the dilution effect, particularly if less virulent parasites replace or compete with virulent groups at higher diversity (22, 23). Because most studies on community structure and disease have relied on correlational approaches (11, 12), identifying the "hidden" importance of each dimension of community diversity, including that of parasites themselves, remains challenging (24, 25).

Here, we sought to build an empirical bridge between research on the dilution effect and parasite coinfections by experimentally evaluating the effects of parasite community structure on pathogen infection success and host fitness. Using a study system involving six trematode parasite species (Fig. 1) and an amphibian host (Pseudacris regilla), we assessed the relative importance of parasite richness and species composition in driving the outcome of host–parasite interactions. We used an experimental approach that included multiple community configurations for most levels of richness, contrasted additive vs. replacement designs, and compared observed and randomized community assemblages (26–28). By incorporating field data on parasite community structures from 134 wetlands and 2,191 hosts, we also evaluated the relationship between parasite richness and parasite abundance in natural assemblages and compared the influence of observed and random community assemblages on the diversity–disease relationship (27, 29). We predicted that cross-reactive immunity within this parasite guild would lead to negative effects on infection success with increasing richness (30, 31). Less clear, however, was how more complex parasite assemblages would affect host pathology; coinfections could reduce parasite infection success yet still enhance pathology (32), emphasizing the need for data on the pathogenicity of each parasite, the order in which they assemble in natural communities, and how their effects combine to influence host fitness. Given the ubiquity of parasite coinfections in natural systems coupled with ongoing changes in ecological communities, including those of parasites, we highlight the conceptual and applied importance of understanding the relationships among parasite diversity, transmission, and disease.
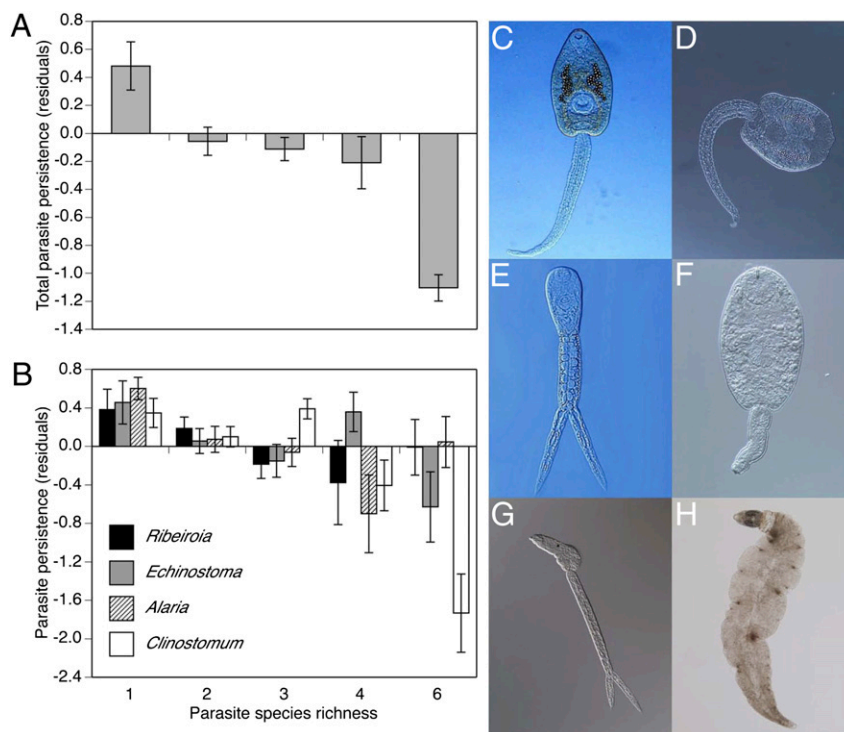
Fig. 1. (*A*) Total parasite persistence and (*B*) persistence of each parasite species in metamorphosed *Pseudacris regilla* exposed to different levels of parasite richness. Persistence values (± 1 SE) represent residuals from regression analyses with time to metamorphosis as a covariate. Infectious cercariae of *Ribeiroia ondatrae* (*C*), *Echinostoma trivolvis* (*D*), *Alaria* sp. 2 (*E*), *Cephalogonimus americanus* (*F*), *Clinostomum attenuatum* (*G*), and an undescribed echinostome magnacauda (*H*).

Results

Parasite Infection Success. Experimental increases in parasite species richness caused a reduction in overall infection success within amphibian hosts ($F_{2, 225} = 22.61$, $P < 0.0001$; lg_2(parasite richness), $B = -0.0479$, $P < 0.0001$; time postexposure: $P < 0.0001$; $n = 228$). Infection success (summed among species) decreased from an average of 40% in the monocultures to 34 and 23% in the four- and six-species treatments, respectively (Fig. 1*A*). This effect appeared linear on the basis of polynomial contrasts in ANOVA. Restricting the data to the factorial portion of the experiment with four of the most common parasites did not change the negative effect of parasite richness ($F_{2, 213} = 17.02$, $P < 0.0001$; lg_2(parasite richness), $B = -0.039$, $P < 0.0002$; time postexposure: $P < 0.0001$; $n = 216$). Although no parasite interactions were detected, inclusion of parasite identity provided an improvement in model fit relative to using richness alone [decrease in Akaike Information Criterion (AIC) from −378.4 to −396.98; *SI Text*]. For richness levels with multiple community structures, a linear mixed effects model indicated that parasite richness also reduced overall infection success when composition was included as a random effect ($B = -0.039$, $t = -2.23$, $P < 0.05$; *SI Text*). Realized parasite richness correlated strongly with richness treatments ($r = 0.94$), indicating the effectiveness of the experimental manipulations in achieving the desired gradients in parasite composition.

Parasite richness consistently reduced infection success when common parasites were analyzed individually (*Ribeiroia B = −0.038, P = 0.0413; Echinostoma B = −0.0713, P = 0.0284; Alaria B = −0.0516, P = 0.0161; and Clinostomum B = −0.128, P < 0.0001*) (Fig. 1*B*). Thus, experimental increases in parasite richness functioned to decrease infection success, including infections by the most pathogenic species (e.g., *Riberioia*). For the factorial portion of the experiment, parasite richness negatively affected persistence of two species (*Ribeiroia*: richness *B* = −0.0578, *P* = 0.0113; *Alaria*: richness *B* = −0.087, *P* = 0.001),

had a marginally negative effect on *Echinostoma* ($P = 0.07$), and no effect on *Clinostomum*. Analyzing the data with parasite composition (rather than parasite richness) provided a comparable fit for changes in *Ribeiroia* infection (~1 ΔAIC), whereas composition (main effects only) had a lower AIC (−93) relative to the richness model (−87) for *Alaria* infection.

Host Pathology. Parasite species richness increased host mortality in the additive experiment ($\chi^2 = 59.3$, df = 1, $P < 0.0001$; $n = 289$). Whereas survival was 100% among controls and averaged 98% in the monocultures, only 30% of hosts in the all-parasite treatment survived through metamorphosis (Fig. 2*A*). This pattern persisted regardless of whether we examined the entire dataset, treatments that included only the most pathogenic trematode *Ribeiroia* ($\chi^2 = 34.59$, df = 1, $P < 0.0001$; $n = 140$), or the factorial experiment ($\chi^2 = 20.23$, df = 1, $P = 0.0014$; $n = 239$). However, parasite species composition strongly influenced mortality, and only *Ribeiroia* had a significant main effect on mortality ($\chi^2 = 9.76$, df = 1, $P = 0.0018$). There was also an interaction between *Echinostoma* and *Clinostomum* ($\chi^2 = 4.53$, $P = 0.0334$), such that *Echinostoma* increased mortality when *Clinostomum* was present ($\chi^2 = 5.65$, $P = 0.0174$).

Results from the additional treatments with *Ribeiroia*, *Alaria*, and *Echinostoma* further emphasized the importance of parasite composition in determining host survival. Total cercarial exposure increased host mortality (Firth corrected $\chi^2 = 62.27$, df = 5, $P < 0.0001$; $n = 134$), but this effect depended on the identity of the parasite species (Fig. 2). Host survival decreased monotonically with progressive increases in *Ribeiroia* exposure, from 100 to 0% ($\chi^2 = 41.72$, $P < 0.0001$; $n = 65$), whereas exposure dosage had no significant effect for *Alaria* or *Echinostoma* (Fig. 2*B*). Examining the experimental results in a substitutive (rather than additive) manner indicated that both richness (one vs. four species) and community composition influenced mortality when

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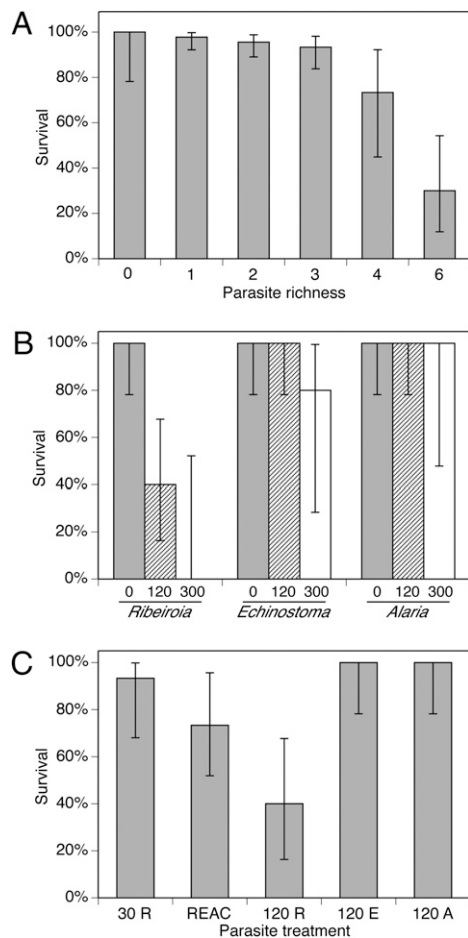
Fig. 2. Survival of *Pseudacris regilla* exposed to (*A*) different levels of parasite richness, (*B*) increasing dosages of three parasites (*Ribeiroia*, *Echinostoma*, and *Alaria*), and (*C*) parasite treatments representing an additive vs. substitutive design. Parasite exposure dosage interacted with parasite identity to determine mortality (Firth-adjusted $\chi^2 = 62.27$, df = 5, $P < 0.0001$; exposure $\chi^2 = 10.67$, $P = 0.0011$, *Ribeiroia* $\chi^2 = 12.23$, $P = 0.0005$, exposure* *Ribeiroia* $\chi^2 = 9.32$, $P = 0.0023$). In *C*, tadpoles were exposed to differing dosages (30 or 120) and combinations of *Ribeiroia* (R) *Echinostoma* (E), *Alaria* (A), and *Clinostomum* (C). The REAC treatment received 30 of each parasite. Data represent proportional survival within each treatment with 95% binomial confidence intervals.

exposure was held constant at 120 cercariae ($\chi^2 = 22.92$, df = 3, $P < 0.0001$; $n = 60$). *Ribeiroia* caused substantially more mortality (60%) than the other monospecific treatments (0%), whereas the mixed species treatment (30 parasites of each of four species) had intermediate mortality (27%, $P = 0.045$; Fig. 2*C*). Thus, substitutive increases in richness reduced mortality only when the added species replaced or reduced the abundance of the most pathogenic species.

Parasite richness reduced host length and mass while delaying host development time [$\lg_{10}$(snout-vent length): $F_{1,266} = 18.74$, $P < 0.0001$; $\lg_{10}$(mass): $F_{1,259} = 5.94$, $P = 0.0154$; time: $F_{1,266} = 5.27$, $P = 0.023$] (Fig. S1), but these effects were sensitive to which data were included. If only treatments with *Ribeiroia* were selected, these patterns persisted for host size ($F_{1,118} = 15.33$, $P = 0.0002$) and development time ($F_{1,118} = 13.24$, $P = 0.0004$), but not for host mass ($F_{1,112} = 2.69$, $P = 0.1034$). In the factorial experiment, richness affected only development time ($F_{1,109} = 5.87$, $P = 0.0171$). Although parasite species richness also increased the frequency of severe malformations ($\chi^2 = 13.48$, df = 1, $P = 0.0002$; $n = 262$), this result owed to the higher

likelihood that diverse treatments included *Ribeiroia* (Fig. S2 and SI Text).

Field Data. Parasite species richness in *P. regilla* hosts from California wetlands averaged 2.26 ± 0.085 (range of 0–5 species), with all but four ponds supporting at least one of the parasite species used in the experiments. Rarefaction analyses indicated that our sampling design was effective in detecting all parasite species, with estimated and observed richness converging for all examined sites (Fig. S3, Table S2 and SI Text). The mean abundance of parasites per host increased monotonically with $\lg_2$(parasite richness) ($F_{1,151} = 21.25$, $B = 0.286$, $P < 0.0001$), supporting an additive (unsaturated) parasite community structure (Fig. 3*A*). The parasite communities of *P. regilla* were also highly nested (observed matrix temperature = 9.44°, P ($T < 9.44°$) = $2.31e^{-29}$ [−11.43σ]) (Fig. 3*B*). Within each level of richness, the observed frequency of parasite community compositions varied (Fig. 3*B*); *Echinostoma* was the most common taxon (present in 91% of ponds), followed by *Ribeiroia* (62%), *Cephalogonimus* (35%), *Alaria* (32%), and *Clinostomum* (6%). Incorporating the information on observed community structures into a weighted regression analysis indicated that nonrandom losses of parasite species led to similar or stronger effects of richness on parasite persistence relative to unweighted communities (SI Text). Weighted regression tended to amplify the effects of richness on host mortality and days to metamorphosis,
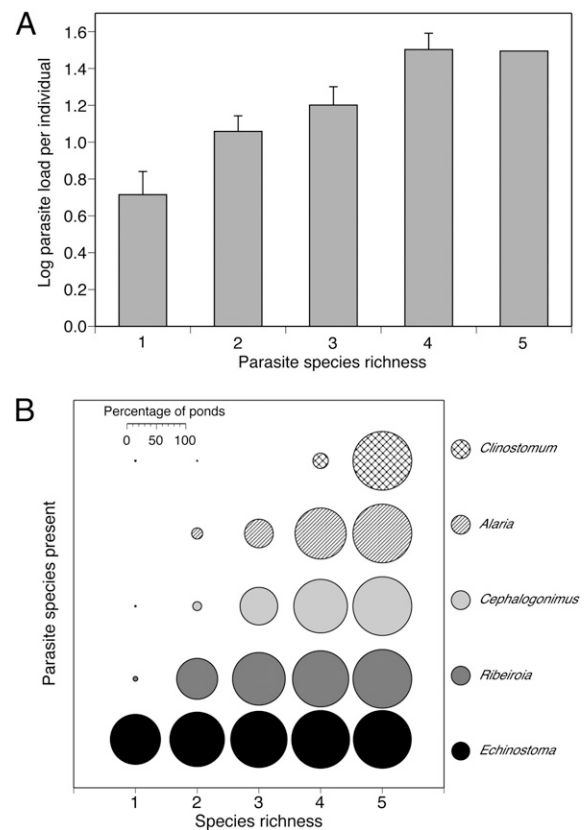
Fig. 3. (*A*) Relationship between mean parasite load (+1 SE) in recently metamorphosed *Pseudacris regilla* and parasite species richness at the wetland. (*B*) Parasite community compositions from recently metamorphosed *P. regilla* representing 134 sampled wetlands; the number of wetlands with one through five parasite species were 38, 53, 39, 23, and 1, respectively. For a given level of parasite richness, the percentage of ponds containing each species is represented by the size of the circle. The parasite communities were highly nested with an observed matrix temperature of 9.44 °C.

whereas richness did not affect host size or mass regardless of whether we used weighted or unweighted regression.

Discussion

By combining field data on parasite community assembly with controlled experiments involving interactions among six parasite species and an amphibian host, this study extends research on the dilution effect to intrahost communities. While biomedical research has traditionally focused on isolated interactions between a single host and a single parasite, growing evidence indicates that interactions among coinfecting parasites can affect host pathology, parasite transmission, and virulence evolution (33). Our work with a guild of amphibian parasites illustrates the importance of this cryptic component of biodiversity in affecting both parasite infection success and host pathology. Higher parasite richness consistently reduced the infection success of parasites within hosts, including those that cause pathogenic disease and host mortality. Concurrently, increases in parasite richness caused either increases or decreases in host pathology as a function of community composition and whether species were added substitutively or additively.

Experimental manipulation of parasite community structure revealed that total infection success decreased consistently with increases in parasite species richness, even when community composition was treated as a random effect. In the six-species treatment, parasite infection success was ~42% lower than in monospecific treatments, and this pattern applied generally to each of the four parasite species studied in detail. Thus, increases in parasite richness had similarly negative effects on the infection success of highly pathogenic and relatively benign parasites alike, suggesting this effect was unlikely to be the result of including or excluding particular species. Using the field data to weight parasite community structures according to their frequency in natural wetlands, we found that this pattern was similar or slightly stronger in observed as opposed to randomly constructed assemblages. Considering that our experiments involved a group of functionally similar and taxonomically related parasites (larval trematodes), many of which localize to different infection sites within the host, the most likely explanation for the decrease in persistence involves cross-reactive immunity (apparent competition) (31, 34) rather than the direct antagonism reported in some free-living microbial communities (4). Previous studies examining pairwise coinfections indicate that antagonistic interactions between similar types of parasites may be common (35), but this study serves to extend this finding to a parasite assemblage. Importantly, however, the outcome of intrahost interactions will likely depend strongly on the types of parasites within a given host (36). Intrahost competition between strains of a particular pathogen, for instance, can lead to increased virulence (37), whereas micro- and macroparasites elicit different immune responses (32), emphasizing the need to extend this approach to a broader range of parasitic communities.

Parasite species richness and coinfection also influenced host fitness. Increases in parasite richness led to a decrease in host survival and growth, particularly in the highest species' richness treatment. However, unlike the results for parasite infection success, this pattern depended strongly on the composition of the parasite assemblage and whether the experiment followed an additive- or replacement-based design (27, 28). Parasite species composition generally and the presence of the most pathogenic species *Ribeiroia* specifically had a disproportionate effect on host fitness, indicating that the addition of specific parasites to the community tended to drive host responses. These results, which illustrate that not all parasites are "pathogens," are consistent with the "sampling effect" from the biodiversity–ecosystem function literature (38, 39): more diverse assemblages had a greater chance of including the most virulent species, contributing to the negative relationship between parasite diversity and host fitness. However, rather than being an experimental artifact, these results emphasize

the important distinction between disease risk, which is often a function of the most virulent pathogen, and parasite diversity, which can include many species with few detectable effects on host fitness (21). Here, larger parasites that actively penetrate amphibian hosts, such as *Ribeiroia*, caused the most tissue damage and host death (40). Interestingly, however, increased parasite richness still caused reductions in host survival and development even when controlling for *Ribeiroia* presence, suggesting an additional effect of richness (e.g., a "complementarity effect") (41). The mechanism underlying this synergistic result is not immediately clear, but could involve saturation of immune defenses with increased infections (42).

These results indicate that the influence of parasite richness on host pathology will depend strongly on whether parasite communities assemble in an additive or substitutive manner. When total parasite exposure remained constant and richness determined the number of species that comprised the total (i.e., a replacement design) (43, 44), increases in diversity led to an increase in host fitness only when pathogenic species were replaced by less-virulent infections. A shift from a monoculture of *Ribeiroia* to a mixed assemblage of four parasites, for instance, each with the same total number of parasites, led to a 40% increase in host survival. This model is consistent with a resource-limited, competition-dominated scenario of community structure in which hosts are saturated with infection (39). When parasite communities were assembled additively, however, with concurrent increases in total parasite load and richness, higher parasite diversity had negative or neutral effects on host fitness. Data from the field surveys and previous parasitological research also support an unsaturated assembly pattern (30, 45, 46), but more research is needed to examine this issue in a broader range of disease systems (31).

By revealing the strongly nested structure of parasite communities within amphibian hosts, the field surveys provide additional evidence of an intrahost dilution effect. Specifically, *Echinostoma* and *Ribeiroia*, which are often reported as the most virulent parasites (47) (although *Echinostoma* caused little pathology in this study), were the most common parasites with other species added to communities as richness progressively increased. These findings parallel a key criterion of the "dilution effect" for host diversity, in which the most competent hosts need to be basally nested components of more diverse assemblages (48, 49). Combining these observations with our experimental findings suggest that, because of antagonism among coinfecting species and the low average level of pathogenicity in this group of parasites, parasite diversity can also function as a mechanism of the dilution effect by reducing the success of virulent species. Whereas such reductions may have only weak effects on individual host fitness, likely because parasite-mediated damage occurs during the initial infection stage (40), competitive effects among parasites could nonetheless cause population-level reductions in pathogen transmission. In this system, for instance, a decrease in parasite persistence in intermediate amphibian hosts will likely reduce transmission to vertebrate definitive hosts (often birds or mammals), ultimately lowering parasite abundance—a pattern that will be further enhanced if parasites also behave antagonistically within other hosts in the life cycle (50).

Continued integration among research on community ecology, the dilution effect, and pathogen coinfections has conceptual as well as applied importance for addressing issues related to ecosystem services, species loss and invasions, and emerging infectious diseases (9, 25, 48). Given that natural communities are composed of a diverse assemblage of parasitic and nonparasitic microorganisms, including heminths, viruses, bacteria, and fungi, there is a growing need to explore the outcome of interactions among these cryptic species across a range of systems and conditions. For instance, experimental studies have demonstrated that skin and soil microbial communities can reduce colonization

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by pathogens of amphibians and plants, respectively (7, 22). In humans, endosymbiotic gut bacteria can help to control or prevent colonization by pathogenic species such as *Clostridium difficile* (23), whereas the endosymbiont community of ticks (*Dermacentor andersoni*) helps to limit the distribution of Rocky Mountain spotted fever (*Rickettsia rickettsii*) (51, 52). These observations suggest that the loss of parasite and microbial biodiversity, for which few quantitative estimates are currently available (53), may play an important yet historically overlooked role in understanding disease emergence.

Materials and Methods

Overview of Approach. Our experiments evaluated the influence of parasite species richness and parasite community composition on two main responses: host pathology, including survival, malformations, size-at and time-to metamorphosis, and parasite infection success (transmission), measured as the proportion of parasites detected at the end of the experiment. Individual amphibian hosts (*P. regilla*) were exposed to infectious cercariae representing one, two, three, four, or six trematode species, which encompassed realistic patterns of parasite richness in amphibian host populations (Fig. 3B). This translated into a fully factorial experiment for four of the common parasite species, which facilitated comparisons of parasite richness and composition while keeping the design tractable. This experiment used an additive design, such that the total number of administered parasites increased directly with richness; however, we conducted complementary treatments involving varying dosages of the three most common parasites to compare additive and replacement designs (43, 44) (*SI Text*). Finally, we used data from field surveys to weight experimental assemblages by their observed frequency in nature to contrast the effects of realistic and randomized community structures.

Parasite Exposures. We field-collected egg masses of Pacific chorus frogs (*P. regilla*), allowed them to hatch in the laboratory, and maintained larvae individually in 1.0-L containers. Once hosts reached early limb development (ref. 54, stage 26), we randomly assigned them to an experimental treatment: no parasites (control) or to 30 cercariae of one parasite species (*n* = 6 treatments), two species (*n* = 6), three species (*n* = 4), four species (*n* = 1), or all six parasites (*n* = 1) (Table S1). Included parasites were *Ribeiroia ondatrae*, *Echinostoma trivolvis*, *Alaria* sp. 2 (55), *Cephalogonimus americanus*, *Clinostomum attenuatum*, and an undescribed echinostome magnacauda (Fig. 1 *C–H*). We obtained cercariae of each parasite by collecting snails (*Helisoma trivolvis*) from sites in California and Oregon (magnacauda only), isolating snails into 50-mL vials for 12–24 h, and administering pooled cercariae to larval amphibians over 14 d (*SI Text*). To evaluate the pathology associated with the common parasites and contrast replacement vs. additive experimental designs (sensu refs. 43, 44), we included complementary treatments that involved exposing hosts to multiple dosages (0, 120, or 300 cercariae) of *Ribeiroia*, *Echinostoma*, or *Alaria* and recorded host survival over 18 d (*SI Text*).

Field Data. We examined parasite infections in 2,191 metamorphosing *P. regilla* from 134 wetlands in the East Bay region of California between 2009 and 2010 (158 total site visits) to quantify patterns of parasite community structure and assembly in natural settings. At wetlands supporting the appropriate snails (*H. trivolvis*), we necropsied a random subset of *P. regilla* hosts (10–60 per site) to determine the identity, richness, and abundance of larval trematodes (*SI Text*). We verified the adequacy of this sampling program to detect parasite richness using sampled-based rarefaction curves and richness estimators (*SI Text*, Table S2, Fig. S3). From these data, we determined the relative frequency of each parasite community permutation

and quantified the relationship between mean parasite abundance (averaged across all hosts examined) and parasite species richness.

Analysis of Infection Success. We analyzed the influence of experimental treatments on the abundance of each parasite species detected upon necropsy (lg_{10} transformed) and the total proportion of parasites detected (summed among species, arcsin-square-root transformed). We used species richness (lg_2 transformed) as our primary explanatory variable in a replicated-regression approach (56). To contrast community composition and richness, we analyzed the effect of each parasite and their interactions on infection success, restricting the analysis to the factorial portion of the experiment. We compared the AIC values of models with richness alone vs. models that included parasite identity. For treatments with multiple community compositions at each level of richness (i.e., one, two, and three species), we also used linear mixed effects models to nest different community compositions within richness (57) (*SI Text*). Days postexposure was included as a covariate because of the established effects of time on parasite persistence (e.g., ref. 58).

Analysis of Host Pathology. We examined the effects of parasite species richness on host survival and malformations using generalized linear models with untransformed values of richness (although results did not change if we used lg_2 richness and excluded controls). To evaluate the role of parasite composition, we reanalyzed the effects of parasite richness while controlling for *Ribeiroia* presence, which was the most pathogenic parasite and expected to contribute to an obvious sampling effect. We also analyzed the factorial portion of the experiment iteratively using either richness or parasite presence as predictors and compared AIC values. We used the additional, short-term treatments to (*i*) test the role of parasite identity, exposure dosage (0, 120, or 300), and their interaction on host survival and (*ii*) contrast the effects of replacement and additive designs by comparing treatments with a total exposure of 120 parasites. Among animals that survived to forelimb emergence (stage 42), we tested the influence of parasite richness on time to metamorphosis, length at metamorphosis, and mass at metamorphosis using general linear models, first on the entire experiment (contrasting treatments with and without *Ribeiroia*), and subsequently on the factorial portion of the experiment.

Analysis of Field Data. We used linear regression to examine the influence of parasite richness at a site (lg_2 transformed) on the mean abundance of parasites per frog (summed among parasite species, lg_{10} transformed). Additionally, we recorded the relative frequency of different parasite community structures and used the nestedness temperature calculator to assess whether low diversity communities represented subsets of more rich communities (59). This information was used to weight the experimental community structures, such that commonly observed communities received a higher regression weight relative to more rare communities (60). By comparing these results with the unweighted regressions, we evaluated the effects of parasite species loss between realistic and randomized assemblages, respectively (27). This analysis was performed only up through four species communities as these were well represented in nature.

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ECOLOGY

Supporting Information

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SI Materials and Methods

Experimental Design and Overview. The manipulation of species richness and community composition in biodiversity experiments is a significant challenge due to the logistical constraints associated with the large number of possible combinations, even with relatively small numbers of species (1–3). We therefore selected treatments for inclusion in the experiment to facilitate comparisons of parasite richness and composition, including parasite interactions, while keeping the design tractable. Importantly, natural macroparasite communities in amphibian populations are relatively depauperate (Fig. 3*B*), which enabled us to include most of the observed parasite community permutations as well as unobserved structures. Individual amphibian hosts (*P. regilla*) were exposed to infectious cercariae representing one, two, three, four, or six trematode species (*Ribeiroia ondatrae*, *Echinostoma trivolvis*, *Alaria* sp. 2 (4), *Cephalogonimus americanus*, *Clinostomum attenuatum*, and an undescribed echinostome magnacauda). This translated into a fully factorial experiment for four of the common parasite species (*Ribeiroia*, *Echinostoma*, *Alaria*, and *Clinostomum*) with two additional single species treatments (*Cephalogonimus* and Magnacauda) and an all-parasite species treatment (see Table S1 for an overview of the experimental treatments). For the main experiment, we used an additive design with equal numbers of each parasite and an increase in total number of parasites with greater parasite richness. We did this because, in a replacement design with a fixed total number of parasites but variable compositions, much of the pathology would be driven by the most virulent parasite rather than by interactions between parasites (5, 6). For instance, a replacement design would constrain the total exposure of each host to 30 parasites, which would result in the addition of five of each parasite species in the six-species treatment compared with 30 in the monoculture treatment. Given that host pathology is positively correlated with exposure dosage (7), a replacement design would confound exposure and parasite community composition (i.e., pathology would decrease with richness because of lower dosages of each parasite). However, to address the influence of exposure dosage on host and parasite responses, we conducted a complementary experiment focused on the three most common parasites (*Ribeiroia*, *Echinostoma*, and *Alaria*). For each, we exposed hosts to increasing dosages of infectious cercariae (0, 120, 300; see Table S1), which allowed us to both evaluate the influence of parasite dosage on host pathology and to analyze a replacement design by comparing the effects of monocultures involving 120 parasites with polycultures (four parasite species) of the same total dosage (Fig. 2*C*). Together, these two experiments allowed us to assess the relative importance of species richness, community composition, and exposure dosage on host pathology and parasite infection success.

Parasite Exposures. We identified parasite cercariae using Schell (8) and Yamaguti (9). Because some of these parasites remain undescribed or are challenging to identify on the basis of morphology alone, we used molecular techniques to verify the identity and/or consistency of parasites used. Specifically, we used the internal transcribed spacer region of ribosomal DNA (ITS 1 and ITS 2) gene fragments and BLAST searches to compare among sequences from our specimens and to those available in GenBank (10–12). This method allowed us to identify *Alaria* sp. 2, *C. americanus*, *E. trivolvis*, and *R. ondatrae* with 99% maximum identification. Sequences of magnacauda were not available in GenBank but we were able to place it in the Echinostomatidae family through a phylogenetic analysis. We also used infections in

frogs from these same field sites to help ensure we were working with appropriate species. Within 4 h of release, cercariae from each parasite species were pooled among infected snails, counted under a dissecting scope, and administered directly into the 1.0-L containers to find and infect hosts. On each of three dates (days 0, 7, and 14), 10 cercariae of a given parasite species were administered in an additive design; thus, hosts in the one-species treatment were exposed to 30 cercariae of species A, hosts in the two-species treatment were exposed to 30 cercariae of species A and 30 cercariae of species B (60 total parasites), and so on. For the complementary experiment, we used the same exposure schedule with the total parasite numbers divided equally among the dates (40 and 100 parasites per exposure for the 120 and 300 parasite treatments, respectively). This was done to better approximate the continuous exposures experienced by hosts in natural environments. All cercariae were added within 12 h of one another to avoid any priority effects in infection (13). Tadpoles in the control treatment were sham exposed to water from uninfected snails. Once hosts reached metamorphosis, they were measured (snout-vent length and wet mass), examined for developmental malformations (1), and necropsied to quantify the number of meta- or mesocercariae of each parasite species. Using a stereomicroscope, we examined the skin (externally and internally), major organ systems (e.g., kidneys, liver), and digestive tract of each animal to quantify all visible infections (15). For analyses of infection and host pathology, we excluded hosts that did not survive the 14-d exposure period.

SI Results

We examined the influence of each parasite species and their interactions on overall parasite persistence and the persistence of each parasite species using general linear models that included terms for the presence of each parasite species and their interactions. Exposure to *Alaria* ($B = -0.061$, $P < 0.0001$) and to *Echinostoma* ($B = -0.054$, $P < 0.0001$) each had negative main effects on overall infection success, whereas there were no significant interactions among parasites or any main effects of *Ribeiroia* or *Clinostomum*. Additionally, *Alaria* and *Echinostoma* had strong effects on the infection success of *Ribeiroia*; the presence of *Alaria* ($B = -0.0622$, $P = 0.016$) and of *Echinostoma* ($B = -0.0630$, $P = 0.015$) each reduced the number of *Ribeiroia* metacercariae detected in metamorphosing hosts at metamorphosis, and parasite composition provided a comparable model fit relative to parasite richness [~1 ΔAkaike Information Criterion (AIC)]. For *Alaria*, the addition of *Ribeiroia* ($B = -0.0622$, $P = 0.016$) or of *Echinostoma* ($B = -0.0630$, $P = 0.015$) had negative effects on mesocercarial recovery, and parasite composition (main effects only) had a lower AIC (-93) relative to the richness model (-87).

For richness treatments with multiple, alternative community compositions (i.e., one-, two-, and three-parasite species; Table S1), we used linear mixed effects models to test the effects of richness (a fixed effect), whereas accounting for composition (a random effect) using the function "lme" in the R package "nlme" (16). We then compared the model with a random effect to a similar model without a random effect (fit using "gls") using the likelihood ratio test (17) to determine whether the random effect was needed. These analyses indicated that parasite richness (lg_2 transformed) negatively affected overall infection success (summed among species) even after accounting for composition ($B = -0.039$, $t = -2.23$, $P < 0.05$), and that the random-effects model provided a superior fit to the model

without a random effect (ΔAIC = 8, df = 2, likelihood ratio = 9.51, $P < 0.005$). Similar analyses performed for host pathology did not detect a significant effect of parasite richness (one, two, or three species), regardless of whether a random effect of composition was included.

We also examined the effects of parasite species richness on the frequency of severe malformations, which are caused by the trematode *Ribeiroia* (14). Whereas parasite species richness increased malformation frequency ($\chi^2 = 13.48$, df = 1, $P = 0.0002$; $n = 262$), this resulted from the higher likelihood that diverse treatments included *Ribeiroia* ("sampling effect"). Indeed, restricting the data to treatments that included *Ribeiroia* eliminated the effect of richness on malformation risk ($\chi^2 = 0.52$, df = 1, $P = 0.47$; $n = 116$). Malformation frequency averaged 34 and 0% in treatments with and without *Ribeiroia*, respectively (Fig. S2).

Rarefaction Analyses. We used sample-based rarefaction curves to determine whether our sampling methods were sufficient for detecting parasite species present within a community. For a subset of 20 randomly selected wetlands, we compared our species richness observations to species accumulation curves and Chao2 biased-corrected species richness estimates using EstimateS v 8.2.0 (18). When the coefficient of variation was greater than 0.5, we used the "classic" Chao2 estimates (19). Samples were composed of the parasites observed within each *Pseudacris regilla* individual dissected at a site. Results indicated that ≥10 *P. regilla* individuals were more than adequate for estimating local parasite richness. Chao2 species richness estimates exactly matched our observed species richness at all 20 of the randomly selected sites (Table S2). All sites showed a clear asymptote between the number of *P. regilla* individuals examined and parasite species richness (Fig. S3).

Weighted Regression Analysis. We weighted each permutation of parasite richness (one through four species) by its relative occurrence in nature, such that common compositions received a higher weight, whereas rare (or unobserved) compositions were given a lower score. Within each level of richness, the sum of community frequencies summed to unity. For *Ribeiroia* persistence, which was the most pathogenic parasite, the coefficient of parasite richness on metacercarial recovery at metamorphosis changed from -0.05783 ± 0.022 in the unweighted analysis ($P = 0.0113$) to -0.0699 ± 0.026 when communities were weighted by their frequency ($P = 0.0263$) (note that the P value is larger because the sample size changes owing to the exclusion of unobserved community structures). For total parasite persistence summed among species, the weighted and unweighted coefficients of richness on parasite persistence were nearly indistinguishable: -0.0326 ± 0.0144 ($P = 0.0257$) and -0.039 ± 0.0104 ($P = 0.0002$), respectively. With respect to host pathology, the use of weighted communities increased the odds ratio of parasite richness on host mortality from 1.469 ± 0.603 ($P = 0.0055$) to 3.0935 ± 1.727 ($P = 0.0068$), indicating the realistic community structures trended toward a greater mortality response with increases in parasite richness. Among growth-related responses, parasite richness significantly affected only days to metamorphosis, and this effect was greater in weighted ($B = 2.019 \pm 0.55$; $P = 0.0004$) as opposed to unweighted community structures ($B = 0.979 \pm 0.43$; $P = 0.022$). This pattern was evident whether we used the untransformed values of parasite richness (which included the control treatment with no parasites) or the lg_2-transformed value of richness (which excluded the controls).

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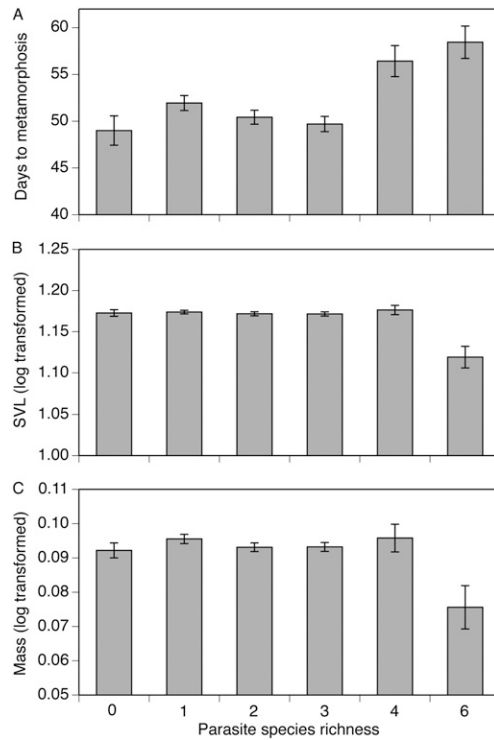
Fig. S1. Effects of parasite species richness on host fitness (days to metamorphosis, snout-vent length, and mass at metamorphosis). Data are means ±1 SE.
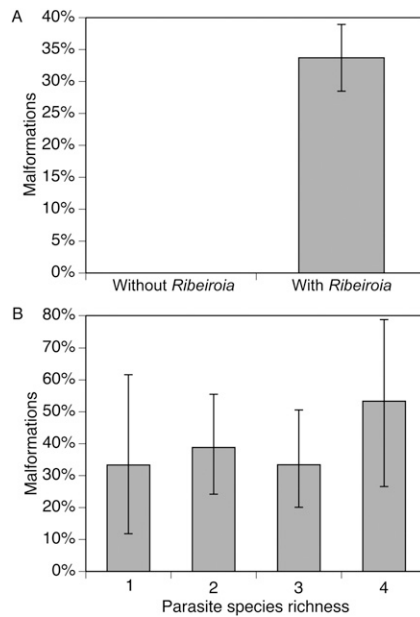

Fig. S2. (*A*) Percentage of metamorphosing frogs with malformations in treatments without and with *Ribeiroia ondatrae*. Data are means ±1 SE. (*B*) Percentage of metamorphosing frogs with malformations in treatments containing *R. ondatrae* at different levels of parasite species richness. Data represent proportional malformations within each treatment with 95% binomial confidence intervals.
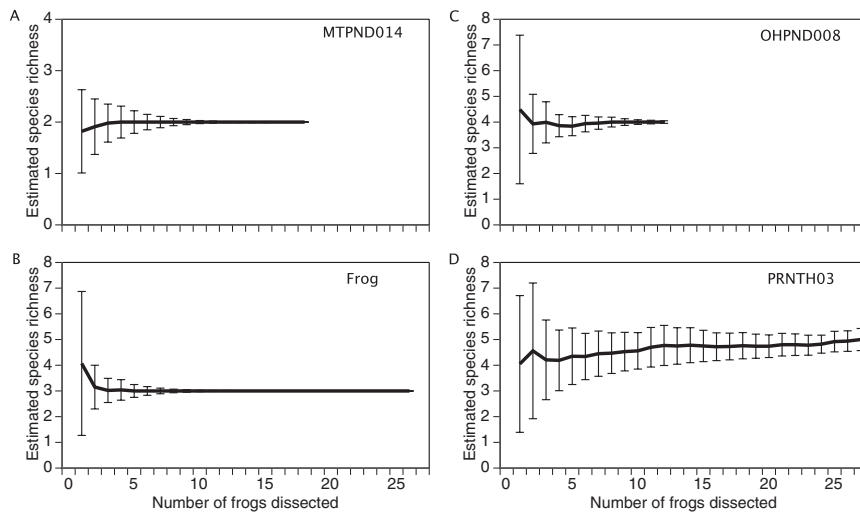
Fig. S3. Species accumulation curves for parasite species richness observed within metamorphosing *Pseudacris regilla* at four sampled wetlands with different observed levels of species richness, (*A*) MTPND014, (*B*) frog, (*C*) OHPND008, and (*D*) PRNTH03. For each plot, the black line represents the rarefaction curve (Chao2) and error bars represent one SD.

Table S1. Summary of experimental treatments used to assess the effects of parasite species richness and parasite community composition on *Pseudacris regilla* tadpoles

Species richness	No. of treatments	No. of replicates	Total parasites	Parasites included in the treatments
0	1	30	0	None
1	6	90	30	*Ribeiroia*, *Echinostoma*, *Alaria*, *Cephalogonimus*, *Clinostomum*, and magnacauda
1	3	45	120	*Ribeiroia*, *Echinostoma*, and *Alaria*
1	3	15	300	*Ribeiroia*, *Echinostoma*, and *Alaria*
2	6	90	60	*Ribeiroia*, *Echinostoma*, *Alaria*, and *Clinostomum*
3	4	60	90	*Ribeiroia*, *Echinostoma*, *Alaria*, and *Clinostomum*
4	1	15	120	*Ribeiroia*, *Echinostoma*, *Alaria*, and *Clinostomum*
6	1	20	180	*Ribeiroia*, *Echinostoma*, *Alaria*, *Cephalogonimus*, *Clinostomum*, and magnacauda

Tadpoles were exposed to one, two, three, four, or six trematode species (*Ribeiroia ondatrae*, *Echinostoma trivolvis*, *Alaria* sp. 2, *Cephalogonimus americanus*, *Clinostomum attenuatum*, and an undescribed echinostome magnacauda).

Table S2. Summary of rarefaction results for 20 randomly selected wetlands

Wetland	Hosts sampled	Total parasites detected	Observed richness	Chao2 richness
I2	11	180	2	2
House	13	85	2	2
OHPND017	15	44	2	2
Murkey bullfrog	16	516	2	2
MTPND014	18	228	2	2
Seascape	22	2,395	2	2
BW1	10	171	2	2
PRNTH02	14	1,141	3	3
OHPND003	20	379	3	3
Newton	19	1,308	3	3
TGIF	16	1,784	3	3
Frog	26	1,944	3	3
Pig	10	384	4	4
OHPND008	12	96	4	4
Downy	10	2,274	4	4
Sheep	60	3,136	4	4
Quick	32	1,517	4	4
Kevin's Pond	29	1,059	4	4
Hidden	21	2,853	4	4
PRNTH03	27	843	5	5

For each wetland, the number of hosts sampled, the total number of parasites observed during dissections, the observed parasite species richness, and the estimated parasite species richness based on Chao2 are presented. Chao2 biased-corrected species richness estimates using EstimateS v 8.2.0 (18).