

# All hosts are not equal: explaining differential patterns of malformations in an amphibian community

Pieter T. J. Johnson<sup>1\*</sup> and Richard B. Hartson<sup>2</sup>

<sup>1</sup>Ecology and Evolutionary Biology, University of Colorado, Ramaley N122, Campus Box 334, Boulder, CO 80309, USA; and <sup>2</sup>Center for Limnology, University of Wisconsin, 680 North Park Street, Madison, WI 53706, USA

## Summary

1. Within a community, different host species often exhibit broad variation in sensitivity to infection and disease. Because such differences can influence the strength and outcome of community interactions, it is essential to understand differential disease patterns and identify the mechanisms responsible.

2. In North American wetlands, amphibian species often exhibit extraordinary differences in the frequency of limb malformations induced by the digenetic trematode, *Ribeiroia ondatrae*. By coupling field studies with parasite exposure experiments, we evaluated whether such patterns were due to differences in (i) parasite encounter rate, (ii) infection establishment, or (iii) parasite persistence within hosts.

3. Field results underscored the broad variation in malformations and infection between host species; while nearly 60% ( $n = 618$ ) of emerging American toads exhibited severe limb deformities such as bony triangles, skin webbings and missing limbs, fewer than 4% ( $n = 251$ ) of Eastern gray treefrogs from the same pond were abnormal. Despite similarities in the phenology and larval development period of these species, they differed sharply in *Ribeiroia* infection. On average, toads supported 75× more metacercariae than did metamorphic treefrogs.

4. Experimental exposures of larval toads and treefrogs to a realistic range of *Ribeiroia* cercariae revealed strong differences in the sensitivity of these species to infection; exposed toads suffered elevated mortality (up to 95%), delayed metamorphosis, and severe limb malformations consistent with field observations. Treefrogs, in contrast, exhibited limited mortality and no malformations, regardless of exposure level. *Ribeiroia* cercariae were substantially less successful in locating and infecting *Hyla versicolor* larvae.

5. Our results indicate that the observed differences in infection and malformations owe to a lower ability of *Ribeiroia* cercariae to both find and establish within larval treefrogs, possibly stemming from a heightened immune response to infection. Because *Ribeiroia* is a highly pathogenic parasite with negative effects on larval and metamorphic amphibian survival, variation in infection resistance among species could have important implications for understanding patterns of species co-occurrence, competition, and community diversity.

**Key-words:** amphibian decline, amphibian deformities, community interactions, disease ecology

## Introduction

All species serve as hosts for one or more parasites, and some infections sharply influence host growth, survival, behaviour and reproduction. Within a host community, however, the abundance and composition of parasites varies substantially among different host species. Differences in species' behaviour, habitat use, phenology, diet, immune function, physiology

and evolutionary history can all influence the types and severity of infections exhibited (e.g. Combes 2001; Hudson *et al.* 2002). Because parasites and pathogens often affect interactions among free-living species, the ecological consequences of differential infection patterns among species can be profound. Parasite infection and disease can influence predator–prey interactions, species competition, food web structure, community stability, and ecosystem processes (e.g. Minchella & Scott 1991; Lafferty & Morris 1996; Lafferty, Dobson & Kuris 2006). For example, introduction of the fungus *Cryptosporidium*

\*Correspondence author. E-mail: pieter.johnson@colorado.edu

*parasitica* Barr to the north-eastern USA in the early 1900s caused the near-complete eradication of the American chestnut (*Castanea dentata* Borkh.) while leaving other tree species unharmed, leading to the wholesale replacement of the dominant forest structure with concomitant changes in community and ecosystem properties (e.g. Eviner & Likens 2007).

While examples from well-studied cases underscore the importance of identifying patterns of differential infection among species and the mechanisms responsible, such information is lacking for most ecological communities. In freshwater ecosystems, larval amphibian communities are exposed to a broad diversity of parasites, including protists, viruses, bacteria and helminths (Reichenbach-Klinke & Elkan 1965; Prudhoe & Bray 1982; Sutherland 2005), and represent an ideal system in which to examine differential patterns of disease. Recently, infection by the digenetic trematode *Ribeiroia ondatrae* Travassos (hereafter, '*Ribeiroia*') has been linked to high levels (> 20%) of limb malformations in North American amphibians, including missing, malformed and supernumerary limbs. Experimental exposures to *Ribeiroia* cause high levels of malformations and mortality in frogs, toads and salamanders (Johnson *et al.* 1999, 2001a, 2006; Stopper *et al.* 2002; Schotthoefer *et al.* 2003). Correspondingly, field surveys have revealed a strong association between elevated infection and deformities among naturally occurring amphibian populations (e.g. Sessions & Ruth 1990; Johnson *et al.* 2001b, 2002; Kiesecker 2002). However, a widely observed yet enigmatic pattern involves the broad variation in malformation frequency observed among amphibian species (Sessions & Ruth 1990; Johnson *et al.* 2001b, 2002; Vandenlangenberg, Canfield & Magner 2003). Severe deformities may be abundant in one species while rare or absent in another, even within the same wetland (e.g. Johnson *et al.* 2001b; Hoppe 2005). For example, among seven anuran species in a Minnesota wetland, Hoppe (2005) reported a 30-fold difference in malformation frequency from the least affected to the most affected species (2.1 vs. 68.1%).

With respect to malformations caused by *Ribeiroia*, the factors responsible for this variation remain speculative, but likely result from differences in (i) the intensity or duration of parasite exposure, (ii) the success of parasites in infecting hosts, or (iii) the host's response to invading parasites. Parasites, like free-living species, are subject to a series of ecological filters when colonizing a new host individual: host encounter and colonization (can the parasite find the host?), host establishment (can the parasite successfully infect the host in the appropriate location), and persistence [can the parasite maintain (or expand) its presence within the host?] (see Combes 2001). Within a wetland, amphibian species exhibit broad variation in breeding phenology and larval development period that affects their exposure to parasites (Schotthoefer *et al.* 2003; Holland *et al.* 2007). Amphibians are most likely to develop malformations when exposed to *Ribeiroia* during active limb development, suggesting that differences in the phenological overlap between amphibian larvae and parasites explains some variation in infection among species and years (Bowerman & Johnson 2003; Schotthoefer *et al.* 2003). Thus,

species or cohorts that complete early larval development well-before or well-after peak parasite abundance can reduce their risk of malformation or mortality. Variation in the relative size, habitat preferences, or behaviour of larval amphibians may also influence the level of infection, as smaller or more active species represent difficult targets for trematode cercariae (Thiemann & Wassersug 2000; Taylor, Oseen & Wassersug 2004; Koprivnikar, Baker & Forbes 2006). Finally, even when hosts are exposed to identical numbers of parasites, differences in parasite specificity or host resistance may determine the amount of successful infection by altering parasite establishment or persistence within host tissues (e.g. Kiesecker 2002; Tinsley & Jackson 2002; Belden & Kiesecker 2005).

In the present study, we coupled field sampling with controlled infection experiments to investigate the often-extraordinary variation in the frequency of limb deformities among amphibian species within a community. We sought to explain why, within a single wetland, deformities are extremely abundant (> 50%) in metamorphosing American toads (*Bufo americanus* Holbrook) but are rare or absent in Eastern gray treefrogs (*Hyla versicolor* LeConte). By regularly sampling a malformation 'hotspot' in central Minnesota, we compared the phenology, frequency of deformities, and the level of *Ribeiroia* infection between American toads and gray treefrogs to determine whether observed patterns resulted from variation in the timing of parasite exposure, the parasite's infection success in different hosts, or the hosts' respective responses to *Ribeiroia* (i.e. immunological or pathological). These alternative scenarios parallel Combes' (2001) parasite 'filters', including the encounter filter and the compatibility filter. To evaluate how each species responded to infection, we exposed laboratory-raised larvae to realistic dosages of *Ribeiroia* and evaluated their survival, development, and growth. Finally, we compared the ability of *Ribeiroia* cercariae to successfully locate, infect, and persist within each amphibian host. In this manner, we used trematode infection in an amphibian community to understand the factors controlling differential disease prevalence among hosts. Considering the broad distribution and high pathogenicity of *Ribeiroia* infection, such patterns have important implications for understanding interspecific competition, amphibian community assemblages, and the viability of affected populations.

## Materials and methods

### STUDY ORGANISMS

*R. ondatrae* has a multi-host life cycle in which stages of the parasite move sequentially among planorbid snails, larval amphibians, and birds or, less often, mammals (Johnson *et al.* 2004). Free-swimming cercariae emerge from infected snails and encyst around the developing limbs of amphibian larvae, often leading to improper limb development or host mortality. Resulting malformations may enhance transmission of *Ribeiroia* by increasing the vulnerability of infected frogs to vertebrate predators, wherein the parasites mature into an adult. Sexually produced eggs of the parasite are released along with faeces of the definitive host, distributing the parasite

across the landscape. Mobile miracidia hatch from eggs, infect a suitable snail host, develop inside the snail ovotestes, and eventually begin releasing free-swimming cercariae.

American toads (*B. americanus*) and Eastern gray treefrogs (*H. versicolor*) are common to a broad range of freshwater habitats in the Midwestern and Eastern USA (Cline 2005; Green 2005). Both species are late spring breeders, with adults congregating to breed during spring rain events between May and June (Oldfield & Moriarty 1994). Larval toads and treefrogs require approximately 40 and 50 days, respectively, to complete development and metamorphose (Cline 2005; Green 2005). In Minnesota and Wisconsin, toads emerge in late June or early July, whereas treefrogs generally emerge in July or early August (Oldfield & Moriarty 1994). Both species (or their congeners) fall prey to a variety of predatory birds and mammals (Olson 1989; Oldfield & Moriarty 1994; Werner *et al.* 2004; Cline 2005; Green 2005), suggesting that either can function to transmit *Ribeiroia*.

#### FIELD STUDIES

Between 10 June and 8 October of 2004, we conducted biweekly sampling at a malformation site in Minnesota (Duck Pond). The pond has a maximum depth of 2.5 m and is surrounded on three sides by residential development. Rams horn snails (*Planorbella trivolvis* Say) act as first intermediate hosts for *R. ondatrae*, whereas Northern leopard frogs [*Lithobates* (= *Rana*) *pipiens* Schreber], eastern gray treefrogs (*H. versicolor*) and American toads (*B. americanus*) function as second intermediate hosts. In 2003, we observed a high frequency (~50%) of limb malformations in *R. pipiens* consistent with the effects of *Ribeiroia* infection (Stopper *et al.* 2002; Schotthoefer *et al.* 2003). Our current goal was to compare the levels of deformities and *Ribeiroia* infection in gray treefrogs and American toads, for which empirical and experimental patterns of infection have not been previously explored. Moreover, gray treefrogs and American toads exhibit similar breeding phenologies, larval development times, and metamorphic sizes, which suggests they ought to exhibit similar levels of infection and deformities. Instead, however, observations from 2003 indicated that toads suffered substantially more malformations than gray treefrogs, underscoring the utility of this system for exploring the mechanisms driving differential disease patterns.

During each visit, we performed 1-m netsweeps with a D-frame (Ward's Natural Science, Rochester, New York, USA) dipnet (1200 µm mesh) every 15 m around the perimeter of the northern and southern shores of the pond (see Johnson *et al.* 2001b). We quantified the abundance of snails (*P. trivolvis*) and anuran larvae in each sweep, and conducted additional time-constrained searches for snails in the littoral zone. Snail transects involved two people wading parallel to the shore in 1 m of water collecting snails through visual examination of substrate (including woody debris) and haphazard dipnet sweeps through submerged vegetation. To determine which individuals were actively producing cercariae (defined here as 'patent' infections), snails were isolated in the dark for 15 h (17.00–08.00 h) in 50 mL centrifuge tubes filled with filtered pond water. Released cercariae were identified with a compound microscope, chilled (5 °C for 24 h), and counted on a gridded Petri dish using a stereomicroscope (Johnson *et al.* 2007). Of the snails that did not release cercariae, we randomly dissected a subset (25–50 individuals) to evaluate infection status. However, because some snails were also infected with an echinostome trematode, which produces morphologically similar infections, we were not able to differentiate immature infections without active cercariae. Remaining snails were measured and released.

We sampled metamorphic toads and treefrogs by hand during time- and space-constrained transects around the perimeter of the pond. Metamorphosing toads, in particular, are easily captured by hand in large numbers, reducing the likelihood of any bias in type of individuals collected (i.e. normal vs. malformed). Captured individuals were carefully inspected for any morphological abnormalities, photographed, and released. On each sampling date, a subset of 10–20 individuals for each species (including normal and malformed individuals, if present) was necropsied to isolate trematode infections. We thoroughly examined the skin (externally and internally), major organ systems, and digestive tract of each animal to quantify all visible infections. *Ribeiroia* is generally found just below the epidermis and above the muscular tissue around the inguinal region, tail resorption area, and, to a lesser extent, along the lower mandible (see Sutherland 2005). We identified metacercariae of *Ribeiroia* based on the presence of esophageal diverticula (Johnson *et al.* 2004). Echinostome metacercariae were isolated from the kidneys of infected animals and identified by characteristic collar spines.

#### LABORATORY EXPOSURES

To evaluate the relative sensitivity of American toads and gray treefrogs to *Ribeiroia* infection, we exposed larvae of each species to one of four cercarial dosages: 0 (control), 20, 40 or 100 cercariae. These levels were selected to bracket the infection intensities observed in field-caught toads from Duck Pond (see *Results*). Multiple egg masses ( $n = 3–5$  of each species) of each species were collected in the field and allowed to hatch in the laboratory. After hatching, we randomly assigned 20 tadpoles of each species to the four conditions for a total of 80 animals per species (exceptions: 30 animals were included in the *H. versicolor* control, and 25 in the *B. americanus* heavy exposure). We raised larvae individually in plastic containers filled with 1.0 L of commercial spring water. Beginning at Gosner (1960) stages 27–28, we exposed larvae every third day to one-fourth of the total intended cercarial dose (0, 5, 10 or 25 cercariae per exposure). Thus, after 10 days, all surviving animals had been exposed to the appropriate number of parasites (see methods in Johnson *et al.* 2001a). This approach ensured that cercarial exposure coincided with early limb development and maximized the likelihood of a malformation response. We conducted parasite exposures with treefrogs and toads concurrently to ensure experimental conditions (e.g. parasites, temperatures, etc.) were identical.

To obtain cercariae for experimental exposures, we collected infected snails (*P. trivolvis*) from Duck Pond, Minnesota (see above), and placed them in 50-mL centrifuge tubes in the dark between 19.00 h and 24.00 h. Because *Ribeiroia* emerges at night, this method ensured cercariae were at maximal infectivity when used in experiments. We isolated and counted cercariae using a stereomicroscope and administered them to amphibian larvae with a pipette. During each exposure event, we combined larvae and cercariae into 100-mL containers to reduce the likelihood of differences in larval amphibian behaviour affecting infection risk. After 8 h, we returned amphibian larvae (along with cercariae-containing water) to the original 1.0 L containers. Control animals were placed in 100-mL containers and exposed to a small amount (~5 mL) of water from an uninfected snail container to control for effects of snail-associated effluent.

We fed amphibian larvae *ad libitum* a 1:1 mixture of finely ground Tetramin® (Tetra Werke, Melle, Germany) and *Spirulina* flakes mixed in spring water. We also provided each animal with a 10-cm strand of cleaned *Egeria densa* Planch. to supplement food availability and offer a habitat refuge. Water and containers were changed

weekly, and animals were maintained in a randomized block design in which blocks were rotated weekly to avoid any positional (shelf) effects. Air temperature during the experiment ranged from 26 to 30 °C and the photoperiod followed a 16:8 h light:dark cycle. As animals reached forelimb emergence (Gosner stage 42), we poured out 90% of the water and discontinued feeding. We considered an individual 'metamorphosed' once 95% of the tail had resorbed (usually within 48 h), at which time it was measured [snout-vent length (SVL)], weighed (wet mass, blotted dry), and examined for morphological abnormalities. We randomly selected and necropsied 10 metamorphosing animals from each treatment to quantify the mean infection abundance of *Ribeiroia* metacercariae. Five animals from each control condition were also necropsied to verify the absence of infection.

#### PARASITE COLONIZATION AND ESTABLISHMENT

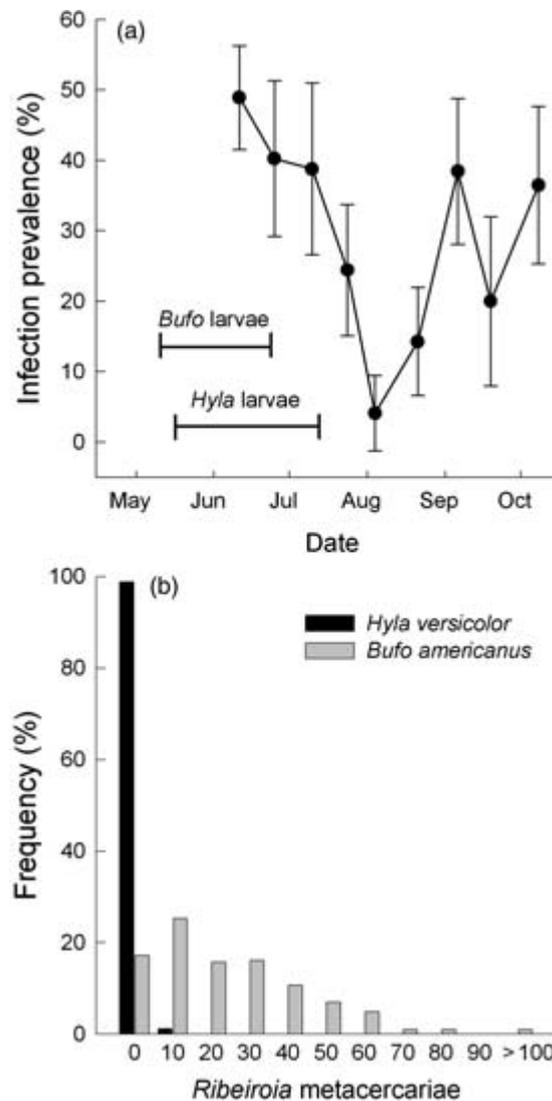
To better understand the results of the main experiment (above) and the factors driving differential infection patterns, we conducted two additional experiments comparing *H. versicolor* and *B. americanus* to quantify (i) what fraction of invading cercariae successfully penetrated each amphibian host (e.g. colonization) and, (ii) of that successful fraction, how did metacercarial persistence vary between species and with time since exposure (e.g. establishment). We individually isolated an additional 40 larvae (stage 28) of each species into 100 mL of spring water with 15 cercariae. After 8 h, larvae were removed and the remaining water from a subset of containers (30 treefrog larvae and 20 toad larvae) was poured through a 25 µm filter to collect any 'leftover' cercariae that were unsuccessful in locating a host. Because the number of recovered cercariae per individual container was generally low, we pooled the contents of 10 containers for each filtration event and examined material trapped on the filter. Thus, if no cercariae were successful in finding a host, we would recover a maximum of 150 cercariae (10 containers × 15 cercariae container<sup>-1</sup>). As a control, we added 15 cercariae to each of ten 100-mL containers devoid of amphibian larvae and filtered the water after 8 h.

Finally, to determine the establishment success of invading cercariae and whether metacercarial abundance varied with time since exposure (potentially indicative of an immune response), we euthanized a subset animals of each species (from the exposures above) on days 2, 4, and 10 and quantified *Ribeiroia* abundance (15 animals on days 2 and 4 and 10 animals on day 10). Thus, this second set of studies allowed us to estimate both the fraction of parasites that failed to penetrate the tadpoles ('leftover' cercariae) and, of those successful in entering the host, what fraction was subsequently eliminated by host defences over time. We thereby sought to identify at which stage(s) differences in the infection process between the two host species were occurring.

## Results

#### FIELD PATTERNS OF INFECTION AND MALFORMATIONS

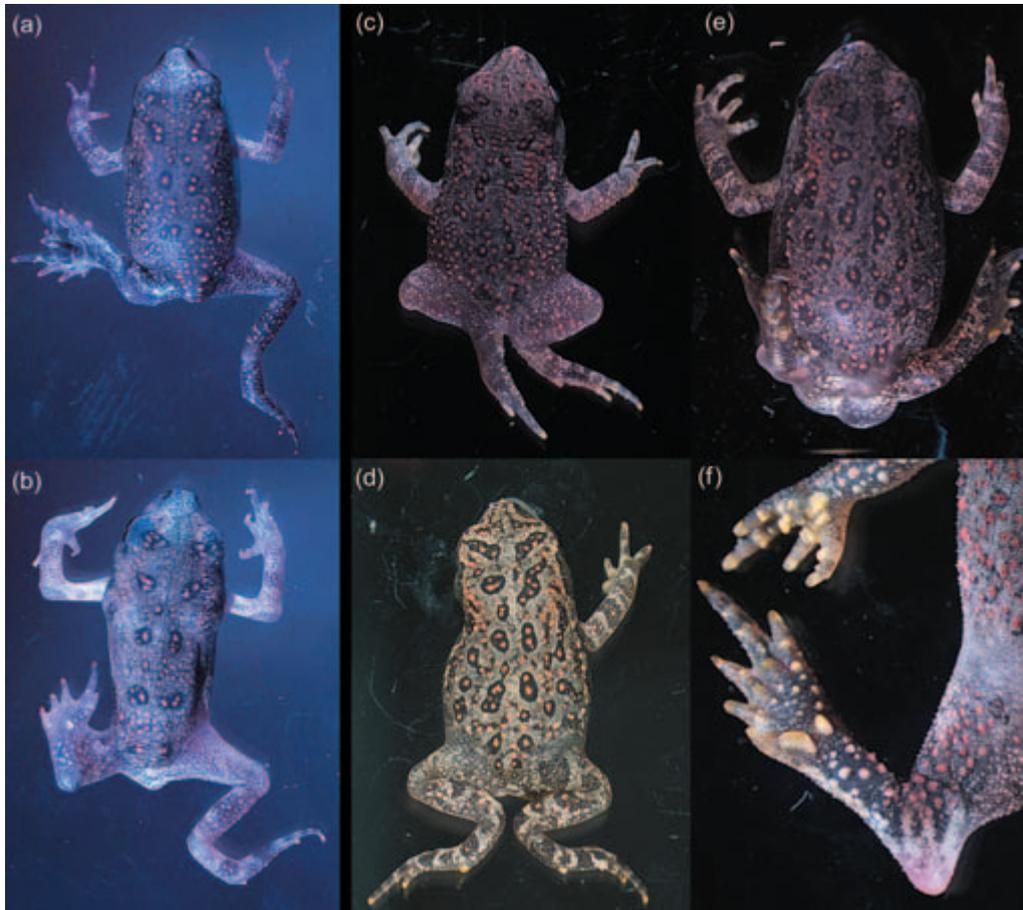
Patent *Ribeiroia* infections in *P. trivolvis* were highly prevalent but variable throughout the season, ranging from a maximum of 49% in June to a minimum of 4% in early August (Fig. 1a). Infected snails released, on average, between 301 and 672 cercariae per sampling date, with a maximum per snail release of 2146 cercariae. For both amphibian species, larval development coincided with periods of active *Ribeiroia* infection in



**Fig. 1.** (a) Seasonal phenology of larval amphibians and infected snails (*Planorbella trivolvis*) from Duck Pond, Minnesota, in 2004. The bracketed horizontal lines represent the estimated duration of larval development for *Bufo americanus* (upper line) and *Hyla versicolor* (lower line) within the pond. The prevalence (percentage) of infected snails ( $\pm$  95% confidence interval) is displayed as a line (note: no samples were collected before 1 June 2004). (b) The frequency distribution of *Ribeiroia* metacercariae isolated from metamorphic *H. versicolor* (black bars,  $n = 84$ ) and *B. americanus* (gray bars,  $n = 186$ ) at Duck Pond.

snails. We observed larval toads on 10 and 24 June (but not thereafter), with the first metamorphs emerging on 24 June. Larval treefrogs were abundant from the first sampling date (10 June) until 9 July, at which time metamorphosis began. Metamorphs of both species were detected through 19 September, albeit at progressively lower densities as individuals dispersed from the pond. Although we did not sample before 10 June, we estimate that peak egg laying likely occurred around 15 May for treefrogs and 5 May for toads (Fig. 1a).

We observed a high and persistent frequency of severe limb malformations in metamorphosing toads from Duck Pond.



**Fig. 2.** Representative malformations observed in metamorphosing American toads (*Bufo americanus*) from experimental exposures (Panels a–b) and from Duck Pond, Minnesota (Panels c–f). (a) Bony triangle in the left hind limb with duplicated foot (anterior mirror image duplication); (b) bony triangle and skin webbing in left hind limb; (c) severe (= complete) skin webbings in both hind limbs; (d) missing right forelimb; (e) double bony triangles in both hind limbs, leading to severe truncation; (f) close-up of bony triangle in left hind limb with ectrodactyly.

Of the 618 individuals inspected, 368 (60%) exhibited one or more malformations. The frequency of malformations in metamorphic toads ranged from a maximum of 69% in July to a minimum of 48% in late August. Malformations in toads were dominated by bony triangles (34%), skin webbings (31%), and missing limbs or limb elements (14%) (Fig. 2; Table 1). In several cases, skin webbings completely inhibited the movement of both hind limbs; other animals exhibited multiple bony triangles within a single limb, such that the overall limb was severely truncated and in close proximity to the body (Fig. 2e). While 90% of abnormalities affected the hind limbs, we also observed extra and missing forelimbs (Table 1). In stark contrast to the malformation pattern in American toads, only 8 of the 251 gray treefrogs examined exhibited morphological abnormalities (3.2%), which included missing eyes, missing digits and micromelia (Table 1).

*Ribeiroia* infection abundance was substantially higher among toads than among treefrogs [ANOVA,  $F_{(1,269)} = 1189.29$ ,  $P < 0.0001$ ]. Infection prevalence in toads was 100%, and the average number of metacercariae per individual ( $\pm 1$  SE) was  $28.8 \pm 1.5$  (range: 3 to 164,  $n = 186$ ). The number of metacercariae

within necropsied toads followed a negative binomial distribution (Fig. 1b). While all toads were infected with *Ribeiroia*, malformed individuals supported significantly more metacercariae ( $\log_{10}$ -transformed) than did normal individuals [ANOVA,  $F_{(1,184)} = 9.41$ ,  $P = 0.002$ ]. It is worth noting, however, that the difference in mean parasite abundance between normal and deformed toads was slight,  $25.6 \pm 2.64$  ( $n = 84$ ) vs.  $31.7 \pm 1.89$  ( $n = 101$ ), respectively, and the effect size of this relationship was weak (partial eta squared = 0.049), indicating that a large sample size is needed to detect a significant difference (minimum of 31 individuals per group). Among necropsied treefrogs, *Ribeiroia* infection prevalence was only 14.3%, and metacercarial abundance averaged  $0.33 \pm 0.18$  ( $n = 84$ ). The maximum number of *Ribeiroia* metacercariae recorded in a single treefrog was 15.

Despite the strong bias of *Ribeiroia* infection in favor of toads, echinostome infections were more common in treefrogs. On average, we found  $19.0 \pm 2.11$  and  $4.8 \pm 0.65$  echinostome metacercariae in the kidneys of treefrogs and toads, respectively [ANOVA,  $F_{(1,269)} = 90.75$ ,  $P < 0.0001$ ]. Echinostome prevalence was 91.7% in *H. versicolor* ( $n = 84$ ) and 75.8% in *B. americanus*

**Table 1.** Composition of malformations in *Hyla versicolor* and *Bufo americanus*. Presented are the numbers (and relative percentages) of each abnormality type in animals from the field (Duck Pond, Minnesota) and experimental exposures to *Ribeiroia* infection (pooled among exposure treatments). Malformation terminology and classification follows Johnson *et al.* (2001b). Because each animal can exhibit >1 abnormality, the total number of abnormalities can exceed the number of abnormal individuals.

Abnormality type	<i>Hyla versicolor</i> Duck Pond 2004	<i>Hyla versicolor</i> Experimental	<i>Bufo americanus</i> Duck Pond 2004	<i>Bufo americanus</i> Experimental
<i>Forelimb</i>				
Ectrodactyly (missing digit)	0 (0.0)	0 (0.0)	5 (0.84)	0 (0.0)
Polydactyly (extra digit)	0 (0.0)	0 (0.0)	1 (0.17)	0 (0.0)
Syndactyly (fused digits)	0 (0.0)	0 (0.0)	2 (0.34)	0 (0.0)
Hemi- and Ectromelia (missing limb elements)	0 (0.0)	0 (0.0)	36 (6.06)	0 (0.0)
Polymelia and Polydactyly (extra limb elements)	0 (0.0)	0 (0.0)	14 (2.36)	0 (0.0)
Cutaneous fusion (skin webbings)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Taumelia (bony triangle)	0 (0.0)	0 (0.0)	1 (0.17)	0 (0.0)
Micromelia (small limb)	0 (0.0)	0 (0.0)	1 (0.17)	0 (0.0)
<i>Hindlimb</i>				
Ectrodactyly (missing digit)	3 (42.86)	0 (0.0)	20 (3.37)	0 (0.0)
Polydactyly (extra digit)	0 (0.0)	0 (0.0)	21 (3.53)	1 (4.0)
Syndactyly (fused digits)	0 (0.0)	0 (0.0)	1 (0.17)	0 (0.0)
Hemi- and Ectromelia (missing limb elements)	1 (14.29)	0 (0.0)	26 (4.38)	0 (0.0)
Polymelia and Polydactyly (extra limb elements)	0 (0.0)	0 (0.0)	43 (7.24)	4 (16.0)
Femoral projection (digit-like outgrowth)	0 (0.0)	0 (0.0)	4 (0.67)	0 (0.0)
Cutaneous fusion (skin webbing)	0 (0.0)	0 (0.0)	185 (31.14)	11 (44.0)
Taumelia (bony triangle)	1 (14.29)	0 (0.0)	204 (34.34)	8 (32.0)
Micromelia (small limb)	2 (28.57)	0 (0.0)	13 (2.19)	1 (4.0)
Other malformed*	0 (0.0)	0 (0.0)	17 (2.87)	0 (0.0)
No. abnormal	8	0	368	14
No. inspected	251	82	618	36
No. abnormalities per abnormal animal	1.33 ± 0.21	0	1.68 ± 0.04	1.60 ± 0.13

\*Among metamorphosing *H. versicolor* from Duck Pond, we also recorded two instances of missing eyes, which are not presented in the table.

( $n = 186$ ). The range of infection was 0 to 110 (*H. versicolor*) and 0 to 87 (*B. americanus*). Malformed toads did not support a greater abundance of echinostome metacercariae than did normal toads [ANOVA,  $F_{(1,184)} = 0.15$ ,  $P = 0.70$ ].

#### LABORATORY EXPOSURES

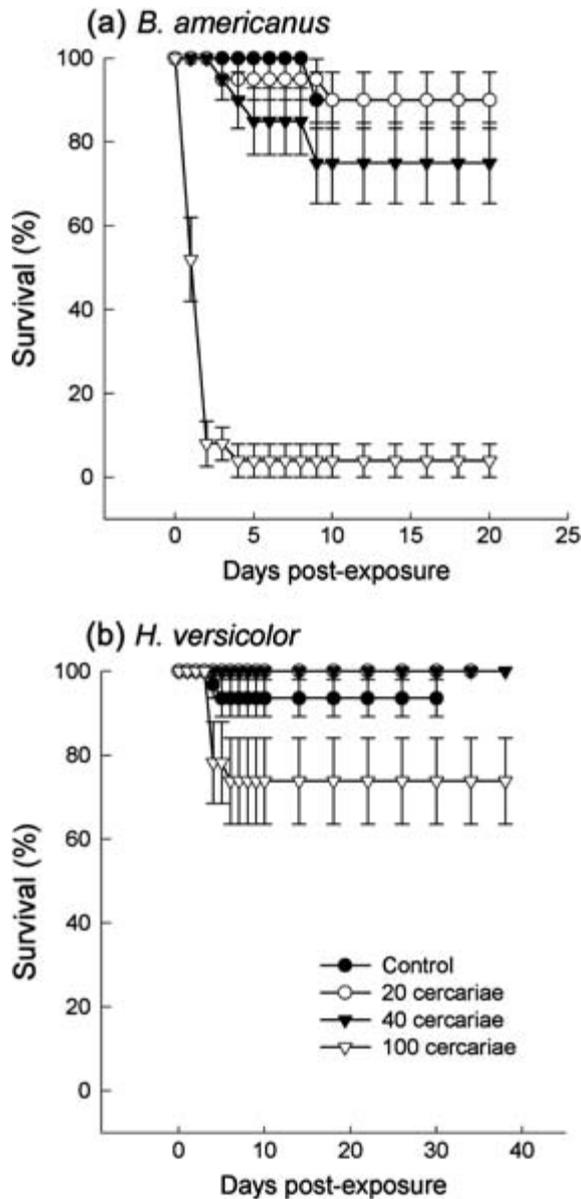
Parasite exposure significantly reduced survival to metamorphosis in both species (logistic regression,  $\chi^2 = 86.40$ ,  $R_N^2 = 0.59$ ,  $P < 0.0001$ ); however, there was a significant species-by-parasite effect, such that *Ribeiroia* had a more pronounced effect on exposed toad larvae than on treefrog larvae (Fig. 3). Of the toads exposed to 40 *Ribeiroia* over 10 days, only 75% survived to metamorphosis, relative to 90% survival in toads from the control treatment. In the 100 parasite exposure treatment, only 5% of larval toads survived, with the majority of animals dying after the first exposure to 25 cercariae (Fig. 3a). Treefrogs, in contrast, exhibited high survival (> 90%) in all but the highest exposure, in which 70% of animals nevertheless reached metamorphosis (Fig. 3b).

Experimental exposure to *Ribeiroia* also induced severe limb malformations in metamorphosing amphibians (logistic regression,  $\chi^2 = 37.29$ ,  $R_N^2 = 0.49$ ,  $P < 0.0001$ ). Intriguingly, however, malformations were observed only in toads. Overall, 38% of metamorphosing toads exposed to *Ribeiroia* developed one or more limb malformations, whereas all treefrogs

(regardless of parasite exposure level) and unexposed toads developed normally (Fig. 4a). Malformations among metamorphic toads were dominated by cutaneous fusion (44%) and bony triangles (32%) in the hind limbs, similar to those observed in the field (Table 1; Fig. 2). Bony triangles were almost always associated with severe truncation of the long bones and additional malformations in the feet. Within this limited sample, however, no extra hind limbs or abnormal forelimbs were observed.

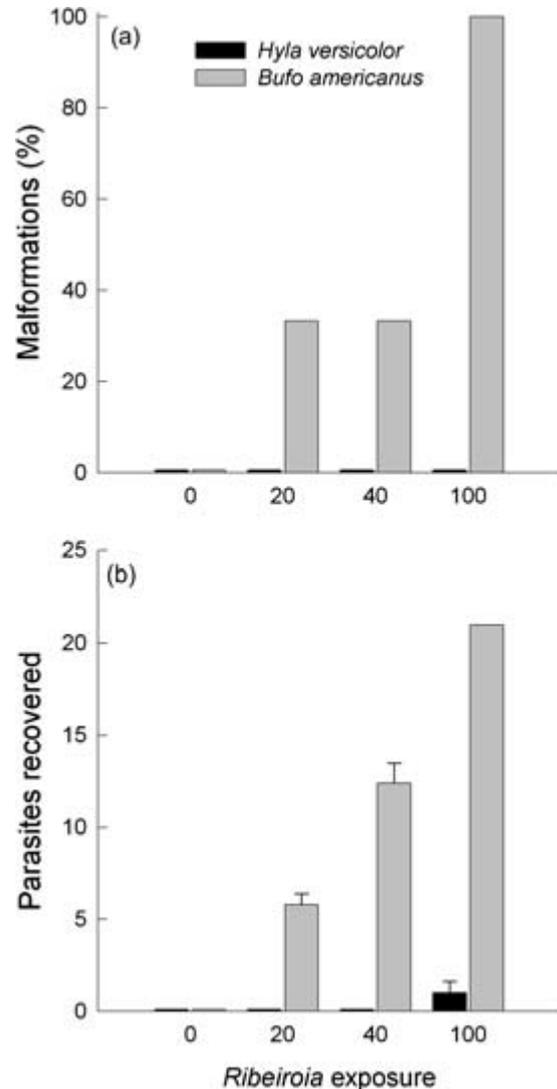
Correspondingly, we found a significant effect of species and parasite exposure level on the number of *Ribeiroia* metacercariae recovered at metamorphosis [ANOVA, exposure  $F_{(1,38)} = 13.916$ ,  $P = 0.001$ ; species  $F_{(1,38)} = 45.914$ ,  $P < 0.001$ ]. Among parasite-exposed toads, we recovered between 21% and 31% of administered parasites as metacercariae, and the number of parasites increased with exposure level. In treefrogs, however, < 1% of possible parasites were detected at metamorphosis (Fig. 4b). No metacercariae were found among *H. versicolor* exposed to 20 or 40 cercariae, while an average of  $1.0 \pm 0.63$  parasites were recovered from larvae exposed to 100 cercariae. No parasites were detected among either species in control treatments.

Finally, *Ribeiroia* exposure significantly reduced metamorphic toad size (SVL) and delayed metamorphosis [MANOVA, Wilks'  $\lambda = 2.44$ ,  $F_{(6,82)} = 82.0$ ,  $P = 0.032$ ]. For both response variables, larval toads exposed to 40 cercariae exhibited



**Fig. 3.** Survival ( $\pm 1$  SE) to metamorphosis of larval amphibians exposed to different dosages of *Ribeiroia* cercariae. (a) *Bufo americanus* (logistic regression for parasite exposure,  $B = 0.06$ ;  $R_N^2 = 0.64$ ;  $\chi^2 = 54.71$ ;  $P < 0.0001$ ; odds ratio = 1.06); (b) *Hyla versicolor* (logistic regression for parasite exposure,  $B = 0.028$ ;  $R_N^2 = 0.207$ ;  $\chi^2 = 8.88$ ;  $P = 0.003$ , odds ratio = 1.03). The percentage of surviving animals is presented daily during parasite exposures (through day 10), after which it is presented every other day (*B. americanus*) or every third day (*H. versicolor*) for clarity.

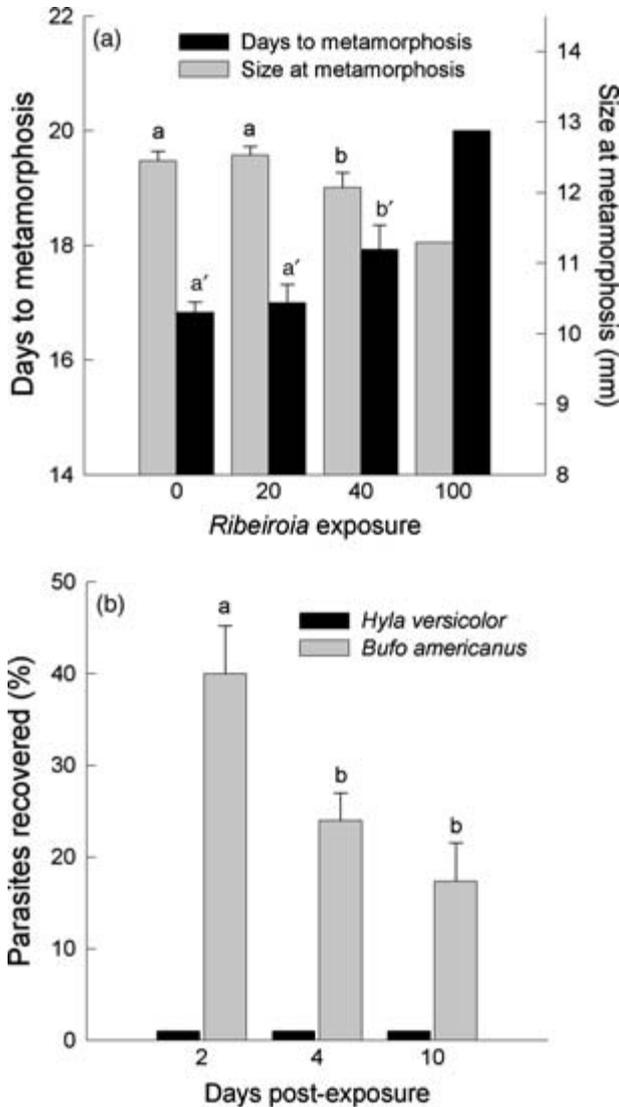
significantly lower values from those exposed to 0 or 20 cercariae, which were statistically equivalent (Fig. 5a). Too few toads ( $n = 1$ ) survived in the 100 cercariae treatment for inclusion in the analysis. Infection did not significantly affect toad mass at metamorphosis, nor did we find any effects of *Ribeiroia* exposure on treefrog size, mass, or time to metamorphosis [MANOVA, Wilks'  $\lambda = 0.88$ ,  $F_{(6,126)} = 1.378$ ,  $P = 0.228$ ].



**Fig. 4.** Malformation frequency and parasite recovery among experimentally infected amphibians. (a) Frequency ( $+ 95\%$  confidence limit) of malformations in *Bufo americanus* at metamorphosis as a function of *Ribeiroia* exposure (logistic regression for parasite exposure,  $B = 0.058$ ,  $\chi^2 = 8.902$ ,  $R_N^2 = 0.238$ ;  $P = 0.014$ ; odds ratio = 1.06). Only one toad survived in the '100' parasite exposure treatment. No abnormalities were observed among *H. versicolor*. (b) Number of *Ribeiroia* metacercariae ( $+ 1$  SE) recovered among metamorphosing *B. americanus* and *H. versicolor* as a function of cercarial exposure [ANOVA, exposure  $F_{(2,14)} = 428.60$ ,  $P < 0.0001$ ]. All groups are significantly different from one other ( $P < 0.0001$ ).

#### PARASITE COLONIZATION AND ESTABLISHMENT

We recovered an average ( $\pm 1$  SE) of  $25.3 \pm 1.7$  'leftover' parasites from containers with gray treefrog larvae (~16% of a possible 150 from each of three exposure trials). However, no remaining cercariae were found on filters following an 8 h exposure with American toad larvae. This included two filtration events, each involving 10 individual larva and 15 cercariae, for a total of 300 possible cercariae. Among control treatments, in which 15 cercariae were added to a container



**Fig. 5.** (a) Time-to- and size-at-metamorphosis of *Bufo americanus* as a function of *Ribeiroia* exposure level [MANOVA, parasite exposure on  $\log_{10}$ -size:  $F_{(2,43)} = 3.82$ ,  $P = 0.03$ ; on  $\log_{10}$ -days:  $F_{(2,43)} = 4.40$ ,  $P = 0.018$ ]. Parasite exposure had no effect on *H. versicolor* size or growth rate. (b) Recovery of *Ribeiroia* metacercariae (percentage + 1 SE) with time post-exposure in larval toads and treefrogs exposed to a single dose of 15 cercariae (at time 0) [ANOVA,  $F_{(2,39)} = 7.071$ ,  $P = 0.003$ ]. No metacercariae were found among any of the exposed treefrog larvae. Differences in letter groupings indicate a significant difference among treatments ( $P < 0.05$ ).

with only water, we recovered 41% of cercariae (61 of 150 possible). This suggests that some cercariae either passed through the filter or became firmly trapped on the filter (and were not rinsed off into the Petri dish). While the inability of this method to completely recover all (or most) of the administered parasites limits its absolute utility, two factors suggest that these results still offer useful information on the relative infection success of cercariae. First, fifteen additional control trials yielded a similar recovery rate [mean ( $\pm 1$  SE) = 56%  $\pm$  (5.9)], suggesting we can apply a correction factor to

the observed patterns to obtain a relative success rate. And second, if anything, this method underestimates the fraction of parasites failing to enter the host, especially for treefrogs. Thus, assuming the filter was consistent in recovering approximately 40% of the total leftover cercariae for each host, our results suggest that an estimated 60% and 100% of cercariae successfully entered gray treefrogs and American toads, respectively. These results are corroborated by microscopy observations. During parasite exposures, we rarely observed leftover cercariae in containers with toads, whereas we frequently noted cercariae with gray treefrog larvae.

In our final study, which examined parasite establishment and persistence in treefrogs and toads at 2, 4 and 10 days post-exposure (PE), we found significant effects of species, time, and time-by-species on the number of parasites recovered [ANOVA, corrected model  $F_{(5,79)} = 47.127$ ,  $P < 0.0001$ ]. No metacercariae were recovered from any of the 40 exposed treefrogs, regardless of days PE, suggesting that the invading parasites failed to establish. In contrast, 35 of 40 exposed toads supported at least one *Ribeiroia* metacercariae. The average abundance of parasites within toads declined significantly with time PE (Fig. 5b).

## Discussion

Differential patterns of parasitic infection among host species and the resulting variation in disease can sharply influence not only the fitness of hosts but their ecological interactions within a community (e.g. Hudson *et al.* 2002; Tompkins, White & Boots 2003; Wood *et al.* 2007). Understanding the mechanisms that drive differential patterns of disease among sympatric host species remains a significant challenge for most host-pathogen systems. Ultimately, these patterns are the endproduct of a series of ecological 'filters', including the host encounter filter and the host-parasite compatibility filter (Combes 2001). Whether host and parasite encounter one another can be influenced by geographical ranges, habitat associations, and behavioural patterns. Compatibility, or whether a parasite can establish and persist within a host, is a function of both host defenses against a parasite (resistance) and the resources required by the parasite (specificity). Parasite-induced malformations in amphibians offer an ideal model system in which to evaluate the relative importance of ecological filters for parasitism because (i) the disease (malformations) is distinctive and readily observed, (ii) parasite infection can be reliably quantified within hosts, and (iii) host-parasite interactions can be manipulated experimentally.

Our field results clearly illustrate the remarkable differences in malformation frequency between American toads and gray treefrogs, even within a single wetland. On average, metamorphic toads exhibited 20 $\times$  the frequency of abnormalities recorded in gray treefrogs. More than half of the emerging toads sampled suffered one or more severe malformations, compared with 3% of sampled treefrogs. This malformation pattern can be explained by differences in *Ribeiroia* infection between the two species: on average, metamorphic toads supported 75 $\times$  more metacercariae than did metamorphosing treefrogs.

Results of the parasite exposure experiments indicated that *Ribeiroia* infection can explain both the frequency and composition of malformations observed in toads at Duck Pond. In both laboratory and field-caught toads, the dominant malformations involved skin webbings and bony triangles, consistent with previous experimental work on *Ribeiroia* infection in toads (Johnson *et al.* 2001a). Although no forelimb malformations were observed among experimentally exposed toads, the overall sample size was limited ( $n = 34$  surviving toads in the experiment), and we suggest that the majority of malformations observed in toads from Duck Pond are consistent with a *Ribeiroia*-induced etiology (see also Johnson *et al.* 2001a, in press).

The low frequency (~3%) of abnormalities in gray treefrogs from Duck Pond is consistent with their low levels of *Ribeiroia* infection. What is more challenging to explain, however, is why metamorphosing gray treefrogs exhibited such low infection levels relative to co-occurring toads. Both gray treefrogs and American toads develop as larvae during a similar time period (Oldfield & Moriarty 1994, this study), which broadly corresponded with high levels of *Ribeiroia* infection in snail hosts (Fig. 1a). Because larval treefrogs are larger than toads and require more time to achieve metamorphosis (Cline 2005; Green 2005), treefrogs should support more rather than fewer parasites. Indeed, this is the precise infection pattern observed for a second group of trematodes, the echinostomes, which were four times more abundant in treefrogs than in toads. *Ribeiroia*, on the other hand, was 75× more abundant in toads. These results suggest that the observed difference in *Ribeiroia* abundance between the two species owes to differences in host-parasite compatibility; treefrogs are either less susceptible to infection, such that cercariae are less successful at colonizing and/or establishing in treefrog larvae, or they are extremely sensitive to infection, such that most animals die rapidly following exposure, leading to a truncated parasite distribution among hosts.

Results of the experimental exposures allow us to differentiate among these alternative hypotheses. While toads exposed to *Ribeiroia* exhibited high mortality and malformations in a dose-dependent manner, treefrogs demonstrated a near-complete absence of pathology. Few parasite-exposed treefrogs died, and none developed malformations or exhibited a reduction in size or developmental rate. Necropsies of metamorphic treefrogs revealed that few of the exposed animals supported *Ribeiroia* metacercariae. Thus, the low frequency of malformations among *H. versicolor* from Duck Pond and in our experiment is explained by the poor ability of parasites to infect treefrogs and establish successfully, rather than to a temporal mismatch between host and parasite or to rapid mortality following infection.

Our experiments focused on the fate of parasites within hosts provide insight into the likely mechanism behind the low infection success of *Ribeiroia* in treefrogs. While most cercariae successfully penetrated and encysted within larval toads, between 16% and 40% of cercariae administered to treefrog larvae failed to encyst, highlighting a reduction in colonization success (although the low parasite recovery in control trials preclude more precise estimates). Results of the

time-sequence necropsies further underscore the differences in infection susceptibility between host species. In toads, we recovered 40% of *Ribeiroia* cercariae as metacercariae 2 days post-exposure; this proportion declined with time, reflecting either the effects of host defenses on encysted parasites or natural mortality of parasites within the host. In treefrogs, however, no parasites were detected among exposed animals, regardless of time-since-exposure. Collectively, these results suggest that the differences in infection and malformations observed between treefrogs and toads owes to the reduced ability of *Ribeiroia* to both invade and especially establish within larval treefrogs. The speed with which invading parasites were eliminated from treefrogs (< 48 h) suggests that elements of innate immunity prevented metacercarial establishment. Invading cercariae generally require 12 to 24 h to construct a protective cyst wall (Smyth & Halton 1983), and elimination of parasites before establishment of this barrier may explain why exposure did not affect host limb development. We consider it unlikely that *Ribeiroia* is more host-specific to toads rather than to treefrogs, considering the low overall specificity of this parasite in selecting second intermediate hosts, including 34 species of fishes and amphibians (Johnson *et al.* 2004).

The low susceptibility of gray treefrogs to *Ribeiroia* infection is unique among the amphibian species examined thus far in the laboratory or in the field, which include more than 20 species of frogs, toads, and salamanders (Johnson *et al.* 2004). Moreover, the Pacific chorus frog (*Pseudacris regilla* Baird and Girard), a close relative of *H. versicolor* (Faivovich *et al.* 2005), is highly susceptible to *Ribeiroia* infection and abnormal limb development (Johnson *et al.* 1999, 2001b, 2002). The underlying mechanism for this elevated resistance remains an open question, but may involve differences in innate immunity. While amphibians possess the same elements of the innate and acquired immune system as other vertebrates (e.g. Carey, Cohen & Rollins-Smith 1999), less is known about their defenses against macroparasites (Tinsley & Jackson 2002), particularly during larval development. Huizinga & Nadakavukaren (1997) found that, following repeated exposures to cercariae of *Ribeiroia marini* Faust, goldfish produced a 'cercaricidal substance' that eliminated 90% of metacercariae within 4–7 days (see also Basch & Sturrock 1969; Simmons 1971). The gray treefrog could produce similar compounds in defence against *Ribeiroia*. Intriguingly, however, treefrogs did not exhibit the same resistance against a second group of trematodes, the echinostomes, which were more abundant in the kidneys of treefrogs than those of toads from Duck Pond. Similarly, other studies have reported a broad variety of larval and adult helminthes from gray treefrogs (Hausfater, Gerhardt & Klump 1990; Belden & Kiesecker 2005; Koprivnikar *et al.* 2006). This suggests that the resistant properties of *H. versicolor* are either uniquely effective against *Ribeiroia* or are localized to its region of encystment (cutaneous and subcutaneous tissue). Subsequent experiments that combine ecological and immunological elements are needed to investigate the mechanisms underpinning the enhanced resistance of *H. versicolor* to *Ribeiroia* infection (e.g. Belden & Kiesecker 2005).

Finally, we suggest that the differential patterns of disease observed here could have important implications for understanding amphibian community structure and parasite transmission. At our field site, few if any of the > 300 deformed toads observed will survive to sexual maturity, and malformed individuals represent only the surviving fraction of exposed toads; the number of larvae that die before metamorphosis is likely to eclipse the proportion that becomes malformed (see Johnson *et al.* 1999, Schotthoefer *et al.* 2003). Indeed, within this experiment, a single dose of 25 cercariae caused ~90% mortality in larval toads within 48 h. On average, an infected snail releases 300 cercariae per day, and some snails produced > 2000 cercariae – enough to kill 70 larval toads within this experiment. Under field conditions, with multiple parasites, food limitation, competitors and predators, the direct and indirect effects of *Ribeiroia* on population viability could be substantial. It is worth noting that in 2005, following several years of high malformations, toads failed to breed at Duck Pond.

Patterns of differential infection and pathology between hosts could also have important ramifications for understanding interactions among amphibian species and the dynamics of ecological communities. For example, the heightened sensitivity of American toads to *Ribeiroia* could alter the outcome of competition with the less-sensitive gray treefrogs. These groups occur sympatrically over much of their ranges and compete in experimental studies (e.g. Wilbur & Alford 1985; Johnson *et al.* in press), suggesting that differential patterns of parasitism could indirectly affect amphibian community composition, the resultant patterns of algal grazing, or even the relative abundance of amphibian predators (e.g. Tompkins *et al.* 2003; Wood *et al.* 2007). Additionally, patterns of differential infection vulnerability could feedback to affect parasite transmission. The abundance and diversity of competent and incompetent hosts is likely to have important effects on the percentage of cercariae successful in forming viable metacercariae available for transmission to definitive (= bird) hosts (e.g. 'dilution effect' Keesing, Holt & Ostfeld 2006). Thus, if *H. versicolor* reduce the local abundance of *Ribeiroia* as a consequence of their enhanced resistance, gray treefrogs could indirectly 'protect' more sensitive amphibian species from infection (see Johnson *et al.* in press). By interrupting transmission between second intermediate (amphibian) and definitive (bird) hosts, gray treefrogs could also reduce the long-term abundance of *Ribeiroia* within an ecosystem, further benefiting more sensitive amphibian species. These results underscore the importance of understanding differential patterns of disease in a host community. As the ubiquitous role of parasites in ecological communities becomes increasingly apparent, ecologists are challenged to broadly incorporate parasites into community ecology and identify the mechanisms through which infection alters ecosystem properties.

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