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RESEARCH ARTICLE

Experimental infection dynamics: using immunosuppression and *in vivo* parasite tracking to understand host resistance in an amphibian–trematode system

Bryan E. LaFonte and Pieter T. J. Johnson*

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

*Author for correspondence (pieter.johnson@colorado.edu)

SUMMARY

Although naturally occurring hosts often exhibit pronounced differences in infection and pathology, the relative importance of factors associated with host life history and immunity in explaining such patterns often remains speculative. Research in eco-immunology highlights the trade-offs between host physiology and immunity, for which natural variations in disease susceptibility offer a valuable platform to test predictions within this framework. Here, we combined use of a novel, *in vivo* assay for tracking parasite fate and an experimental manipulation of host immune function (*via* chronic corticosterone exposure) to assess the role of host immunity in regulating susceptibility of amphibian hosts to three larval trematodes: *Ribeiroia ondatrae*, *Echinostoma trivolvis* and *Alaria* sp. 2. Results from the *in vivo* parasite-tracking assay revealed marked differences in initial parasite penetration and subsequent host clearance. Relative to infections in a highly susceptible species (*Pseudacris regilla*), the virulent trematode *R. ondatrae* was ~25% less successful at penetrating larvae of three hylid frog species and was cleared >45× faster, such that all parasites were rapidly cleared from hylid hosts over 72 h following a Weibull distribution. Immune suppression of *Hyla versicolor* sharply reduced this resistance and increased infection of all three trematodes by 67 to 190%, with particularly strong increases for *R. ondatrae*. Diminished resistance correlated with a 62% decrease in circulating eosinophils. Correspondingly, 10 days after corticosterone exposures ended, infections declined dramatically while eosinophil levels returned to normal. In light of ongoing declines and deformities in amphibian populations, these findings have application potential for mitigating disease-driven effects.

Key words: amphibian declines, disease ecology, disease susceptibility, eco-immunology, host–parasite interaction, immune suppression, wildlife disease.

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INTRODUCTION

Ongoing efforts in the fields of eco-immunology and disease ecology have helped advance our understanding of what drives variation in disease susceptibility (Hawley and Altizer, 2011; Martin et al., 2011), which often varies markedly among host individuals, populations and species (Johnson et al., 2012). The rapidly growing discipline of eco-immunology has highlighted the importance of trade-offs between immune function and competing physiological functions, such as reproduction and growth (Hamilton and Zuk, 1982; Sheldon and Verhulst, 1996; Hamilton and Poulin, 1997; Martin et al., 2011). For example, species of birds that invest in rapid growth and early reproduction (i.e. 'fast' pace of life) tend to invest less in immune function than species with a slower pace of life (Lee et al., 2008; but see Martin et al., 2007). Understanding variation in disease susceptibility in non-model systems has become a growing priority (Pedersen and Babayan, 2011), particularly in systems where infectious disease represents a conservation threat. For instance, a recent study of cellular and humoral immune activity in endangered *Zalophus wollebaeki* (Galapagos sea lions) from two colonies revealed that animals in a human-impacted colony suffered greater immune-stimulatory pressure, which could increase the likelihood of disease emergence (Brock et al., 2013).

Continued advancement of eco-immunological research requires enhanced integration of field and experimental investigations. Field surveys are ideally suited for initially identifying patterns of variation in disease susceptibility (Altizer et al., 2001; Farmer et al., 2005; Johnson and Hartson, 2009); however, it can be difficult to interpret such patterns as they represent the product of host exposure, parasite infection success and subsequent persistence – each of which can be influenced by host defences as well as parasite adaptation (Combes, 2001; Davis et al., 2008). Controlled experiments enable investigators to manipulate pathogen exposures and host immunity, often allowing more precise mechanisms to be revealed, particularly when such experiments are guided by prior field patterns. For instance, Altizer et al. (Altizer et al., 2001) found variations in the susceptibility of different populations of monarch butterflies to the protozoan parasite *Ophryocystis elektroscirrha* using field surveys. Then, a subsequent study by Lefèvre et al. (Lefèvre et al., 2011) employed an experimental approach to show how host populations varied in their resistance but not tolerance to this pathogen.

Amphibian hosts provide a valuable and timely opportunity to study interspecific variation in disease susceptibility. As a group, amphibians are declining at an alarming rate (Houlahan et al., 2000; Stuart et al., 2004), and infectious diseases are believed to be an important contributor to this pattern (Daszak et al., 2003; Blaustein et al., 2012). Amphibians host a broad range of disease-causing agents; ranaviruses (e.g. frog virus 3 and *Ambystoma tigrinum* virus), the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al., 1999) and the malformation-causing trematode *Ribeiroia*

ondatrae (Price, 1931) are three well-studied amphibian pathogens. Amphibian immunity can be broken down into the innate, generally non-specific components such as macrophages, neutrophils, basophils, eosinophils, natural-killer cells, complement proteins, lysozymes and anti-microbial peptides, as well as the more specific adaptive immune system, which is composed of B- and T-lymphocytes (reviewed by Rollins-Smith and Woodhams, 2012). Although the amphibian immune system features most of the same components found in other vertebrate taxa, it is important to emphasize that immunity in larval amphibians remains incompletely developed such that the composition of various immune components varies throughout development (e.g. eosinophils and monocytes peak near metamorphic climax) (Davis, 2009; Das and Mahapatra, 2012). To better understand the role of host defenses in explaining patterns of disease, researchers have increasingly examined how the immune system may influence variation in host susceptibility to *Bd* (Rollins-Smith et al., 2002; Woodhams et al., 2007; Richmond et al., 2009), trematodes (Kiesecker, 2002; Belden and Kiesecker, 2005) and ranaviruses (Forson and Storfer, 2006; Hoverman et al., 2010). For example, amphibian susceptibility to *Bd*, which varies from highly susceptible (e.g. *Litoria caerulea*) to highly resistant species (e.g. *Limnodynastes tasmaniensis*), correlates strongly with *in vitro* effectiveness of anti-microbial peptide skin extracts from each host species (Woodhams et al., 2007).

Considerably less is known about the role of host immunity in regulating amphibian–trematode interactions, despite the well-documented effects of several trematodes on host morphology and survival. Infection with *R. ondatrae*, for instance, causes elevated mortality and an increased incidence of severe limb malformations in a range of amphibian hosts (Johnson et al., 1999; Johnson et al., 2002; Johnson et al., 2012; Schotthoefer et al., 2003a). Similarly, exposure of early-stage tadpoles to echinostomes can cause renal dysfunction, leading to edema or mortality (Schotthoefer et al., 2003b; Holland et al., 2007; Johnson and McKenzie, 2008). In natural systems, host species exhibit substantial variation in both infection and pathology associated with these trematodes. At least some of this variation can be explained by differences in host life history: larger-bodied hosts appear to be better able to tolerate infections, while amphibians with longer development periods have a more extended opportunity to clear infections (although this can be offset by increased exposure during aquatic development) (Johnson et al., 2012). However, life-history-associated variables such as body size and lifespan are often only indirect proxies of resource allocation (Stearns, 1989), and we still know comparatively little about the immunological mechanisms driving variation in susceptibility. Some species (e.g. *Hyla versicolor*) show remarkable resistance to invading *R. ondatrae* parasites, despite having a relatively small body size and short life span (Johnson and Hartson, 2009; Johnson et al., 2012). These observations underscore the importance of moving beyond phenomenological approaches in favor of more mechanistic studies that explore host immune function.

In the present study, we sought to characterize the exposure–infection–clearance dynamics of *R. ondatrae* in detail by pairing controlled laboratory exposures with a novel, *in vivo* method of tracking infection through time. We did this by exposing larvae of four frog species to fluorescently labeled parasites and monitoring infections over 72 h. In order to identify the role of host immunity in controlling parasite persistence, we tested the extent to which a broad-spectrum immune suppressant (exogenous corticosterone) altered the susceptibility of an otherwise highly resistant host to infection with *R. ondatrae*. We compared these results with effects of chronic corticosterone exposure on infections with two other common trematodes, *Echinostoma trivolvis* (Cort, 1914) and *Alaria* sp. 2 (Locke et al., 2011), and over time (4 and 14 days after parasite exposure). Finally, to better understand the mechanisms underlying observed changes in susceptibility, we compared leukocyte profiles among treatment groups to identify which, if any, leukocytes contribute to the immune response against these larval trematodes. Given the wide range of hosts that are susceptible to *Ribeiroia* infection, our results help begin to explain the mechanisms underlying the infection process within highly resistant hosts and further our understanding of resistance in general.

MATERIALS AND METHODS

Study system

The digenetic trematodes *R. ondatrae*, *E. trivolvis* and *Alaria* sp. 2 (hereafter: *Ribeiroia*, *Echinostoma* and *Alaria*, respectively) utilize amphibians as second intermediate hosts and frequently co-occur in freshwater ecosystems (Johnson and Hoverman, 2012). When the free-swimming cercarial stages of these parasites encounter an amphibian host, they lose their tails and form encysted metacercariae (*Ribeiroia* and *Echinostoma*) or unencysted mesocercariae (*Alaria*). *Ribeiroia* encysts subcutaneously, typically near the hind-limb buds or the oral disc (Johnson et al., 1999; Johnson et al., 2004), while *Echinostoma* enters through the cloaca and encysts in the nephric tissues of its amphibian host (Beaver, 1937; Fried et al., 1997; Thiemann and Wassersug, 2000). *Alaria* mesocercariae can be found throughout the host and exhibit little tissue specificity (Fried, 1997; Johnson et al., 1999). Each of these parasites is trophically transmitted, such that they develop into a reproductive adult following consumption of the infected amphibian by the parasites' appropriate definitive hosts (birds or mammals).

Experimental exposures

Amphibian egg masses were collected from field sites in University Park, Pennsylvania [*Hyla versicolor* (LeConte, 1825)], and Lake Penhollow, Oregon [*Pseudacris regilla* (Baird and Girard, 1852)]. Individuals of a single species were combined to minimize any clutch-specific effects. We raised hatching larvae in 20 l containers with ~50 individuals per container until they reached Gosner stage 27 (Gosner, 1960). *Hyla versicolor* is known to be resistant to *Ribeiroia* infection (Johnson and Hartson, 2009; Johnson et al., 2012). However, past studies have established that *P. regilla* is highly susceptible to *Ribeiroia* infection and a large proportion of the parasites will persist through metamorphosis (Johnson et al., 1999; Johnson et al., 2012; Johnson and Buller, 2011; Paull et al., 2012). We therefore included *P. regilla* as an 'outgroup' species for comparison.

We also collected early-stage larvae of two additional hylid species, *Hyla cinerea* (Schneider, 1799) and *Hyla gratiosa* (LeConte, 1856), from separate ephemeral ponds near Tampa, Florida. Because we were unable to obtain these species as eggs, we performed preliminary dissections to verify the absence of any trematode infections. Of the 13 species of frogs, toads and salamanders that have been experimentally challenged with *Ribeiroia*, only the two species of the gray treefrog complex have shown strong resistance to *Ribeiroia* (Johnson and Hartson, 2009; Johnson et al., 2012). We chose to include *H. cinerea* and *H. gratiosa* to determine whether the gray treefrog complex is unique or whether their congeners also exhibit strong resistance. These two species are native to the southeastern United States and there are no published accounts of either species being naturally or experimentally infected with *Ribeiroia*. During laboratory experiments, we maintained amphibian larvae in individual plastic containers (1.0 l TakeAlongs Squares,

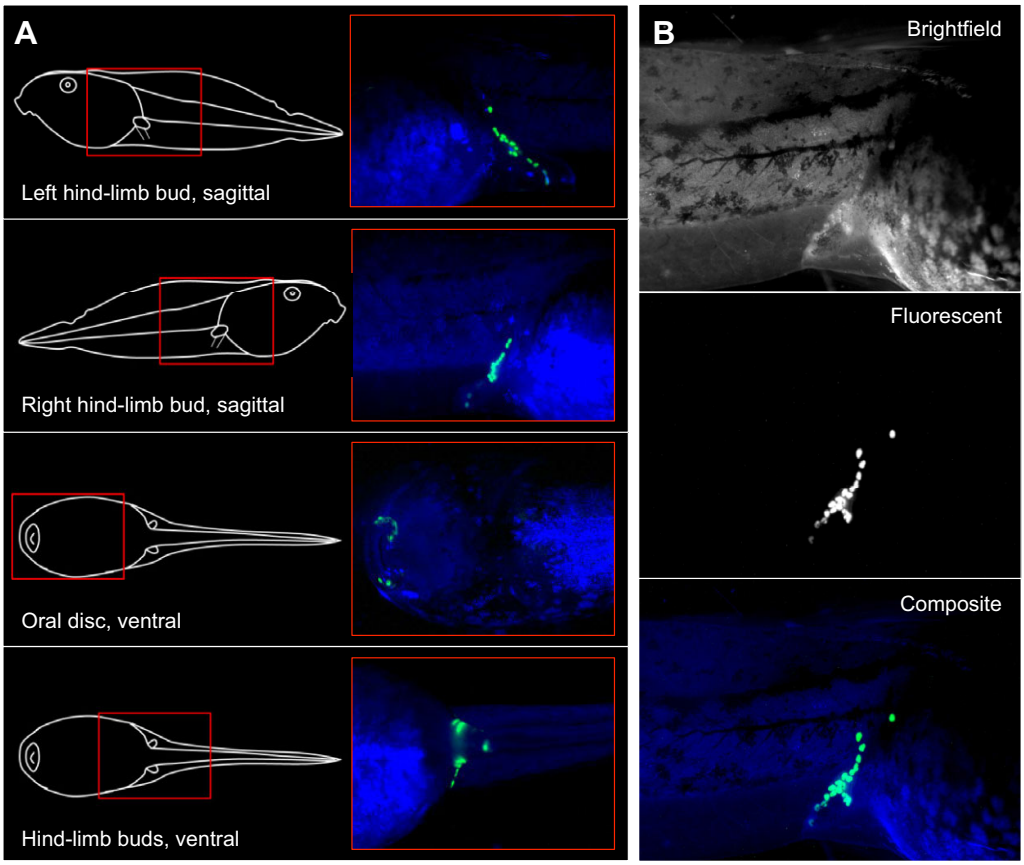

Fig. 1. (A) Illustrations of tadpoles detailing the primary locations of infection (red boxes) with corresponding example images of larval amphibians infected with fluorescently labeled parasites. Top to bottom: sagittal view of the left hind-limb bud, sagittal view of the right hind-limb bud, ventral view of the oral disc/gill region and ventral view of the hind-limb buds. (B) Example of how raw images are layered together to visualize infection. Top to bottom: an unfiltered brightfield image, a GFP2-filtered fluorescent image and the resulting composite image (parasite tissue appears green and host tissue appears blue).

Rubbermaid, Atlanta, GA, USA) and fed tadpoles an *ad libitum* diet consisting of equal parts ground TetraMin (Tetra, Melle, Germany) and ground *Spirulina*.

We obtained parasite cercariae from naturally infected *Helisoma trivolvis* (Say, 1817) collected from field sites in the San Francisco Bay Area, California. We identified *Ribeiroia*, *Echinostoma* and *Alaria* based on morphological features of the cercariae (Schell, 1985; Johnson et al., 2004). Previous molecular work from this region has helped validate these identifications (Johnson and Hoverman, 2012). To obtain cercariae for experimental exposures, we isolated infected snails in centrifuge tubes with 40 ml of treated tap water and harvested parasites within 5 h of emergence to ensure we only used highly active cercariae. Using a Pasteur pipet under a stereomicroscope, we separated cercariae into groups of 30 for use in host exposures.

Fluorescent parasite clearance assay

To finely characterize dynamics of *Ribeiroia* infection and clearance within four host species, including the refractory *H. versicolor*, the susceptible *P. regilla* and two previously untested hylid species (*H. cinerea* and *H. gratiosa*), we employed a novel parasite clearance assay in which we exposed hosts to 30 fluorescently labeled cercariae and used fluorescent microscopy to monitor parasite persistence over 72 h. Work by Keeney et al. (Keeney et al., 2008) and our own preliminary trials have shown that fluorescent labeling does not affect cercarial longevity; however, it is worth noting that the effects of fluorescent labeling are not yet fully understood. Nevertheless, potential side effects of fluorescent labeling were not a major concern for the clearance assay given that all of the parasites were labeled. The *Ribeiroia* cercariae used in this experiment were fluorescently labeled prior to host exposure using a fatty acid analog probe (BODIPY FL C_{12}, Invitrogen, Carlsbad, CA, USA) following Keeney et al. (Keeney et al., 2008). From the purchased dye, a 100 mmol l^{-1} working stock solution was created by dissolving the dye in dimethyl sulfoxide (D8418, Sigma-Aldrich, St Louis, MO, USA). This stock solution was added to centrifuge tubes containing ~1000 free-swimming cercariae (aggregated from multiple infected snails) in 40 ml of carbon-filtered, UV-sterilized water to achieve a 100 nmol l^{-1} concentration. Parasites were incubated in the dye solution for 45 min prior to being administered into the individual 1.0 l tadpole containers at the appropriate dosage (30 cercariae per host).

We quantified *Ribeiroia* infection intensities within living hosts using a fluorescent stereomicroscope equipped with a green fluorescent protein 2 (GFP2) filter (Leica MZ FLIII, Leica Microsystems, Wetzlar, Hesse, Germany) and a CCD digital camera (MicroPublisher 3.3 RTV, QImaging, Surrey, BC, Canada). We conducted each observation by imaging the sagittal view of the right hind-limb bud region, the sagittal view of the left hind-limb bud region, the ventral view of the hind-limb buds and the ventral view of the oral disc/gill region (Fig. 1A). These views were chosen to encompass the primary sites of *Ribeiroia* infection; however, we also visually examined hosts and imaged additional regions as necessary. For each region, we created both a brightfield, unfiltered image and a fluorescent, GFP2-filtered image using image acquisition software (QCapture 3.1.2, QImaging) and subsequently created a composite of these images using ImageJ 1.44p (National Institutes of Health, Bethesda, MD, USA) (Fig. 1B). Prior to each observation, tadpoles were temporarily anesthetized by immersion in a 0.125% (m/v) solution of neutral buffered MS-222 (Western

Chemicals, Ferndale, WA, USA) for 15–20 s. Anesthetized tadpoles were placed in a Petri dish containing a small amount of water, which enabled us to gently position them for imaging. Anesthesia typically lasted between 10 and 15 min before normal motor function resumed. We quantified *Ribeiroia* infections at 1 h post-exposure (PE), 3 h PE, 6 h PE and every 6 h thereafter until all parasites had been cleared with the exception of *P. regilla*, for which infections were quantified at 1 h PE, 36 h PE and 72 h PE. After the final observation time, tadpoles were euthanized and dissected (as described below) to confirm the accuracy of *in vivo* observations.

Immune suppression experiment

To understand of the role of host immunity in affecting the outcome of parasite exposure and subsequent clearance patterns, we manipulated parasite exposure and immune competence in the highly resistant species, *H. versicolor*, using exposure exogenous corticosterone, a broad-spectrum immunosuppressant. This design enabled us to tease apart the relative roles of host immunity and pathogen exposure when interpreting differential leukocyte counts (Davis et al., 2008). We chose to employ chronic corticosterone exposure to achieve broad immune suppression because it has been shown to be effective in suppressing immune function of larval *H. versicolor* (Belden and Kiesecker, 2005). We isolated tadpoles into individual containers filled with 500 ml of treated tap water and randomly assigned them to a treatment group within a 2×2×3 factorial design involving parasite exposure (exposed or unexposed), sampling time (4 or 14 days post-exposure) and immune treatment (control, vehicle and corticosterone). For these immune treatments tadpoles were maintained in water only (control), 0.02% ethanol in water (vehicle), or 4.0×10^{-5}% corticosterone (catalog no. 27840, Sigma-Aldrich) + 0.02% ethanol in water (corticosterone). We chose these concentrations to maintain consistency with the methods of Belden and Kiesecker (Belden and Kiesecker, 2005). Each immune treatment was replicated 21 times with parasite exposure and five times without parasite exposure at two sampling times for a total of 156 experimental units. We performed water changes and re-applied immune treatments every other day.

After 14 days, we exposed hosts in the parasite exposure treatment to 30 cercariae of each of three different trematode species commonly found in wetland ecosystems: *Ribeiroia*, *Echinostoma* and *Alaria* (Johnson and Buller, 2011; Johnson and Hoverman, 2012). In accordance with the natural emergence times of these parasites, hosts were always exposed first to *Echinostoma*, then to *Alaria*, and lastly to *Ribeiroia*. All three parasite species were added to the host containers within 24 h of each other. At 4 days PE, we randomly selected half of the tadpoles from each treatment to be euthanized and necropsied for parasite quantification. Concurrently, to study the effects of immune recovery, we discontinued corticosterone exposures 4 days PE and hosts from all treatments were maintained in water only for the remainder of the experiment. After 14 days PE, all remaining hosts were euthanized to quantify infections. We prepared blood smears from a random subset of five individuals per treatment group at both time points (4 and 14 days PE) by making a sagittal incision through the heart and using a heparinized glass capillary tube to collect as much blood as possible. We transferred blood from the capillary to a clean glass microscope slide and spread it out using a clean glass coverslip. Once air-dried, we immersed slides in Wright's stain (R9350, Ricca Chemical, Arlington, TX, USA) for 5 s, then waited 2 min before rinsing in de-ionized water, and allowed them to dry overnight. For each blood smear, we identified and counted the number of lymphocytes, neutrophils, eosinophils, basophils and monocytes per 2000 erythrocytes following the methods of Hadji-Azimi et al. (Hadji-Azimi et al., 1987) and Davis (Davis, 2009). In cases of mortality, tadpoles were necropsied soon after being discovered, but no blood was collected. The necropsy process required to quantify parasites precluded us from directly measuring corticosterone levels within hosts. However, Belden et al. (Belden et al., 2005) reported that treatment with 0.1 μmol l^{-1} corticosterone (the same concentration used here) resulted in whole-body corticosterone levels that were ~20–30 ng g^{-1} body mass above baseline.

Analyses

We analyzed experimental data using R v.2.15.12 (R Development Core Team, 2012) with the 'survival', 'MASS' and 'eha' packages. We analyzed parasite penetration success (i.e. number of parasites at initial observation) by host species using ANOVA. We analyzed parasite survival by host species using both an accelerated failure time (AFT) and a proportional hazards survival regression model with exponential, Weibull and Rayleigh distributions. Data were censored in cases of host mortality and in cases where parasites survived until the experiment was terminated. We used corrected Akaike's information criterion (AIC_c) values to determine that the Weibull distribution was most appropriate. Because parasites were nested within individual hosts, we initially ran the analyses both with and without a frailty term (i.e. a random effect of individual host). However, the frailty coefficient was non-significant (coefficient=0.002, P=0.82), thus we opted to use the Weibull survival model without a frailty term for the remaining analyses. Using this model, we determined parasite survival functions for each host species using:

$$S_i(t) = e^{-\lambda_i t^p}, \quad (1)$$

where p is the Weibull shape parameter and λ_i is the Weibull scale parameter for host species i. These terms were determined as:

$$p = \frac{1}{k}, \quad (2)$$

$$\lambda_i = \left(\frac{1}{e^{(\alpha_0+\alpha_i)}}\right)^p, \quad (3)$$

where k is the scale output from the AFT model, α_0 is the AFT regression coefficient for the intercept and α_i is the AFT regression coefficient for host species i. From these survival functions we estimated median parasite survival time ($t_{½}$) in each host species as:

$$t_{1/2} = \left(\frac{-\ln(1/2)}{\lambda_i}\right)^{1/p}. \quad (4)$$

For the immune suppression experiment, we analyzed infection abundance as a function of immune treatment, time, parasite species and their interactions with a random effect for host individual using a generalized linear mixed model with a negative binomial distribution and a log-link function). Because all hosts in the parasite exposure group were exposed to the three parasite species concurrently, including host individual as a random effect helped account for any individual-specific variation in infection or immune responses, although this meant that we were unable to analyze the effects of the specific parasites on host responses, including mortality, growth and eosinophil concentrations. We analyzed host mortality as a function of parasite exposure and immune treatment using a generalized linear model with a binomial distribution (survived or died) and logit-

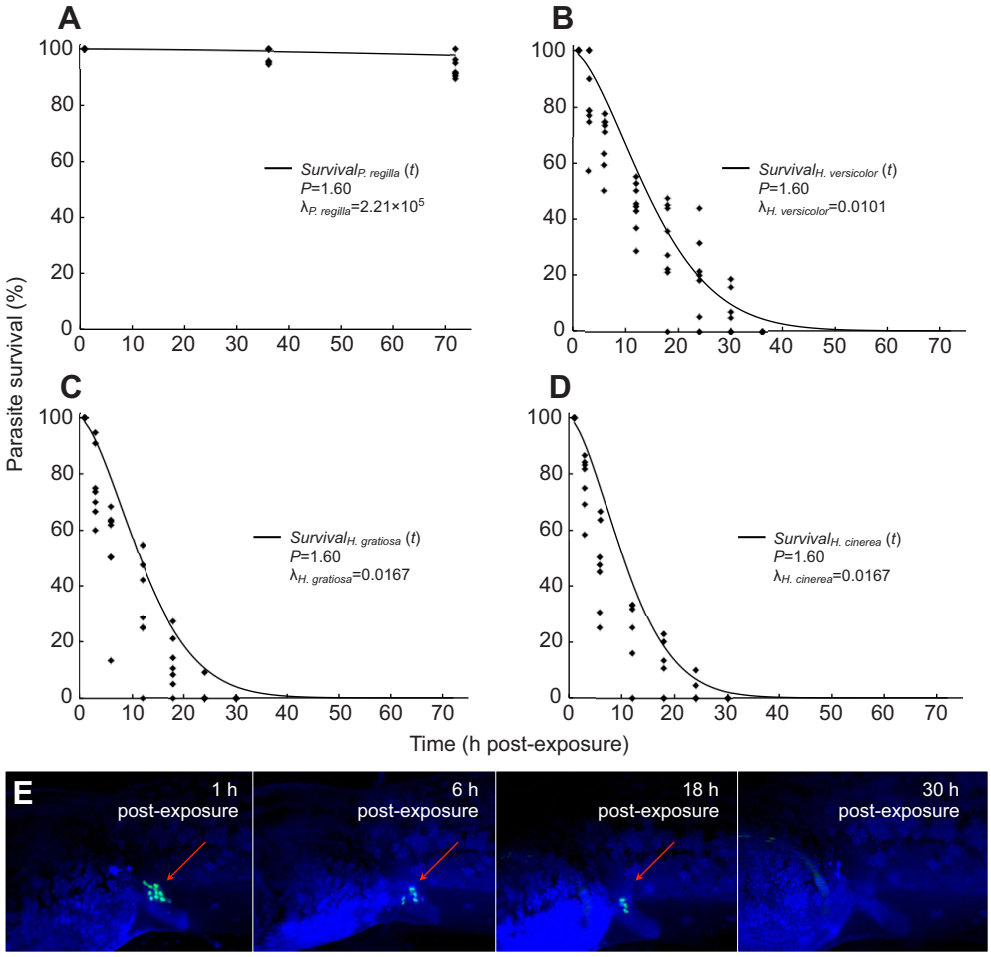

Fig. 2. Host-specific plots of parasite survival measured in individual hosts *versus* time post-exposure with corresponding survival functions derived from the Weibull model (*P*, shape parameter; λ_i, host-specific scale parameter) for (A) *Pseudacris regilla*, (B) *Hyla versicolor*, (C) *Hyla gratiosa* and (D) *Hyla cinerea*. (E) A time series of composite images showing a sagittal view of the left hind-limb bud region within a single *H. gratiosa* host to illustrate the process of *Ribeiroia* clearance.

link function. Finally, we analyzed host size (measured as snout–vent length) and eosinophils (per 2000 erythrocytes) as function of immune treatment, parasite exposure, sampling time and their interactions using a general linear model with a Gaussian distribution and an identity-link function.

RESULTS

Fluorescent parasite clearance assay

A comparison of distributions revealed that Weibull was more appropriate than either Rayleigh (ΔAIC_c=34.2) or exponential (ΔAIC_c=117.0). Our results show that after 1 h, of the 30 *Ribeiroia* cercariae each host was exposed to, 76% successfully penetrated the out-group host *P. regilla* while 55% penetrated *H. versicolor*, 56% penetrated *H. gratiosa* and 51% penetrated *H. cinerea* (F=5.17, d.f.=3, P<0.01). All parasites within hylid hosts were lost over the next 72 h. In contrast, 93% of the parasites that initially penetrated *P. regilla* remained at 72 h PE. The results also clearly indicated that, unlike in *P. regilla* (Fig. 2A), rapid clearance of *Ribeiroia* occurred in *H. versicolor* (Fig. 2B), *H. gratiosa* (Fig. 2C) and *H. cinerea* (Fig. 2D). Using the Weibull survival model, which provided a good fit to the data (see Fig. 2), we estimated an acceleration factor (γ_i) relative to *P. regilla* ($\gamma_{P.\ regilla}\equiv1$) of 0.022 for *H. versicolor* (coefficient=–3.83, P<0.001), 0.016 for *H. gratiosa* (coefficient=–4.14, P<0.001) and 0.016 for *H. cinerea* (coefficient=–4.14, P<0.001). Correspondingly, we estimated a median parasite survival time of 14.0 h in *H. versicolor*, 10.3 h in *H. gratiosa* and 10.3 h in *H. cinerea*. In contrast, median parasite survival time in *P. regilla* hosts was estimated to be 646.1 h (26.9 days).

Immune suppression experiment

There were no significant differences in infection between hosts in the control and vehicle treatments, and we therefore combined these groups for all subsequent analyses. Our analysis revealed a three-way interaction between immune treatment, time and parasite species (interaction coefficient=–2.37, t=–3.62, P<0.001). Corticosterone exposure consistently increased infection, but the strength of this effect varied by time and parasite species. At 4 days PE, corticosterone-treated tadpoles had 191% more *Ribeiroia* (coefficient=3.39, z=9.86, P<0.001), 80% more *Echinostoma* (coefficient=0.80, z=13.59, P<0.001) and 67.8% more *Alaria* (coefficient=0.63, z=9.41, P<0.001) relative to the controls (Fig. 3). We saw strong evidence of clearance of all parasite species following cessation immune suppression, but this varied by parasite species: *Echinostoma* infections were reduced by 63% (coefficient=–1.01, z=–11.42, P<0.001) and *Alaria* infections were reduced by 56% (coefficient=–0.81, z=–7.91, P<0.001), while *Ribeiroia* infections were reduced by 94% (i.e. almost completely eliminated; coefficient=–2.85, z=–7.82, P<0.001; Fig. 3).

Growth and survival

Survival was 100% in hosts that were not exposed to parasites, while survival was 84.6% in those that were exposed to parasites (coefficient=–9.90, χ^2=6.85, P<0.001). Of those hosts exposed to parasites, we observed 8.7, 10.6 and 25.0% mortality in the control, vehicle and corticosterone treatments, respectively (although these differences were not significant). However, host growth varied as a function of sampling time and parasite exposure. Overall, hosts

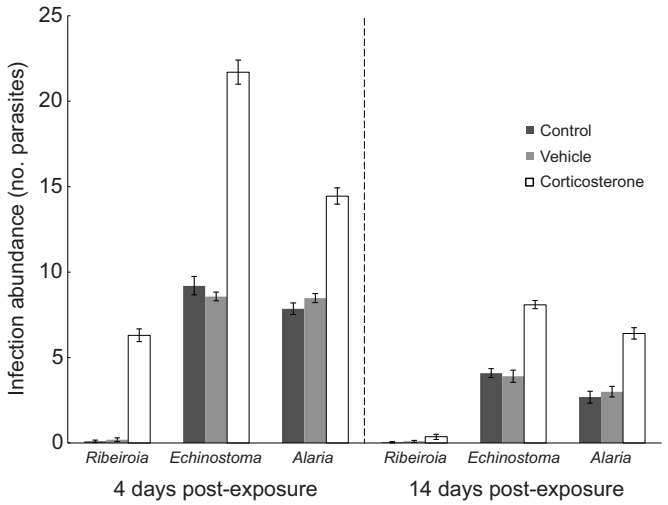

Fig. 3. Mean (±1 s.e.m.) infection abundance of each parasite species within experimental *H. versicolor* hosts grouped by immune treatment at 4 and 14 days post-exposure. Note: corticosterone and vehicle exposures were discontinued 4 days post-exposure.

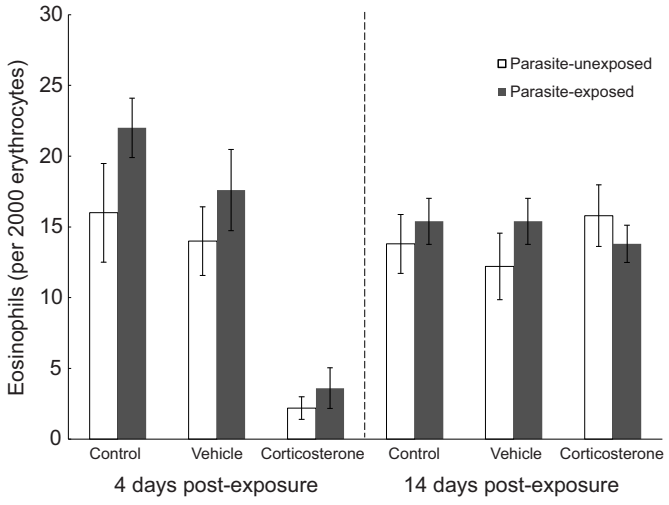

Fig. 4. Mean (±1 s.e.m.) number of eosinophils per 2000 erythrocytes for parasite-unexposed and -exposed *H. versicolor* hosts as a function of immune treatment at 4 and 14 days post-exposure. Note: corticosterone and vehicle exposures were discontinued after 4 days post-exposure.

grew by 22% between 4 and 14 days PE (coefficient=–1.57, χ^2=192.0, P<0.001), but we observed a significant interaction between parasite exposure and sampling time. From 4 to 14 days PE, parasite-unexposed hosts grew by 54.2% whereas parasite-exposed hosts only grew by 14.5% (coefficient=–0.91, χ^2=77.5, P<0.001). There were no main effects or interactions involving immune treatment.

Leukocyte profiles

Eosinophil concentrations (per 2000 erythrocytes) differed as a function of both immune treatment and parasite exposure. Hosts treated with exogenous corticosterone exhibited 62% fewer eosinophils than controls (coefficient=–4.63, χ^2=27.6, P<0.001), whereas we found no difference between the control and vehicle control groups (Fig. 4). Overall, parasite-exposed individuals exhibited a 17% increase in eosinophils relative to parasite-unexposed individuals (coefficient=–1.15, χ^2=4.17, P<0.05; Fig. 4). Additionally, we found an interaction between immune treatment and sampling time. We observed a marked increase in eosinophils among corticosterone-treated individuals – but not those in the control or vehicle control treatment – from 4 to 14 days PE (coefficient=–1.92, χ^2=31.4, P<0.001; Fig. 4).

DISCUSSION

Although variation in disease susceptibility among host species is widespread, understanding the mechanisms underlying such patterns remains a persistent challenge in eco-immunological research. Our use of a novel method to track parasite infections *in vivo* within amphibian hosts in combination with experimental immune suppression provides direct evidence of parasite clearance by hosts and offers insights into the dynamics, immunological mechanisms and variation in such patterns among host and parasite species. The results of the parasite clearance assay revealed that, in addition to *H. versicolor*, two other hylid hosts (*H. gratiosa* and *H. cinerea*) also exhibit remarkably rapid clearance of the trematode parasite *Ribeiroia*. Using the fluorescent labeling method to monitor infections *in vivo*, we show that infection variation among species owes to differences in both initial penetration success and subsequent clearance. Penetration success at 1 h PE ranged from 51 to 55% for the three hylid species and was 76% for *P. regilla*. Subsequent clearance dynamics were similar in each of the hylid species and stand in stark contrast to what was observed in the non-hylid outgroup species (*P. regilla*). Based on our Weibull survival model, which provided a close fit to the observed data, median parasite survival of *Ribeiroia* was between 10.3 and 14.0 h in the hylids compared with 26.9 days in *P. regilla* (a >45-fold difference). In addition to sub-lethally tracking infection, this assay has broad potential value for other applications, including labeling parasites from multiple exposure events and clearly differentiating processes such as penetration, encystment and clearance, which collectively influence concepts such as resistance (Råberg et al., 2009).

Through further manipulations of host immune function, we show that the lack of *Ribeiroia* infection observed in hylids owes mainly to active host resistance rather than to lack of suitability of the hosts for infection. In one such hylid host, chronic corticosterone exposure inhibited the ability to dramatically clear *Ribeiroia* as well the more subtle ability to limit infection with two other trematode species (*Echinostoma* and *Alaria*). At 4 days PE, corticosterone-treated hosts exhibited 191% greater *Ribeiroia* infection than hosts in the control treatment – for which infection was nearly undetectable at 4 days PE. Furthermore, immunity in these hosts recovered once the corticosterone treatment was discontinued, such that we observed a near-complete elimination of *Ribeiroia* 10 days later as well as a more subtle reduction in *Echinostoma* and *Alaria*. Our fluorescent observations revealed that parasites appear to be completely eliminated from the host tissue; however, the precise mechanism for this clearance is still not fully understood. Thus, while relatively little is known about amphibian host immune responses to larval trematodes, particularly with respect to clearance dynamics, our results provide evidence of host clearance for all three of the trematode species included here, an effect that was consistently weakened by the broad-spectrum immunosuppressant corticosterone. Moreover, we found evidence that exposure to parasites caused an increase in host mortality (15.4% in exposed hosts versus 0% in unexposed hosts) and a decrease in host growth

(18.2% in exposed hosts *versus* 59.9% in unexposed hosts). Taken together, these results suggest that, despite strong resistance to infection establishment and persistence, parasite exposure nonetheless incurs costs on host fitness. Host resistance varied according to parasite species. For instance, resistance to *Ribeiroia* infection by *H. versicolor* was 195.6% stronger than to *Echinostoma* and 194.9% stronger than to *Alaria*. Although this finding is in line with evidence from field surveys that show that *H. versicolor* is not highly resistant to all larval trematodes, only *Ribeiroia* (Johnson and Hartson, 2009), the underlying mechanism is still unknown. Given that *Ribeiroia* encysts subcutaneously, perhaps there is a skin-specific mechanism at play.

Results from the differential leukocyte counts provide evidence that chronic corticosterone treatment induced eosinopenia (i.e. lower than normal levels of circulating eosinophils). In fact, there were 62% fewer circulating eosinophils in corticosterone-treated hosts relative to controls. This result is consistent with the findings of Belden and Kiesecker (Belden and Kiesecker, 2005) and Davis and Maerz (Davis and Maerz, 2010), who found that amphibians treated with exogenous corticosterone exhibited a decrease in circulating eosinophils, as well as numerous other experimental findings involving glucocorticoid-induced apoptosis of eosinophils in other vertebrates (Wallen et al., 1991; Druilhe et al., 2003). Furthermore, our results indicate that the corticosterone (rather than parasite exposure) was responsible for the observed eosinopenia. Parasite exposure resulted in 17% higher eosinophil concentrations compared with unexposed hosts, indicating that eosinophil levels actually increased in response to parasite exposure. Given the design of our study, we were unable to determine whether this was due to any particular parasite species, which should be addressed by future research. Correspondingly, 10 days after cessation of corticosterone exposure, circulating eosinophils among previously immuno-suppressed hosts increased to levels comparable with those hosts not exposed to corticosterone. *Ribeirioia* infections decreased drastically during this same period, offering correlational support for the role of eosinophils in regulating trematode infection abundance. Differences in eosinophil quantity or quality should be investigated further given that they may help explain the interspecific variation in resistance to *Ribeiroia* as well as other larval trematodes. However, we cannot rule out the additional or alternative involvement of other immune components (e.g. anti-microbial skin peptides, humoral immunity), which are also subject to the immuno-suppressive effects of corticosterone (Rollins-Smith, 2009).

Combes (Combes, 2001) discusses the use of two ‘filters’, which together determine a pathogen’s range of suitable hosts. The first is the ‘encounter filter’, which requires that a pathogen and a host come into contact with one another in nature. Field studies by Johnson and Hartson (Johnson and Hartson, 2009) provide evidence that *Ribeiroia* and *H. versicolor* meet the requirements of the ‘encounter filter’ as they co-occur in the same habitats, even though *H. versicolor* exhibits dramatically reduced infections relative to concurrent amphibian host species. Correspondingly, while the geographic distributions of *Ribeiroia* and *H. versicolor* have extensive overlap, there are few reports of infection or parasite-induced malformations within this host group (Johnson et al., 2012). The second filter is the ‘compatibility filter’, which requires that a host must possess adequate resources for the pathogen and, at the same time, the host’s immune system cannot be so successful that transmission becomes impossible. Drawing upon ecological theory and application in invasion biology, this filter can be further subdivided into colonization (i.e. can the parasite colonize the host?), establishment (i.e. can the parasite establish a successful infection?) and persistence/spread (i.e. can the parasite persist and/or spread within the host?) (Combes, 2001; Kolar and Lodge, 2001; Johnson and Hartson, 2009). Based on the results of our fluorescent clearance assay and the immune suppression experiment, infectious stages of *Ribeiroia* are clearly capable of invading the host and establishing infections in the short-term; however, persistence appears to be inhibited actively by host defenses. Thus, in the current case, the host–parasite combination is not compatible because of failed persistence, and our data suggest that this outcome is a host- rather than parasite-driven process.

An emerging theme in eco-immunological research involves the potential link between host life history and investment in immunological defenses (Hamilton and Zuk, 1982; Sheldon and Verhulst, 1996; Hamilton and Poulin, 1997; Martin et al., 2011). While our results help to elucidate some of the short-term mechanisms underlying strong host resistance by hylid frogs to *Ribeiroia*, it is less clear why this host group in particular exhibits such strong and specific defenses. From a life history standpoint, the hylid treefrogs are predicted to have intermediate levels of resistance owing to a relatively fast-pace of life (Johnson et al., 2012). Instead, however, they are the most resistant group of hosts documented thus far, showing lesser susceptibility even relative to frogs with no co-evolutionary history of *Ribeiroia* infection (e.g. African clawed frogs, *Xenopus laevis*) or to separate amphibian orders such as the salamanders and newts (Johnson et al., 2012). Johnson and Hartson (Johnson and Hartson, 2009) advanced that this unusual pattern could be due to the tetrapoid genome of *H. veriscolor*, which could provide additional copies of certain immune defense genes. Our documentation, as well as that of Johnson et al. (Johnson et al., 2012), of comparably strong resistance in other, non-tetraploid hylid species indicates the explanation lies elsewhere. While the detrimental effects of *Ribeiroia* infection, including host mortality and debilitating limb malformations (Goodman and Johnson, 2011), undoubtedly provide a strong selective pressure for the evolution of resistance, why this trait has developed in the hylids specifically and why it appears to be highly specific against *Ribeiroia* remains uncertain. Given that hylid frogs have unique dermal compounds associated with host defense (e.g. hylaseptin P1) and that the primary route of *Ribeiroia* infection is transcutaneous, future work should investigate the added potential role of active or passive skin defenses (Prates et al., 2004; Conlon, 2011).

Taken together, our results demonstrate that not only are active host defenses capable of conferring resistance to larval trematodes, but also that these defenses are sensitive to stress. Although interspecific variation in host anti-parasite behaviors can also lead to differences in infection success (see Daly and Johnson, 2011; Szuroczki and Richardson, 2012), this explanation is unlikely to account for our observations given the similarity in initial infection success among species, the comparable body sizes of host species at the time of infection, and the remarkable differences in clearance rate post-infection (e.g. between *Hyla* and *Pseudacris*). These results also have immediate relevance to host–parasite dynamics in nature. Amphibian declines have been increasingly reported since the 1980s (Stuart et al., 2004) and emerging disease is one important implicated factor (Carey et al., 1999; Daszak et al., 1999; Pounds et al., 2006). Importantly, both natural and anthropogenic factors can alter host stress hormones and the potential efficacy of their immune response. In particular, the introductions of predators (Relyea and Mills, 2001; Relyea, 2003; Relyea, 2005), pathogens (Parris and Cornelius, 2004), UV-B radiation (Kiesecker and Blaustein, 1995; Belden et al., 2003) and environmental contaminants (Carey et al., 1999; Newcomb

Homan et al., 2003; Rohr et al., 2008; McMahon et al., 2011) have each been shown to increase stress in amphibians. Thus, changes in stress hormones in nature could cause similar changes in infection and result in increased host pathology, depending on timing and concentration relative to the experiments conducted here.

LIST OF SYMBOLS AND ABBREVIATIONS

AFT	accelerated failure time
AIC_c	corrected Akaike's information criterion
Bd	*Batrachochytrium dendrobatidis*
GFP2	green fluorescent protein 2
k	AFT scale output
p	Weibull shape parameter
PE	post-exposure
$S_i(t)$	parasite survival as a function of time within host species i
$t_{½}$	median parasite survival time
α_0	AFT regression coefficient for the intercept
α_i	AFT regression coefficient for host species i
γ_i	acceleration factor for host species i relative to *P. regilla*
λ_i	Weibull scale parameter for host species i

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AUTHOR CONTRIBUTIONS

B.E.L. and P.T.J.J. contributed to the conception, design, execution and interpretation of the findings of this work. B.E.L. wrote the first draft of the manuscript and both authors provided subsequent editorial input.

COMPETING INTERESTS

No competing interests declared.

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