esa

ECOSPHERE

Using an ecosystem-level manipulation to understand host-parasite interactions and how they vary with study venue

KEVIN B. LUNDE,[1,3,]† VINCENT H. RESH,[1] AND PIETER T. J. JOHNSON[2]

[1]*Environmental Science, Policy, and Management, University of California Berkeley, Berkeley, California 94720 USA*
[2]*Ecology and Evolutionary Biology Department, University of Colorado, Boulder, Colorado 80309 USA*

Citation: Lunde, K. B., V. H. Resh, and P. T. J. Johnson. 2012. Using an ecosystem-level manipulation to understand host-parasite interactions and how they vary with study venue. Ecosphere 3(10):84. http://dx.doi.org/10.1890/ES12-00001.1

Abstract. We investigated interactions between the virulent trematode *Ribeiroia ondatrae*, which has been linked to amphibian malformations across the United States, and its amphibian host (*Pseudacris regilla*) using a hierarchical approach involving multi-year regional field surveys, replicated pond enclosures, and an unreplicated ecosystem manipulation of parasite infection. Results of this multi-tiered approach provide strong evidence of the causal linkage between infection and malformations while offering additional insights about the influence of experimental venue on effect size. Among years and across 18 wetlands in northern California, *Ribeiroia* infection was a strong predictor of malformation frequency, which ranged from 0 to 77% at all sites ($n = 6{,}511$). Correspondingly, the addition of >500 *Ribeiroia* infected snails to an experimentally divided wetland using a three-year Before After Control Impact (BACI) design caused sharp increases in *Ribeiroia* infection and severe malformations in *P. regilla* during the manipulation year (but not pre- or post-manipulation). These results were complemented by the findings from a replicated ($n = 16$) enclosure/exclosure study conducted on both sides of the divided wetland (Hog Lake), which showed increased infection and malformations only among larvae within cages that allowed parasite entry and only on the manipulated side. No differences in mortality were observed among animals as a function of cage type. A comparison of the slope between observed infection and malformations as a function of venue (previous laboratory work and the two spatial scales of this study (cages and whole-pond)) indicated that small-scale experiments exhibit stronger effects relative to results from larger spatial extents. Multi-year sampling also indicated that malformed frogs were unlikely to return as breeding adults, highlighting the potential for population-level impacts associated with high *Ribeiroia* infections. Taken together, these results provide support for the causal relationship between *Ribeiroia* infection and amphibian malformations under realistic conditions while simultaneously emphasizing the influence of study venue on the strength of this relationship.

Key words: abnormalities; amphibian decline; amphibian malformations; aquatic ecology; disease ecology; ecosystem manipulation; experimental venue; lentic; *Ribeiroia*; whole-lake.

Received 3 January 2012; revised 29 May 2012; accepted 30 May 2012; final version received 31 August 2012; **published** 3 October 2012. Corresponding Editor: K. Lafferty.

[3] Present address: San Francisco Bay Regional Water Quality Control Board, 1515 Clay Street, Suite 1400, Oakland, California 94612 USA.

† **E-mail:** klunde@berkeley.edu

INTRODUCTION

Over the past 15 years, amphibians with severe malformations have been reported in wetlands across the United States and Canada (Ouellet 2000, Johnson et al. 2010). Most often, these

reports describe frogs with missing, extra, or malformed limbs. Although there are many potential causes of such abnormalities on a national scale, laboratory and field studies indicate that the trematode parasite *Ribeiroia ondatrae* is an important proximate cause of such malformations, particularly in the western USA (Blaustein and Johnson 2003). Other potential causes of limb deformities include chemical contaminants (e.g., pesticides) and aquatic predators such as dragonfly larvae (Ouellet 2000, Bowerman et al. 2010, Reeves et al. 2010). Typical of digenetic trematodes, *Ribeiroia ondatrae* (hereafter "*Ribeiroia*") has a complex life cycle that involves snail, amphibian, and bird hosts (Johnson et al. 2004). In laboratory studies, exposure to *Ribeiroia* increased mortality and/or induced limb deformities in 13 species of frogs, toads, and salamanders (Kiesecker 2002, Stopper et al. 2002, Schotthoefer et al. 2003, Johnson et al. 2012). However, only one study (Kiesecker 2002) has examined *Ribeiroia*-malformation dynamics using field experiments and none has conducted an ecosystem manipulation of this system to test the causal relationship under natural conditions.

Here, we used an ecosystem manipulation to explore host-parasite interactions under realistic conditions that allow for important co-factors to moderate these dynamics. In natural wetlands, numerous biotic and abiotic factors can affect the strength—or alter the direction—of interactions between hosts and parasites (e.g., Thieltges et al. 2008, Marcogliese and Pietrock 2011). For example, while amphibian hosts in laboratory experiments are often raised in isolation under artificial conditions, hosts in nature occur alongside numerous factors with the potential to alter transmission or pathology, including predators, competitors, and physicochemical variation. Aquatic predators, for example, can reduce amphibian avoidance behavior and thereby infection risk (Thiemann and Wassersug 2000), or actively consume free-swimming parasites and reduce amphibian infections (Schotthoefer et al. 2007, Orlofske et al. 2012). Predators and other 'natural stressors' can also amplify patterns of mortality observed under laboratory conditions, as found in studies of certain contaminants in aquatic systems (e.g., Relyea and Mills 2001). Collectively, these factors illustrate the complexity of parasite transmission patterns in natural environments and underscore the difficulties in translating the results of simple laboratory studies to disease dynamics within real ecosystems.

In the current study, we used a multi-tiered approach to evaluate the importance of parasite exposure for amphibian hosts and how this relationship varies with study venue and spatial scale. In the first tier, we collected data from 18 wetlands in northern California (including 43 year $\times$ wetland assessments) to evaluate the relationship between *Ribeiroia* infection and amphibian malformations among sites, between years, and across life history stages (e.g., metamorphic vs. adult amphibians). These data provided a foundation for the second objective, in which we used a whole-ecosystem experiment to test the influence of infection on amphibian malformations under realistic conditions. Specifically, we used a Before-After-Control-Impact (BACI) study design over three years to examine the influence of parasite enhancement within an experimentally divided pond and complemented this approach with a replicated enclosure/exclosure cage study nested within both pond treatments. Finally, to investigate how study venue influenced host-parasite interactions, we compared data from previously published laboratory experiments, the 18 monitored wetlands, and the 16 in situ cages at Hog Lake to determine whether the relationship (slope) between parasite infection and malformations varied at these three spatial scales (see Skelly and Kiesecker 2001, Skelly 2002).

Materials and Methods

Long-term monitoring and regional field surveys

To assess variation in the relationship between *Ribeiroia* infection and malformations, we sampled Pacific chorus frogs (*Pseudacris regilla*) from 13 ponds with *Ribeiroia* and 5 without between 2006 and 2010. Ponds were distributed across Northern California (Lassen, Mendocino, Sonoma, Alameda, Contra Costa, Santa Clara, and San Mateo Counties) and were sampled multiple times over the study period ($n = 43$ site $\times$ years) (Table 1). We focused on *P. regilla* as an indicator species because it is common in many pond habitats, often locally abundant, and frequently exhibits a strong malformation response to

Table 1. Site list and results from the regional survey of limb malformations and *Ribeiroia* infection within Pacific chorus frogs (*Pseudacris regilla*) from 18 ponds in Northern California from 2006 to 2010.

Pond name	Year	County	Latitude	Longitude	Sample size	Malformation prevalence (%)	*Ribeiroia* abundance
Barn Pond	2008	Santa Clara	37.09376	−121.740352	166	6.0	1.7
Barn Pond	2009	Santa Clara	37.09376	−121.740352	35	0.0	1.1
Barts Pond	2009	Mendocino	39.000606	−123.09814	78	76.9	48.6
BW1	2006	Lassen	40.505292	−121.438115	177	3.4	2.3
BW1	2007	Lassen	40.505292	−121.438115	98	6.1	39.0
BW1	2008	Lassen	40.505292	−121.438115	169	2.4	4.9
Coon Lake South	2007	Mendocino	39.01621	−123.07546	105	4.8	0.0
Coon Lake South	2008	Mendocino	39.01621	−123.07546	141	0.7	0.0
Coon Lake South	2009	Mendocino	39.01621	−123.07546	211	2.8	0.0
Corte Madera	2007	San Mateo	37.3669	−122.21178	265	3.8	0.0
Frog Pond	2006	Santa Clara	37.08548	−121.73949	114	32.5	27.9
Frog Pond	2007	Santa Clara	37.08548	−121.73949	34	32.4	5.2
Frog Pond	2008	Santa Clara	37.08548	−121.73949	155	25.2	27.7
Frog Pond	2009	Santa Clara	37.08548	−121.73949	135	34.8	19.5
Hidden Pond	2006	Santa Clara	37.08881	−121.73949	156	57.7	20.3
Hidden Pond	2007	Santa Clara	37.08881	−121.73949	229	28.8	18.0
Hidden Pond	2008	Santa Clara	37.08881	−121.73949	232	35.8	23.1
Hidden Pond	2009	Santa Clara	37.08881	−121.73949	243	32.5	16.1
Hog Lake	2006	Mendocino	39.0316	−123.0789	257	48.6	22.6
Hog Lake	2007	Mendocino	39.0316	−123.0789	115	53.0	70.4
Hog Lake	2008	Mendocino	39.0316	−123.0789	419	5.0	2.4
Hog Lake	2009	Mendocino	39.0316	−123.0789	462	5.6	5.4
Hog Lake	2010	Mendocino	39.0316	−123.0789	211	2.4	0.2
Morgan Territories 1	2008	Contra Costa	37.82087	−121.79494	19	0.0	0.0
Morgan Territories 11	2008	Contra Costa	37.82048	−121.79209	125	0.8	0.0
Morgan Territories 15	2008	Contra Costa	37.8326	−121.85107	116	2.6	1.0
Pepperwood 1	2007	Sonoma	38.5844	−122.6997	112	3.6	2.2
Pepperwood 1	2008	Sonoma	38.5844	−122.6997	135	5.9	1.0
Pepperwood 1	2009	Sonoma	38.5844	−122.6997	154	19.5	16.1
Pepperwood 3	2007	Sonoma	38.58261	−122.69759	99	29.3	48.6
Pepperwood 3	2008	Sonoma	38.58261	−122.69759	194	7.2	14.5
Pepperwood 3	2009	Sonoma	38.58261	−122.69759	163	2.5	0.0
Pepperwood 4	2007	Sonoma	38.57521	−122.7073	65	6.2	1.6
Pepperwood 4	2008	Sonoma	38.57521	−122.7073	233	3.4	0.0
Pepperwood 4	2009	Sonoma	38.57521	−122.7073	93	0.0	8.0
Pico Pond	2008	Santa Clara	37.09119	−121.73645	70	7.1	2.0
Pyramid Reservoir 1	2007	Mendocino	39.04307	−123.07945	63	17.5	66.2
Pyramid Reservoir 1	2008	Mendocino	39.04307	−123.07945	130	7.7	1.0
Pyramid Reservoir 1	2009	Mendocino	39.04307	−123.07945	17	5.9	0.9
Rickabough Glade	2008	Mendocino	39.049321	−123.0584	138	10.9	11.0
Riley Ridge	2007	Mendocino	39.0125	−123.0577	123	2.4	0.0
Riley Ridge	2008	Mendocino	39.0125	−123.0577	171	2.3	0.0
Riley Ridge	2009	Mendocino	39.0125	−123.0577	84	0.0	0.0
Summary statistics					6,511	14.8	12.3

infection (Lunde and Johnson, *in press*). During each visit, we attempted to capture 50 or more animals, inspected them visually for limb abnormalities in the field (Johnson et al. 2001), and released the majority of frogs back into the pond. A random subset of ~10 normal and 10 abnormal frogs were necropsied to quantify *Ribeiroia* infection (Sutherland 2005). To further evaluate whether limb malformations might increase the mortality of metamorphic frogs, a result that could have population-level impacts, we compared the infection and malformations from metamorphic amphibians emerging from the pond in late summer (June-July) with those from adult amphibians returning to breed in late winter (December-January) at two ponds between 2006 and 2009. Hog Lake and Hidden Pond were selected for this comparison because each supported high levels of *Ribeiroia* infection and malformations in one or more sampling years (Table 1).

Hog Lake: whole-ecosystem manipulation

We selected Hog Lake (latitude: 39.0316, longitude: −123.0789) as the site for a wetland manipulation owing to its intermediate size

(3,200 m^2), shallow depth (maximum of 1 m), spatial isolation (nearest neighbor $>$ 1.1 km), and previous history of supporting *Ribeiroia* and amphibian malformations, all of which facilitated implementation of our experimental design and the collection of high resolution data on snails, parasites, and larval amphibians. This seasonal wetland, which is located within the University of California Hopland Research and Extension Center, supports a relatively simple aquatic community (two amphibians, *P. regilla* and *Taricha torosa*, and one snail species, *Helisoma trivolvis*).

Prior to the pond filling with rainwater in December 2007, we divided Hog Lake into two, comparably sized wetlands approximately 1,600 m^2 each using a 60 m $\times$ 1.8 m Ethylene Propylene Diene Monomer (EPDM) pond liner with 1 mm thickness (e.g., Fig. 3C). Eight 100-cm^2 sections were removed from the liner and replaced with 35 μm Nitex bolt cloth to allow exchange of water but not tadpoles, snails, or parasite cercariae between the two sides. We then randomly assigned the west side of the pond to the *Ribeiroia* enhancement treatment while the east side remained as an unmanipulated control. Furthermore, because *Ribeiroia* abundance and malformations exhibit strong interannual variability (see *Results*), the split-plot design helps to reduce confounding effects of individual wetland variability. This design follows methods similar to those used in large-scale lake manipulations (e.g., Brezonik et al. 1993, Sass et al. 2006, Helmus and Sass 2008) and allows for side-by-side comparisons of the two treatments. Although the unreplicated nature of our split-plot design makes it difficult to generalize a treatment effect, it provides a natural context for our smaller-scale replicated studies. The addition of infected snails was chosen as the optimal study design rather than parasite removal because the latter would require removal of all snails, adding a confounding effect by simultaneously eliminating an important pond herbivore and potential competitor of *P. regilla*. We note, however, that as this study involved manipulation of a single ecosystem, extrapolation of the results to other wetlands should be made with caution.

To experimentally enhance infection, we added 517 *Ribeiroia*-infected snails to the west side of the pond (*Ribeiroia* addition) over four events between 25 April and 2 June 2009. Approximately 60% of the infected snails added were allowed to move freely through the pond while the remaining 40% were isolated within enclosures (45 cm diameter $\times$ 50 cm high, 2 mm mesh size). Snail enclosures were used to derive coarse estimates of snail survivorship over the study and to prevent accidental trampling during biweekly sampling. Infected snails were either collected from Bart's Pond ($n = 79$), which is also located on the Hopland Research and Extension Center property, or infected in the laboratory ($n = 438$) using parasite eggs from a colony of *H. trivolvis* snails originally from Hog Lake. To experimentally infect snails, we exposed snails to *Ribeiroia* eggs obtained from surrogate definitive hosts (*Rattus rattus*) (for methods see Johnson et al. 2007, Paull and Johnson 2011). Ten weeks following exposure, we confirmed snails were infected by isolating individuals into 50 ml vials and quantifying release of *Ribeiroia* cercariae over a 24 hr period. Based on pre-manipulation data from 2006–2008, the total number of infected snails within Hog Lake ranged from 0 to 825 (mean: 155.4 $\pm$ 114.8), varying by year and by sample date within year. Thus, the addition of $\sim$500 infected snails was expected to create a significant yet realistic increase in average *Ribeiroia* infection load within amphibians similar to what was observed in 2006 and 2007 (see Lunde 2011 for additional details). However, this addition represented a negligible increase to overall *H. trivolvis* density and was unlikely to influence snail-tadpole competition. For instance, the previous cohort's population of snails in half of Hog Lake ranges from 5,000 to 15,000 (i.e., added snails represented $<$5% of the adult cohort population) (Lunde 2011). As indicated in the results, this addition led to an increase in amphibian infection that was well within the range of what has been observed within Hog Lake and other ponds in the area (see Table 1 and *Results*).

We compared infection and malformation prevalence between the two pond treatments before (2008), during (2009), and after (2010) the manipulation according to a Before-After-Control-Impact (BACI) sample design (Smith 2006). Between May and June of each year, we collected data on the malformation prevalence in *P. regilla* larvae and frogs, which serve as a

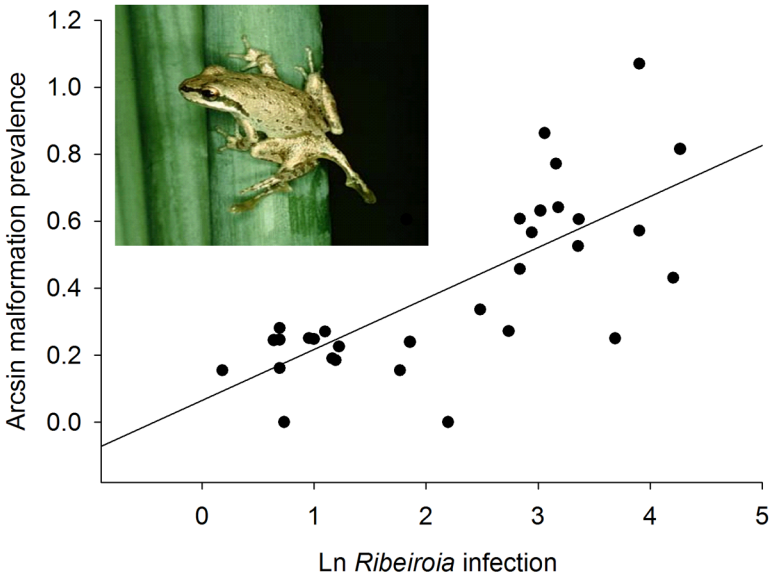

Fig. 1. Relationship between *Ribeiroia* metacercariae infection (natural log transformed) and malformation prevalence (arcsin-square-root transformed) among Pacific chorus frogs (*P. regilla*) at ponds with *Ribeiroia* in Northern California ($R^2 = 0.52$, $p < 0.001$, $n = 32$). Ponds sampled on different years were treated as independent for this analysis. Photograph copyright Steve Holt (stockpix.com).

temporal comparison for the experiment. Larvae were collected using fixed-area netsweeps with a D-frame dip net (1200 μm) and examined in the laboratory for malformations and developmental stage (Gosner 1960), whereas metamorphic frogs were inspected for malformations in the field with a subset dissected to quantify *Ribeiroia* (see Lunde 2011). Malformation frequency was determined from metamorphic frogs and larvae of Gosner (1960) stage 30 or greater (i.e., when the hind limbs were developed enough for inspection). To determine whether water quality varied between the two treatments, we measured surface water temperature (°C) and specific conductance (conductivity calibrated to 25°C) using a YSI MP 556 meter, and pH using an Oakton pHTstr 3. Turbidity (NTU) was measured with a HACH 2100P turbidity meter. Water samples for total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were frozen within 8 hrs of collection, then thawed and filtered using glass fiber filters (Whatman GF/D 2.7 μm) prior to analysis (Cukjati and Seibold 2010).

Hog Lake: cage study

To complement the large-scale manipulation, we conducted a replicated ($n = 16$) enclosure/exclosure study nested within both sides of Hog Lake. The study involved two different enclosure treatments: cages made with 35-μm mesh that prevented parasite entry ("closed") and cages made with 500-μm mesh that allowed entry ("open") of *Ribeiroia* cercariae from the surrounding habitat. This experimental design is similar to that of Kiesecker (2002) but involved finer mesh based on pilot studies that indicated cercariae could pass through 53-μm mesh. Enclosures measured $0.5 \times 0.5 \times 1$ m in size (1 m^3) and were closed on the bottom and top to prevent predator entry (e.g., Fig. 4B). Importantly, the two mesh sizes would identify the proximate cause of any observed malformations at Hog Lake given that water-based contaminants (e.g., pesticides) should be able to enter all cages regardless of mesh size, predators (e.g., dragonfly larvae) should be precluded from all cages, while parasite cercariae can enter only the cages with the large mesh size (Johnson and Bowerman 2010). Eight cages were installed on each pond side: four that allowed parasite entry and four that prohibited parasite entry. On 25 June 2009, each cage was stocked with 25 *P. regilla* larvae that had been previously collected as egg masses,

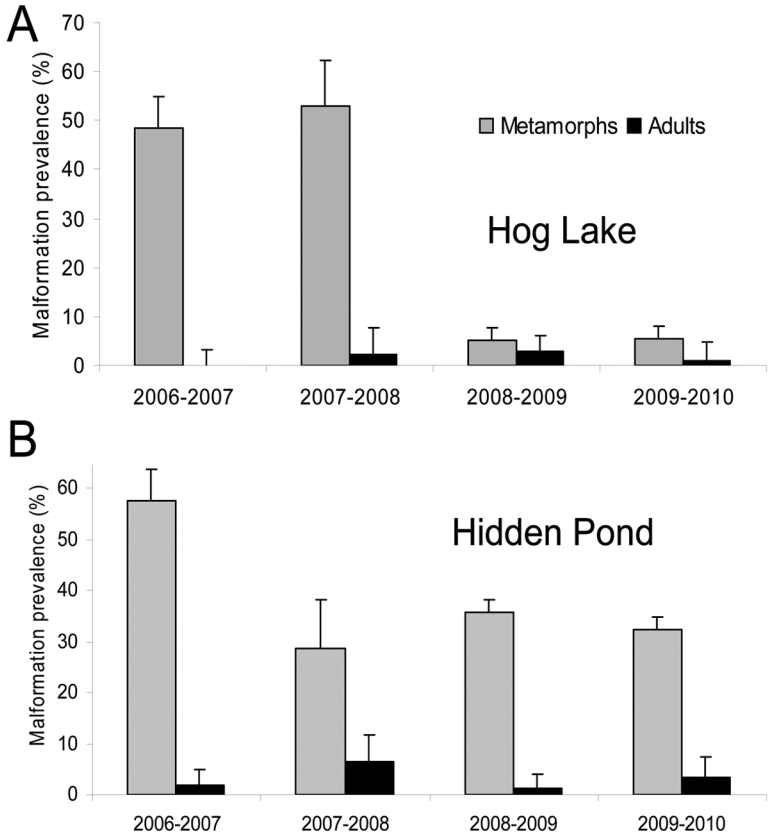

Fig. 2. Comparison of malformation prevalence of *P. regilla* metamorphic frogs from summer and adult frogs during the following winter at (A) Hog Lake and (B) Hidden Pond over four consecutive years. Error bars represent the 95% binomial confidence interval based on sample size.

hatched, and raised to Gosner (1960) stages 26–28, which are stages known to be susceptible to *Ribeiroia* exposure. To ensure adequate resource availability, 50 g of frozen spinach was added to each cage weekly for the duration of the study. After 21 d, we collected the following data from each cage: survival (number of animals recovered), mean Gosner stage, mean wet mass (after removing excess moisture), malformation prevalence, and mean *Ribeiroia* infection abundance (number of metacercariae per larvae).

Comparison of host pathology across spatial scales and venues

Contrasts across spatial scales and study venues involved a comparison of the slope of the relationship between observed *Ribeiroia* infection (measured as infection abundance upon necropsy) and the prevalence of malformations within that population, be it an experimental treatment or a collection of animals from nature. Included in our comparisons were the data from the 18 northern California wetlands presented here, from the 16 cages installed in Hog Lake in 2009 ($n = 15$ due to 100% mortality in one cage), and from previously published laboratory experiments with *P. regilla* (Johnson et al. 1999, Johnson and Buller 2011). For the laboratory studies, we included data only from *P. regilla* that survived to metamorphosis and only from treatments exposed to *Ribeiroia* between Gosner stages 26 and 28. Given the high degree of interannual variability observed in natural systems, we treated each wetland-by-year sampling event as an independent replicate for the 18 wetlands ($n = 43$) (however, results were nearly identical if we restricted the analysis only to the first visit of each wetland, $n = 18$). We expected that *P. regilla* frogs outside of enclosures (e.g., free-living individuals) might have a different

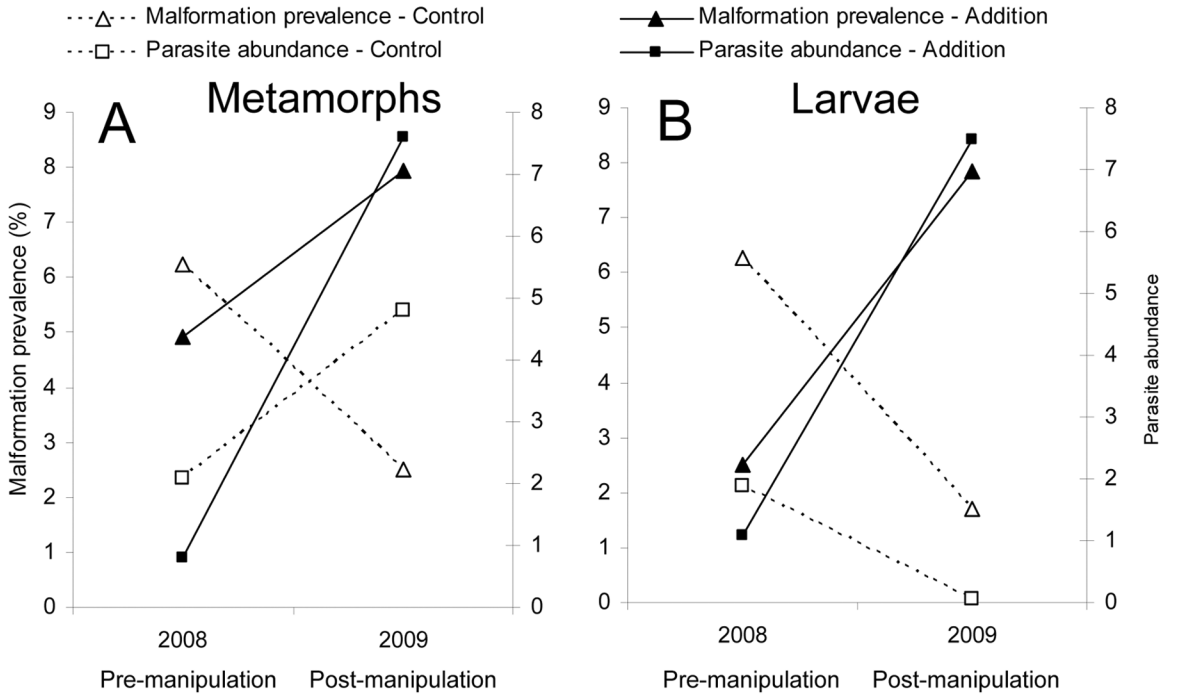

Fig. 3. Effects of *Ribeiroia*-infected snail addition on (A) *P. regilla* metamorphic frogs, and (B) *P. regilla* larvae. (C) Photograph of Hog Lake following installation of the cage study in June 2009. *Ribeiroia* infected snail addition occurred in the left half of Hog Lake.

malformation response because frogs were exposed to parasites before and after the most sensitive developmental window to infection and because severely malformed larvae might survive to metamorphosis in a cage, but not in a pond. We further predicted that caged animals

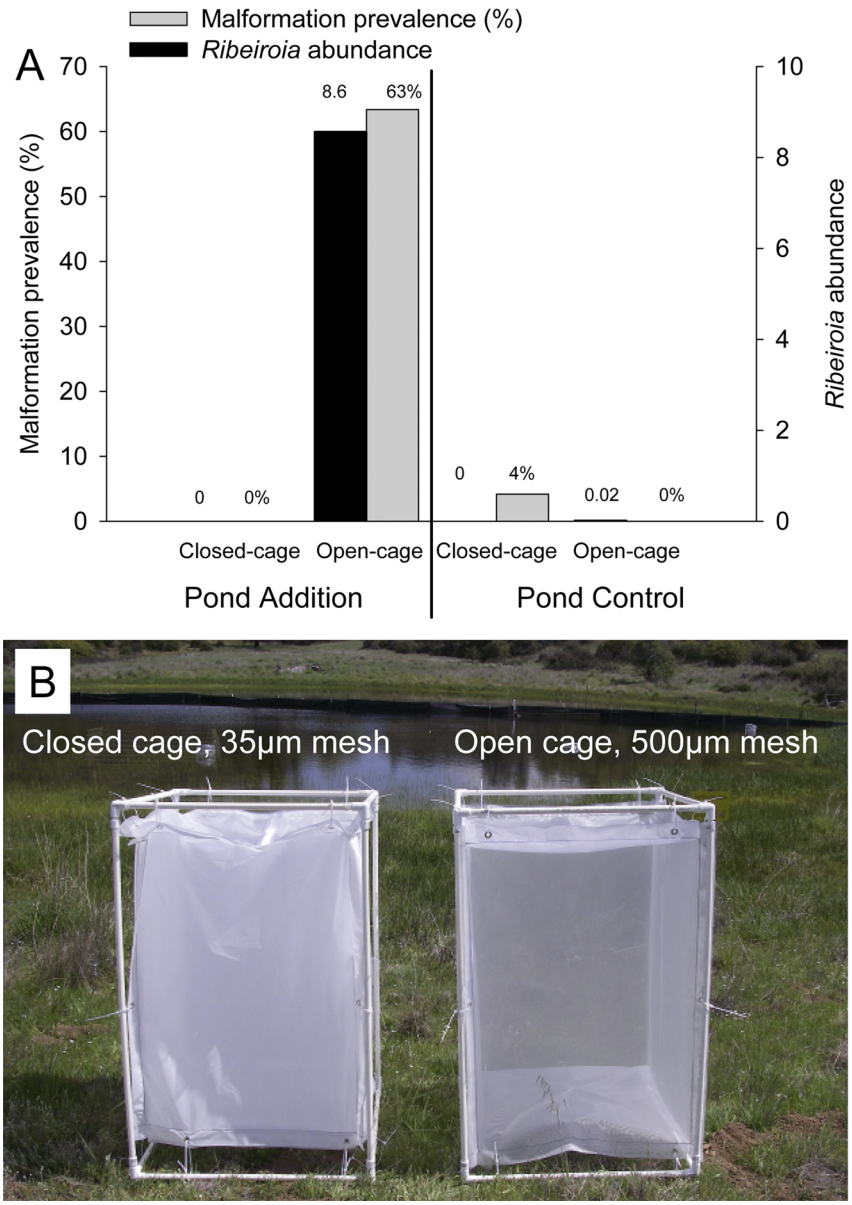

Fig. 4. (A) Results from cage study within Hog Lake. "Pond Addition" refers to the half-pond treatment of adding *Ribeiroia* infected snails while "Pond Control" refers to the control half-pond that reflects ambient *Ribeiroia* levels. (B) "Closed-cage" (left) refers to the cage mesh of 35 µm that prevented trematodes from entering the cages and "Open-cage" (right) refers to 500 µm mesh that allowed parasites to enter. Both mesh sizes would have allowed potential pesticides or chemicals to enter, whereas neither mesh size allowed predators to enter (e.g., larval dragonflies, leeches).

would have a different response relative to lab-exposed animals if differences in stress from being in the field (e.g., exposed to cues of predators or environmental variability) altered immune responses or behaviors in ways that affected infection (e.g., Relyea and Mills 2001).

Statistical analyses

To analyze data from the Hog Lake manipulation study, we used Generalized Linear Models (GLM) and Generalized Linear Mixed Models (GLMM) because they (1) allow for non-normally

distributed response variables, such as malformation presence and parasite count data, and (2) facilitate the nesting of samples by time or space (GLMMs only) (see Bolker et al. 2009, Zuur et al. 2009). Specifically, we sought to compare changes in response variables as a function of pond manipulation (*Ribeiroia* addition vs. control), time period (pre-manipulation year [2008], manipulation year [2009], and post-manipulation year [2010, sampled only for metamorphic frogs]), and their interaction. We were particularly interested in interactions between the treatment and time period, which would suggest that the addition of infected snails had an effect but only during the manipulation year. The primary response variables were malformations (present or absent in an individual) and *Ribeiroia* infection abundance (number of metacercariae per individual). We analyzed these responses for larval and metamorphic *P. regilla*, although sample sizes and sampling frequency varied by species and life history stage. For metamorphic *P. regilla*, we included data on malformations and infection annually from 2008–2010, whereas for larvae we included multiple sampling events per year for 2008 and 2009. Thus, for larval amphibians, we nested individual amphibians by sampling date within a year (random effect) using GLMM, thereby helping to account for the lack of independence among animals sampled on the same date (which was not specifically of interest). Analyses were performed using the "lmer" function within the lme4 package in the statistical program R (R Development Core Team 2011).

For the cage study within Hog Lake, we analyzed the data in two ways. Because animals within closed cages that prevented parasite entry had no infection and no malformations, it was not possible to use the GLMM approach above while incorporating cage treatment (i.e., the closed cages had no variance in the response variables of interest). Thus, we used the summary data for each cage (averaged among animals) and standard general linear model approaches. The primary response variables of interest were survival (proportion of animals recovered at the end of the study relative to the number stocked, arcsine-square-root transformed), malformations (proportion of surviving animals with one or more malformations, arcsine-square-root transformed), *Ribeiroia* infection (average number of metacercariae among animals surviving the study period), and host stage/mass (average mass and Gosner stage of animals surviving the study, $\log_{10}$-transformed). For comparative purposes, we also used the GLMM approach to analyze *Ribeiroia* infection and malformations among animals from the open cage treatments only, with pond side (manipulated vs. control) as a fixed effect and cage identity as a random effect.

Finally, to analyze the data from our field surveys and as a function of venue/spatial scale, we used least squares regression to compare the relationship between malformation frequency (arcsine-square-root transformed) and *Ribeiroia* infection abundance ($\ln(x + 1)$ transformed). We used the pooled data from animals (e.g., malformation frequency) rather than individual frog data because the sample sizes for necropsy and malformations varied (i.e., only a subset of examined frogs from each site or treatment were necropsied). To compare the relationship between infection and malformations as a function of venue, including *P. regilla* from laboratory experiments, from the cage study, and from whole wetlands, we included venue and venue-by-infection as additional predictors in regression models. This analysis thus examined variation in the slope of the relationship between observed level of infection (parasite burden) and host pathology (malformations) as a function of venue. We did not run this analysis for host mortality because this would require that we recover hosts that died in natural ponds and in cages to be able to attribute their deaths to infection and quantify any their parasite burdens, which was not possible.

Results

Long-term monitoring and regional field surveys

Multi-year sampling of wetlands with *Ribeiroia* revealed extremely high variation in infection and *P. regilla* malformations. Overall, based on the examination of 6,511 *P. regilla* from 18 wetlands between 2006 and 2010, malformation frequency at wetlands with *Ribeiroia* ranged from 2 to 77% and showed a strong functional relationship to infection abundance, which averaged between 0.2 and 70 cysts per frog among ponds and site-years ($R^2 = 0.52$, $p < 0.001$, $n = 32$;

Fig. 1). Common malformations at ponds with *Ribeiroia* were extra limbs (polymely), bony triangles (taumelia), extra digits (polydactyly), and skin webbings; these malformation types and relative proportions were similar to observations from previous surveys in the same species (Johnson et al. 2002). In contrast, *P. regilla* from five ponds without *Ribeiroia* exhibited a low frequency (<5%) of predominantly minor abnormalities (e.g., missing digits, partially missing limbs). Malformation prevalence varied at *Ribeiroia* positive sites over the study period. At Hog Lake, for example, 53% of emerging *P. regilla* in 2007 were malformed and supported a *Ribeiroia* abundance of 70 metacercariae per frog. The next year malformation prevalence fell to 5.5%, with a corresponding decrease in parasite infection abundance to 1.5 metacercariae per frog. At all sites supporting *Ribeiroia*, interannual variation of malformation prevalence (standard deviation) was 9 percentage points and ranged from 1 to 26.

Repeated, multi-year comparisons of malformation frequency between metamorphosing and adult *P. regilla* at two independent ponds (Hog Lake and Hidden Pond) suggested that malformed frogs rarely survive to sexual maturity (Fig. 2). Based on a paired t-test, adult frogs had consistently lower frequencies of malformations relative to metamorphosing frogs from the previous spring ($t = -4.28$, 2-tailed $p = 0.0036$, average difference: 31.04%). Even following years in which malformations were extremely common (>50%) in metamorphosing frogs, adult frogs consistently exhibited a low level (<5%) of abnormalities (Fig. 2).

Hog Lake: whole-ecosystem manipulation

The addition of infected snails to Hog Lake significantly influenced population-level patterns of infection and malformations in amphibian hosts (Fig. 3). According to the pre-addition shedding data, the 438 lab-infected snails and 79 naturally-infected snails produced an average of 44 and 94 cercariae per night, respectively. The four-week survivorship of caged snails was ~50% for the laboratory-raised snails and ~80% for the wild-infected snails. Therefore, we estimated the parasite-enhancement treatment increased the daily input of *Ribeiroia* cercariae by approximately 16,000 (assuming shedding patterns remained consistent over the course of the experiment, which was not recorded). By comparison, dissections of 2,354 snails from Hog Lake in 2006–2008 yielded a *Ribeiroia* infection prevalence of 0.18% (95% CL: 0.05, 0.43%). When combined with snail density data (Lunde 2011), the abundance of infected snails in one half of Hog Lake was estimated as 78 (range: 0 to 412). Mature natural infections of *Ribeiroia* tend to yield approximately 100 to 400 cercariae per night, suggesting that background daily release of cercariae likely ranges between 7,800 and 31,200. Thus, the addition of ~16,000 cercariae per night as part of the manipulation increased cercarial production by 50 to 200%, assuming snail infection patterns in 2009 were similar to the average of previous years. Based on the low levels of infection observed in metamorphosing *P. regilla* from the reference side of the lake, however, the background density of infected snails in 2009 was likely less than in past years.

Correspondingly, the parasite-addition treatment caused increases in *Ribeiroia* infection abundance and the frequency of severe limb malformations within larval and metamorphic *P. regilla* from the manipulated half of Hog Lake (Fig. 3). On the addition side, malformation prevalence in *P. regilla* larvae increased from 2.5% ($n = 200$) to 7.8% ($n = 293$) while infection levels correspondingly increased from 1.1 to 7.5 cysts per larvae. In contrast, on the control side of Hog Lake, malformation prevalence decreased from 6.3% ($n = 287$) to 1.7% ($n = 349$) and this change was also associated with an ambient decrease in *Ribeiroia* load from 1.9 to 0.06 cysts per larvae (Fig. 3). Based on the statistical analysis for larval amphibian data in 2008 and 2009 (no samples collected in 2010), both infection and malformation risk revealed significant interactions between treatment and year (GLMM infection: treatment × period $z = -6.08$, $p < 0.0001$; GLMM malformations: treatment × period $z = -3.61$, $p < 0.005$) (sample date within each year was treated as a random effect). The effect of treatment was significant and positive only in 2009 (infection $z = 8.73$, $p < 0.0001$; malformations $z = 3.24$, $p < 0.005$).

Similarly, metamorphic frogs had significantly higher infection levels and a greater prevalence of limb malformations following the infected snail addition (Fig. 3). On the addition side, malformation prevalence increased from 4.9% (*n*

= 163) in 2008 to 7.9% ($n = 265$) in 2009 and the parasite load increased from 0.8 to 7.6 cysts per frog over the same time period. Within the control treatment, malformation prevalence decreased from 6.3% ($n = 144$) in 2008 to 2.5% ($n = 198$) in 2009, even though *Ribeiroia* infection showed an increase from 2.1 to 4.8 cysts per frog. In the year following the manipulation (2010), malformations and infection were low on both sides of the wetland (e.g., 1.0% ($n = 104$) on the control side and 3.7% ($n = 107$) on the former addition side). Based on the statistical analysis, pond treatment interacted significantly with study period to determine malformation risk (GLM, treatment × period $z = -2.046$, $p = 0.04$). Re-analyzing the data after separating it by study period (pre-, during- and post-manipulation), *Ribeiroia* addition treatment led to an increase in malformations only during the manipulation in 2009 (GLM, treatment $z = 2.369$, $p = 0.02$) (no significant effects in either 2008 or 2010). Patterns of *Ribeiroia* infection for metamorphosing frogs exhibited a similar interaction between treatment and period (GLM, treatment × period $z = -3.53$, $p = 0.0004$), such that infection increased in the treatment side only during the manipulation year (2009 treatment effect $z = 3.22$, $p = 0.001$). During the pre-manipulation year *Ribeiroia* infection was slightly greater on the reference side (2008 $z = -2.51$, $p = 0.0121$), whereas during the post-manipulation year infection was equivalent between the two sides (2010 $z = -0.18$, $p = 0.85$). Water chemistry as well as amphibian and snail density data at the pond were generally similar between the two treatments throughout the 2009 experiment (Table 2).

Hog Lake: cage study

Results from the replicated enclosure/exclosure cage study conducted in 2009 supported findings from the whole-lake results and provided additional information about the effects of study method on the relationship between host infection and malformations. The cage study confirmed that the addition of *Ribeiroia* was responsible for the increase in infection levels in *P. regilla* larvae, and that larvae exposed to *Ribeiroia* developed a wide range of severe limb malformations (Fig. 4, Table 3). No parasites were recovered from *P. regilla* larvae raised within closed cages, demonstrating the effectiveness of the closed cage mesh size of 35 μm. In support of the link between *Ribeiroia* and malformations, only one abnormality (2%) was observed in animals from the closed cages, which was edema related and dissimilar in type from malformations in the open cages and wild caught frogs. In contrast, 63% of larvae recovered from the open cages from the addition side exhibited malformations, and all animals were infected with *Ribeiroia* metacercariae (7.1 cysts per larvae; Fig. 4). Similar to the whole pond results, *Ribeiroia* levels from larvae maintained within the open cages from the control treatment were

Table 2. Mean parameters measured between May and July 2009 within the two halves of Hog Lake that comprise each parasite treatment.

Parameter	Control treatment	Addition treatment
Water chemistry		
Turbidity (NTU)	1.6	2.7
Temperature loggers† (°C)	21.8	21.7
Specific conductance (μS/cm)	154.5	131.6
Dissolved oxygen (% saturation)	146.6	141.0
Dissolved oxygen (mg/L)	12.1	11.6
pH	8.5	8.8
Total nitrogen (mg/L)	0.887	0.883
Total phosphorus (mg/L)	0.044	0.030
Dissolved organic carbon (mg/L)	16.0	15.5
Chromium (mg/L)	0.0004	0.0011
Lead (mg/L)	0.0027	0.0023
Biological variables		
Helisoma sp. density 2008 (no./m^2)	1.4	0.3
Helisoma sp. density 2009 (no./m^2)	59.0	30.0
Pseudacris regilla density (no./m^2)	17.4	13.1
Taricha torosa density (no./m^2)	2.3	10.2

† Continuous collection of temperature data with logging every 30 minutes from 1 May to 16 July.

Table 3. Effects of Hog Lake parasite addition and nested cage study on *P. regilla* survival and development.

Cage number	Cage treatment	Hog Lake treatment	Survival (%)	Malformed (%)	*Ribeiroia* cyst load	Weight (g)	Gosner stage
1	closed	addition	40	0	0	0.633	39
4	closed	addition	20	0	0	0.601	39
6	closed	addition	24	0	0	0.708	39
7	closed	addition	32	0	0	0.747	39
2	open	addition	0	...	...	...	...
3	open	addition	28	57.1	6.1	0.496	37
5	open	addition	48	45.5	6.3	0.488	39
8	open	addition	32	87.5	8.8	0.525	38
9	closed	control	4	0	0	...	35
12	closed	control	20	0	0	0.672	40
14	closed	control	36	0	0	0.588	39
15	closed	control	28	16.7	0	0.495	38
10	open	control	56	0	0.1	0.570	39
11	open	control	28	0	0	0.529	38
13	open	control	40	0	0	0.482	39
16	open	control	40	0	0	0.533	39

Notes: All cages were initially stocked with 25 *P. regilla* larvae. Hog Lake treatment refers to *Ribeiroia* addition or control that was achieved by adding infected snails to the addition side and leaving natural infection levels on the control side. Ellipses indicate absence of data due to lack of any survivors in the cage (Cage 2) or the weight measurement of a single survivor was known to be inaccurate (Cage 9).

very low (0.02 cysts per larvae) and no malformed animals were observed. Cage treatment (closed vs. open) and pond treatment (*Ribeiroia* addition vs. control) interacted significantly to influence infection and malformation risk (Malformations: ANOVA, $F_{3,11} = 28.73$, pond × cage $p < 0.0001$; *Ribeiroia* infection: ANOVA, $F_{3,\ 11} = 583.85$, pond × cage $p < 0.0001$). While pond treatment had no effect on infection or malformation levels in animals within the closed cages (all $p > 0.3$), larvae in the open cages showed significant increases in both response variables on the addition side (Malformations: cage effect, $F_{1,\ 5} = 62.79$, $p = 0.0005$; *Ribeiroia* infection: cage effect, $F_{1,5} = 605.26$, $p < 0.0001$). Closed cages tended to increase host mass ($F_{3,10} = 3.18$, $p < 0.005$) while neither cages nor pond treatment altered developmental stage ($F_{3,11} = 0.944$). Survival, which averaged 30% among cages, was not affected by cage treatment or pond manipulation ($p > 0.5$). Generalized linear mixed effects models using only the data from the open cages provided comparable results.

Water chemistry within the cages showed some significant differences between treatments (Table 4). Conductivity was higher on the control side compared to the addition side, which led to a significant difference between the addition open and control open cages ($F = 9.8$, $p = 0.001$). Temperatures were slightly higher in the open cages on the control side compared to the closed cages on the addition side ($F = 4.1$, $p = 0.032$). Dissolved oxygen did vary among individual cages but no significant differences were observed between treatments ($F = 0.4$, $p = 0.7$).

Comparison of host pathology across spatial scales and venues

The relationship between parasite infection and malformations across the three spatial scales

Table 4. Water chemistry conditions (mean ± standard deviation) measured within Hog Lake cages on 16 June 2009 (mid-study).

Cage treatment	Hog Lake treatment	Specific conductance (μS/cm^2)	Temperature (°C)	Dissolved oxygen (%)
Closed	addition	184 ± 5	23.2 ± 0.4	171 ± 25
Open	addition	181 ± 7	24.0 ± 1.0	170 ± 54
Closed	control	193 ± 2	23.9 ± 0.9	181 ± 14
Open	control	195 ± 1	25.3 ± 1.0	196 ± 16

Note: Hog Lake treatment refers to *Ribeiroia* addition or control that was achieved by adding infected snails to the addition side and leaving natural infection levels on the control side.

[laboratory (0.001 m^3); cages (0.5 m^3); and whole ecosystems (800 m^3)] differed as a function of venue ($F_{5,62} = 77.38$, $p < 0.0001$ with infection-by-venue interaction). Separating the data by venue, *Ribeiroia* infection (ln-transformed) always had a strong positive effect on malformation frequency (arcsin-square root-transformed (all $p < 0.0001$ with R^2 ranging from 0.62 to 0.95), but the slope of this relationship varied (lab: 0.468; cage: 0.439; field: 0.142) (Fig. 5). For example, comparable levels of *Ribeiroia* infection were recorded in open cages from the addition side of Hog Lake and within wild-caught larvae, but the malformation prevalence in the cages (63%) was 8 times higher than that in wild larvae (7.8%). Re-running the analyses to compare each pair of venues indicated that cage and laboratory results were not different ($p > 0.05$), but the dose-response for field was significantly lower than the other two ($p < 0.001$).

Discussion

By combining an ecosystem manipulation, in situ cage studies, and a survey of 6,511 frogs from 18 wetlands in the region, this study offers an integrated perspective into the causes and consequences of parasite infection under ecologically relevant conditions. The experiment at Hog Lake, which represents one of the few studies to manipulate aquatic parasites at an ecosystem scale (see also Nassi et al. 1979, Sato et al. 2012), showed that large-scale manipulations of trematode populations are feasible. The study also demonstrated that the relationship between *Ribeiroia* infection and host pathology varied in strength as a function of study venue (field surveys, lab experiments, and field enclosure cages). Lastly, this study indicated that animals with severe limb malformations caused by parasite infection were both widespread across the landscape and unlikely to return as breeding adults, which has potential implications for species conservation and the management of aquatic systems.

The complementary use of experimental and survey-based approaches across a gradient of spatial scales and methods provided evidence that *Ribeiroia* infection is a major cause of limb malformations in *P. regilla* populations. Among the regional wetlands sampled between 2006 and 2010, we observed a clear and consistent relationship between infection abundance and malformation frequency (Fig. 1). Documented malformations, which were dominated by extra limbs, bony triangles, skin webbings, and extra digits, were similar to the types of limb abnormalities induced in laboratory-exposed *P. regilla* and to those observed other field sites with *Ribeiroia* (Johnson et al. 2002, 2012). Abnormalities at wetlands without *Ribeiroia* tended to be rare (<5% prevalence) and dominated by missing digits and partially missing limbs. The multiyear monitoring at many sites illustrated the remarkable spatial and interannual variability in parasite abundance and the resulting frequency of malformations, which varied from <1% to 70% (Table 1). Possible causes of interannual variation in parasite abundance included changes in snail populations or in definitive host (bird) activity at the ponds.

Results of the large-scale parasite manipulation offered further evidence of the causal link between infection and malformations under more controlled conditions. While most previous work on this system and for aquatic parasites generally has involved laboratory, mesocosm or cage studies (Kiesecker 2002, Schotthoefer et al. 2003, Raffel et al. 2010, Rohr et al. 2010, Johnson et al. 2012), ecosystem-scale experiments play an important role in ecology by testing the validity of findings reported from more artificial settings or correlative studies (Tilman 1989, Carpenter et al. 1995, 2011, Schindler 1998, Vredenburg 2004, Sato et al. 2012). By using a BACI design in an experimentally divided lake, we found that the addition of ~500 *Ribeiroia* infected snails, which represented a small fraction of the overall snail biomass but a significant increase in parasite cercarial release, resulted in a seven-fold increase in observed infection abundance among larval and metamorphic *P. regilla*. This increase in infection was accompanied by a two- to four-fold increase in malformations. Importantly, this increase occurred only on the manipulated side of Hog Lake and only during the manipulation year, with no significant difference either in the year before or the year after the experiment.

Finally, results from the in situ enclosure/exclosure cage study offered additional insights into the effects of *Ribeiroia* infection on amphibians while helping to offset the limited replica-

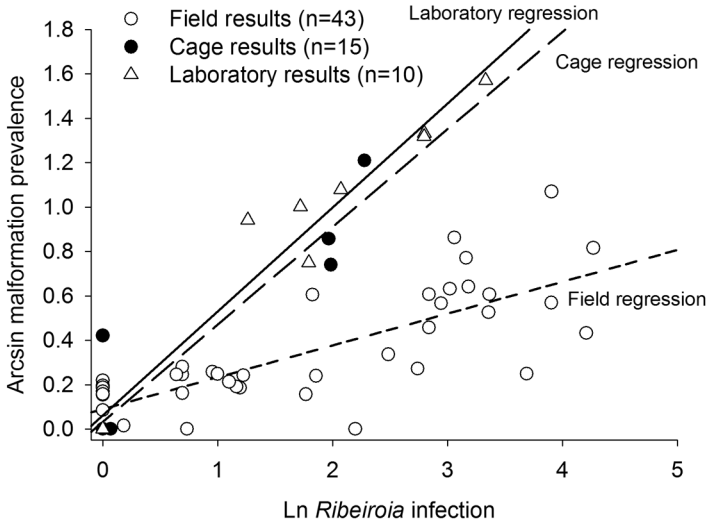

Fig. 5. Comparison of the dose response effect of *Ribeiroia* on *P. regilla* malformation prevalence at three spatial scales: laboratory results (straight line) from Johnson et al. (1999) and Johnson and Buller (2011); cage results (long dash) from animals in the Hog Lake cage study conducted in 2009; field results (short dash) derived from frogs collected from ponds across Northern California between 2006 and 2010.

tion inherent to many whole-ecosystem manipulations (e.g., Carpenter et al. 1996, Schindler et al. 1996, Pace et al. 2004, Sass et al. 2006, Kidd et al. 2007). The cage results showed an even stronger effect of infected snail addition, with a mean malformation prevalence of 63% in the open (500-μm mesh) cages in the addition treatment and an average of 7 parasite metacercariae per host. In contrast, few to no malformations or infections were observed in the closed (fine mesh) cages in the addition treatment or in either cage type on the un-manipulated half of Hog Lake (one abnormal animal was recovered in a closed cage from the control treatment). Had the malformations at Hog Lake been caused by pesticides or a water-based contaminant, we would have predicted comparable malformations in all cages, regardless of mesh size or wetland treatment, whereas had the abnormalities been caused by predators they should have been absent from all cages (i.e., predators could not enter the exclosures). Thus, these results illustrated the sharp differences in parasite abundance between the two sides of Hog Lake, reinforcing the functional linkage between infection and malformations and demonstrating the efficacy of using mesh size as a treatment for allowing or preventing cercarial entry (see also Kiesecker 2002, Johnson and Bowerman 2010).

Comparisons of the relationship between parasite abundance and malformation frequency across study scales revealed intriguing differences as a function of venue (e.g., Winkler and Van Buskirk 2012). While *Ribeiroia* infection was a consistent predictor of malformations in *P. regilla*, the slope of this relationship varied across laboratory experiments, in situ pond cages, and regional field surveys. The two small-scale venues (field cages [0.5 m^3] and laboratory microcosms [1 L]) exhibited a similar and generally steeper relationship between observed parasite load and malformation risk relative to frog populations sampled from wetlands across northern California. The congruence between laboratory infection and field cages suggests that laboratory microcosms offered a good approximation for understanding the influence of infection on malformations in nature, despite the lack realism associated with the absence of cues from predators, conspecifics and heterospecifics, all of which have the potential to influence host behavior and immunity (Thiemann and Wassersug 2000, Taylor et al. 2004, Belden and Kiesecker 2005, Rohr et al. 2008). This result is in contrast with work on agricultural pesticides, which can show sharply amplified effects of exposure

(mortality) in the presence of predator cues (Relyea and Mills 2001), but note we were unable to comapre mortality response across venues in this study.

The shallower slope between infection and malformation frequency recorded for *P. regilla* frogs in free-living populations (regional data) could have several explanations. The most likely involves the timing and duration of parasite exposure. In the field, larval *P. regilla* are exposed to *Ribeiroia* cercariae throughout larval development of ~40 to 60 d (Rorabaugh and Lannoo 2005), whereas animals used in experiments were exposed for only 10 d (laboratory) or 21 d (field cages). Experimental hosts were also exposed primarily during early limb development at Gosner (1960) stages 26–30, which is a 'critical window' for causing malformations (Johnson et al. 2011); field hosts, in contrast, continue to accumulate parasites well beyond this developmental window, thereby lessening the apparent per-capita effect of each parasite on malformation likelihood. Two additional factors that could influence this pattern are (a) the occurrence of non-parasite associated abnormalities in field populations such as sublethal predators (Bowerman et al. 2010), especially if affected animals have low parasite infection levels, and (b) increased mortality of malformed hosts that support high infections, either directly due to the infection or indirectly due to predators (Johnson et al. 1999). Either of these outcomes could function to weaken the slope and/or increase the intercept in the infection-malformation relationship. These results also suggest caution when extrapolating effect sizes from laboratory or short-term cage studies to whole ecosystems and communities.

The long-term effects of *Ribeiroia* exposure and malformations on amphibian population viability remain largely unexplored (Ouellet 2000, Johnson et al. 2010). Results from this study indicated that *Ribeiroia* infection and malformation epidemics are widespread across the region, with observed malformations affecting between 2 and 77% of sampled *P. regilla* metamorphs when *Ribeiroia* is present. In some wetlands, 90–100% of larval *P. regilla* are malformed (Johnson et al. 2002; L. Guderyahn, *unpublished data*) with few individuals metamorphosing and likely leading to complete recruitment failure. Here, repeated sampling by host life stage (metamorph vs. adult) suggested that most malformed frogs fail to reach sexual maturity (Rorabaugh and Lannoo 2005). Similarly, Goodman and Johnson (2011*a*, *b*) found that frogs with parasite-induced malformations exhibited poorer performance both under field and laboratory conditions, likely contributing to a significantly lower survival post-metamorphosis. Laboratory studies have also reported that direct exposure to *Ribeiroia* cercariae can cause 60–100% mortality in larval amphibians (Johnson et al. 1999, 2002, 2012, Schotthoefer et al. 2003, Rohr et al. 2010), highlighting the potential for both direct and indirect mortality stemming from infections.

Counter to our predictions and this previous body of work, however, parasite exposure did not increase mortality in caged *P. regilla* larvae, nor was it associated with differences in size or developmental stage over the exposure period. This could suggest that, under more natural conditions where hosts are more freely available to avoid or tolerate infection, the risk of direct mortality from infection is lowered. However, given the relatively low level of infection in caged animals (mean of 7 cysts per larvae) relative to the average infections in many regional ponds (mean of 17 cysts per frog: range = <1 to 71), the lack of detectable parasite-induced mortality among caged larvae is consistent with the effects of low dosages of *Ribeiroia* exposure documented in previous laboratory experiments (e.g., Johnson et al. 2012). In addition, mortality in the control cages from both pond treatments was much higher than observed in control treatments from laboratory studies, which may have obscured the small mortality effects predicted in response to the infections experienced here. Clearly, infection and malformations can cause individual host mortality, sometimes at high levels, but whether this translates into population-level effects remains conjectural. Longer-term studies that take a detailed look at the effects of infection and malformations on host life history while accounting for population 'rescue' from neighboring ponds are needed to address this issue. While the focal species of this study (*P. regilla*) is considered 'stable' throughout much of its range, perhaps in part because of its ability to rapidly recolonize extirpated wetlands, several other western species of conservation concern have been reported

with *Ribeiroia* infection and limb malformations, including the California red-legged frog (*Rana draytonii*), the western toad (*Anaxyrus boreas*), and the Oregon spotted frog (*Rana pretiosa*) (Johnson et al. 2002; Johnson, *unpublished data*).

Acknowledgments

We thank A. Merenlender for assistance with the study design, K. Fraiola, S. Poor, T. McWilliams for assistance installing the pond divider, B. Keiffer, S. Feirer for logistical support, J. Ball for help with the cage study, S. Paull for providing parasite eggs, M. Power, S. Kupferburg, and the Angelo Coast Range Reserve for providing amphibian eggs, I. Buller, B. LaFonte, E. Kellermanns, M. Aghaee with dissection of amphibians, and M. Aghaee, M. Baragona, C. Castanon, A. Cuneo, J. Dugan, C. Dunn, L. Gomez, K. He, J. Henderson, S. Ly, M. McKillop, M. Miranda, S. Osman, D. Preston, K. Thi, J. Wang, J. Xin for assistance in the field or with snail dissections. This project was supported by grants from National Science Foundation (DEB-0553768 to PTJJ), the David and Lucile Packard Foundation (to PTJJ), the University of California Berkeley Committee on Research Faculty Research Grant (to VHR), and graduate fellowships from National Science Foundation Graduate Research Fellowship Program (NSF-GRFP) and U.S. Environmental Protection Agency's Science to Achieve Results (EPA-STAR) program (to KBL). Although the research described in the article has been funded wholly or in part by the U.S. Environmental Protection Agency's STAR program, it has not been subjected to any EPA review and therefore does not necessarily reflect the views of the Agency, and no official endorsement should be inferred.

Literature Cited

Belden, L. K. and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria* sp. trematode cercariae. Journal of Parasitology 91:686–688.

Blaustein, A. R. and P. T. Johnson. 2003. The complexity of deformed amphibians. Frontiers in Ecology and the Environment 1:87–94.

Bolker, B. M., M. E. Brooks, C. J. Clark, S. W. Geange, J. R. Poulsen, M. H. H. Stevens, and J. S. White. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. Trends in Ecology & Evolution 24:127–135.

Bowerman, J., P. T. J. Johnson, and T. Bowerman. 2010. Sublethal predators and their injured prey: Linking aquatic predators and severe limb abnormalities in amphibians. Ecology 91:242–251.

Brezonik, P. L., et al. 1993. Experimental acidification of Little Rock Lake, Wisconsin: chemical and biological changes over the pH range 6.1 to 4.7. Canadian Journal of Fisheries and Aquatic Sciences 50:1101–1121.

Carpenter, S. R., et al. 2011. Early warnings of regime shifts: A whole-ecosystem experiment. Science 332:1079–1082.

Carpenter, S. R., S. W. Chisholm, C. J. Krebs, D. W. Schindler, and R. F. Wright. 1995. Ecosystem experiments. Science 269:324–327.

Carpenter, S. R., J. F. Kitchell, K. L. Cottingham, D. E. Schindler, D. L. Christensen, D. M. Post, and N. Voichick. 1996. Chlorophyll variability, nutrient input, and grazing: Evidence from whole-lake experiments. Ecology 77:725–735.

Cukjati, J. and C. Seibold. 2010. Kiowa Environmental Chemistry Laboratory procedure manual. Mountain Research Station & Institute of Arctic and Alpine Research, University of Colorado, Boulder, Colorado, USA.

Goodman, B. A. and P. T. J. Johnson. 2011*a*. Disease and the extended phenotype: Parasites control host performance and survival through induced changes in body plan. PLoS One 6:e20193.

Goodman, B. A. and P. T. J. Johnson. 2011*b*. Ecomorphology and disease: cryptic effects of parasitism on host habitat use, thermoregulation, and predator avoidance. Ecology 92:542–548.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Helmus, M. R. and G. G. Sass. 2008. The rapid effects of a whole-lake reduction of coarse woody debris on fish and benthic macroinvertebrates. Freshwater Biology 53:1423–1433.

Johnson, P. T. J. and J. Bowerman. 2010. Do predators cause frog deformities? The need for an eco-epidemiological approach. Journal of Experimental Zoology Part B, Molecular and Developmental Evolution 314B:515–518.

Johnson, P. T. J. and I. D. Buller. 2011. Parasite competition hidden by correlated coinfection: Using surveys and experiments to understand parasite interactions. Ecology 92:535–541.

Johnson, P. T. J., J. M. Chase, K. L. Dosch, R. B. Hartson, J. A. Gross, D. J. Larson, D. R. Sutherland, and S. R. Carpenter. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proceedings of the National Academy of Sciences USA 104:15781–15786.

Johnson, P. T. J., E. Kellermanns, and J. Bowerman. 2011. Critical windows of disease risk: Amphibian pathology driven by developmental changes in host resistance and tolerance. Functional Ecology 25:726–734.

Johnson, P. T. J., K. B. Lunde, E. G. Ritchie, and A. E. Launer. 1999. The effect of trematode infection on

amphibian limb development and survivorship. Science 284:802–804.

Johnson, P. T. J., K. B. Lunde, E. G. Ritchie, J. K. Reaser, and A. E. Launer. 2001. Morphological abnormality patterns in a California amphibian community. Herpetologica 57:336–352.

Johnson, P. T. J., K. B. Lunde, E. M. Thurman, E. G. Ritchie, S. N. Wray, D. R. Sutherland, J. M. Kapfer, T. J. Frest, J. Bowerman, and A. R. Blaustein. 2002. Parasite (*Ribeiroia ondatrae*) infection linked to amphibian malformations in the western United States. Ecological Monographs 72:151–168.

Johnson, P. T. J., M. K. Reeves, S. K. Krest, and A. E. Pinkney. 2010. A decade of deformities. Advances in our understanding of amphibian malformations and thier implications. Pages 511–536 *in* D. W. Sparling, G. Linder, C. A. Bishop, and S. K. Krest, editors. Ecotoxicology of amphibians and reptiles. Second edition. Society for Environmental Toxicology and and Contaminants (SETAC), Pensacola, Florida, USA.

Johnson, P. T. J., J. R. Rohr, J. T. Hoverman, E. Kellermanns, J. Bowerman, and K. B. Lunde. 2012. Living fast and dying of infection: host life history drives interspecific variation in infection and disease risk. Ecology Letters 15:235–242.

Johnson, P. T. J., D. R. Sutherland, J. M. Kinsella, and K. B. Lunde. 2004. Review of the trematode genus *Ribeiroia* (Psilostomidae): Ecology, life history and pathogenesis with special emphasis on the amphibian malformation problem. Advances in Parasitology 57:191–253.

Kidd, K. A., P. J. Blanchfield, K. H. Mills, V. P. Palace, R. E. Evans, J. M. Lazorchak, and R. W. Flick. 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences USA 104:8897–8901.

Kiesecker, J. M. 2002. Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? Proceedings of the National Academy of Sciences USA 99:9900–9904.

Lunde, K. B. 2011. Investigations of altered aquatic ecosystems: biomonitoring, disease, and conservation. Dissertation. University of California Berkeley, Berkeley, California, USA.

Lunde, K. B. and P. T. J. Johnson. In press. A practical guide for the study of malformed amphibians and their causes. Journal of Herpetology.

Marcogliese, D. J. and M. Pietrock. 2011. Combined effects of parasites and contaminants on animal health: parasites do matter. Trends in Parasitology 27:123–130.

Nassi, H., J. P. Pointier, and Y. J. Golvan. 1979. Evaluation of a trial to control *Biomphalaria glabrata* in Guadeloupe by using a sterilizing trematode. Annales de Parasitologie Humaine et Comparee 54:185–192.

Orlofske, S. A., R. Jadin, D. L. Preston, and P. T. J. Johnson. 2012. Parasite transmission in complex communities: Predators and alternative hosts alter pathogenic infections in amphibians. Ecology 93:1247–1253.

Ouellet, M. 2000. Amphibian deformities: Current state of knowledge. Pages 617–661 *in* D. W. Sparling, G. Linder, and C. A. Bishop, editors. Ecotoxicology of amphibians and reptiles. Society for Environmental Toxicology and and Contaminants (SETAC), Pensacola, Florida, USA.

Pace, M. L., J. J. Cole, S. R. Carpenter, J. F. Kitchell, J. R. Hodgson, M. C. Van de Bogert, D. L. Bade, E. S. Kritzberg, and D. Bastviken. 2004. Whole-lake carbon-13 additions reveal terrestrial support of aquatic food webs. Nature 427:240–243.

Paull, S. H. and P. T. J. Johnson. 2011. High temperature enhances host pathology in a snail-trematode system: possible consequences of climate change for the emergence of disease. Freshwater Biology 56:767–778.

R Development Core Team. 2011. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raffel, T. R., J. T. Hoverman, N. T. Halstead, P. J. Michel, and J. R. Rohr. 2010. Parasitism in a community context: trait-mediated interactions with competition and predation. Ecology 91:1900–1907.

Reeves, M. K., P. Jensen, C. L. Dolph, M. Holyoak, and K. A. Trust. 2010. Multiple stressors and the cause of amphibian abnormalities. Ecological Monographs 80:423–440.

Relyea, R. A. and N. Mills. 2001. Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). Proceedings of the National Academy of Sciences USA 98:2491–2496.

Rohr, J. R., T. R. Raffel, and C. A. Hall. 2010. Developmental variation in resistance and tolerance in a multi-host-parasite system. Functional Ecology 24:1110–1121.

Rohr, J. R., et al. 2008. Agrochemicals increase trematode infections in a declining amphibian species. Nature 455:1235–1239.

Rorabaugh, J. C. and M. J. Lannoo. 2005. *Pseudacris regilla* (Baird and Girard, 1852[b]) Pacific treefrog. Pages 478–484 *in* M. J. Lannoo, editor. Amphibian declines: the conservation status of United States species. University of California Press, Berkeley, California, USA.

Sass, G. G., J. F. Kitchell, S. R. Carpenter, T. R. Hrabik, A. E. Marburg, and M. G. Turner. 2006. Fish community and food web responses to a whole-lake removal of coarse woody habitat. Fisheries 31:321–330.

Sato, T., T. Egusa, K. Fukushima, T. Oda, N. Ohte, N. Tokuchi, K. Watanabe, M. Kanaiwa, I. Murakami, and K. D. Lafferty. 2012. Nematomorph parasites indireclty later the food web and ecosystem function of stremas through behavioural manipulation of their cricket hosts. Ecology Letters.

Schindler, D. W. 1998. Replication versus realism: The need for ecosystem-scale experiments. Ecosystems 1:323–334.

Schindler, D. W., P. J. Curtis, B. R. Parker, and M. P. Stainton. 1996. Consequences of climate warming and lake acidification for UV-B penetration in North American boreal lakes. Nature 379:705–708.

Schotthoefer, A. M., A. V. Koehler, C. U. Meteyer, and R. A. Cole. 2003. Influence of *Ribeiroia ondatrae* (Trematoda: Digenea) infection on limb development and survival of northern leopard frogs (*Rana pipiens*): effects of host stage and parasite-exposure level. Canadian Journal of Zoology 81:1144–1153.

Schotthoefer, A. M., K. M. Labak, and V. R. Beasley. 2007. *Ribeiroia ondatrae* cercariae are consumed by aquatic invertebrate predators. Journal of Parasitology 93:1240–1243.

Skelly, D. K. 2002. Experimental venue and estimation of interaction strength. Ecology 83:2097–2101.

Skelly, D. K. and J. M. Kiesecker. 2001. Venue and outcome in ecological experiments: manipulations of larval anurans. Oikos 94:198–208.

Smith, E. P. 2006. BACI design. Pages 141–148 *in* A. H. El-Shaarawi and W. W. Piegorsch, editors. Encyclopedia of environmetrics. John Wiley & Sons, Chichester, UK.

Stopper, G. F., L. Hecker, R. A. Franssen, and S. K. Sessions. 2002. How trematodes cause limb deformities in amphibians. Journal of Experimental Zoology 294:252–263.

Sutherland, D. 2005. Parasites of North American frogs. Pages 109–123 *in* M. J. Lannoo, editor. Amphibian declines: the conservation status of United States species. University of California Press, Berkeley, California, USA.

Taylor, C. N., K. L. Oseen, and R. J. Wassersug. 2004. On the behavioural response of *Rana* and *Bufo* tadpoles to echinostomatoid cercariae: Implications to synergistic factors influencing trematode infections in anurans. Canadian Journal of Zoology 82:701–706.

Thieltges, D. W., K. T. Jensen, and R. Poulin. 2008. The role of biotic factors in the transmission of free-living endohelminth stages. Parasitology 135:407–426.

Thiemann, G. W. and R. J. Wassersug. 2000. Patterns and consequences of behavioural responses to predators and parasites in *Rana* tadpoles. Biological Journal of the Linnean Society 71:513–528.

Tilman, D. 1989. Ecological experimentation: strengths and conceptual problems. Pages 136–157 *in* G. E. Likens, editor. Long-term studies in ecology. Springer-Verlag, New York, New York, USA.

Vredenburg, V. T. 2004. Reversing introduced species effects: Experimental removal of introduced fish leads to rapid recovery of a declining frog. Proceedings of the National Academy of Sciences USA 101:7646–7650.

Winkler, J. D. and J. Van Buskirk. 2012. Influence of experimental venue on phenotype: multiple traits reveal multiple answers. Functional Ecology doi: 10.1111/j. 1365-2435.2012.01965.x.

Zuur, A. F., E. N. Ieno, N. Walker, A. A. Saveliev, and G. M. Smith. 2009. Mixed effects models and extensions in ecology with R (statistics for biology and health). Springer, New York, New York, USA.