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# Developmental variation in resistance and tolerance in a multi-host–parasite system

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## Summary

**1.** Organisms can protect themselves against parasitism by reducing either parasite burden (resistance) or damage caused by parasites at a given burden (tolerance), but little is known about resistance and tolerance to multiple parasites among wild animal populations and species. The fitness effects of parasitism can be broken down into two components: (i) cost of parasite exposure, the difference in fitness between organisms that were not exposed to parasites and those that were exposed but not infected, and (ii) tolerance, the change in host fitness as a function of parasite burden. For amphibians, a taxon that is being decimated by disease, knowing which species and ontogenetic stages are least resistant or least tolerant to which parasites would help target research and management efforts.

**2.** We reared American toad (*Bufo americanus*) and green frog (*Rana clamitans*) tadpoles in a common garden environment and quantified survival and trematode burdens after exposure to 20 *Echinostoma trivolvis*, *Ribeiroia ondatrae* or plagiorchid trematode cercariae.

**3.** All three trematodes caused significant *B. americanus* mortality, but *R. ondatrae* was the only species to induce significant mortality of *R. clamitans* and was the most deadly of the parasites. Young tadpoles had greater parasite-induced mortality than older tadpoles.

**4.** Mortality patterns were driven by *B. americanus* having lower resistance and a higher cost of parasite exposure than *R. clamitans*, older tadpoles having higher tolerance than younger tadpoles, *B. americanus* and *R. clamitans* exhibiting lower resistance and a higher cost of parasite exposure, respectively, to *R. ondatrae* relative to the other tested trematodes, and skin-penetrating trematodes inducing a higher cost of parasite exposure than non-skin-penetrators. Host size was not predictive of resistance but was a positive predictor of the cost of resistance and tolerance; however, size alone could not fully account for host differences in tolerance.

**5.** Interactions among developmental stage and host and trematode species emphasize the plasticity and context-dependency of defence strategies and the importance of considering resistance and tolerance to multiple parasites in wild host species.

**Key-words:** *Bufo americanus*, disease, *Echinostoma trivolvis*, *Rana clamitans*, *Ribeiroia ondatrae*

## Introduction

When faced with parasites, hosts can utilize two strategies to maximize fitness. They can reduce parasite burden by avoiding or directly attacking parasites, or they can minimize the harm caused by a given parasite load (Read, Graham & Raberg 2008; Schneider & Ayres 2008; Raberg, Graham & Read 2009). Since the late 1800s, these strategies have been referred to as resistance and tolerance, respectively, by plant biologists (Cobb 1894). Operationally, resistance has been defined as the inverse of the number of parasites per host given constant parasite exposure, whereas tolerance has been defined as the change in host fitness with a change in parasite burden (i.e. the slope of the relationship between fitness and burden) (Read, Graham & Raberg 2008; Raberg, Graham & Read 2009). Only recently have animal biologists empirically deconstructed variation in pathogen control (resistance) and damage control (tolerance), but thus far these studies have only been conducted on laboratory and domesticated animals (Read, Graham & Raberg 2008; Raberg, Graham & Read 2009).

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Furthermore, these studies have emphasized variation within a species among genetic lines, despite the framework allowing for comparisons of tolerance and resistance among populations, species and communities if individuals are reared under a common garden environment. Consequently, we know little about variation in tolerance vs. resistance in wild-caught animals or at levels of biological organization above genetic lines.

Plant biologists and theoreticians have long highlighted the value of distinguishing between resistance and tolerance because of the diverse selection pressures they can impose on hosts and pathogens (Fineblum & Rausher 1995; Pilson 2000; Roy & Kirchner 2000; Tiffin & Inouye 2000; Rausher 2001; Kover & Schaal 2002; Restif & Koella 2004). For instance, resistance should reduce parasite prevalence because it reduces pathogen fitness, whereas tolerance should have a neutral or even positive effect on prevalence (Roy & Kirchner 2000; Restif & Koella 2004; Best, White & Boots 2008). Hence, resistance is expected to cause much greater antagonistic coevolution between host and parasite (Miller, White & Boots 2006; Boots 2008); nevertheless, there is very little empirical evidence to support this assertion. Given the multiple parasites and environmental contexts to which most hosts are exposed, hosts are also expected to face an array of trade-offs and selection pressures that could prevent the fixation of resistance or tolerance traits (Roy & Kirchner 2000; Best, White & Boots 2008). Hence, to more thoroughly understand the context-dependency of resistance and tolerance strategies, animal biologists must quantify resistance and tolerance to multiple parasites through development and across environmental conditions (Ayres & Schneider 2008; Ayres, Freitag & Schneider 2008; Du *et al*. 2008; Raberg, Graham & Read 2009). However, host strategies for managing infections might be limited if resistance and tolerance are not independent traits. Indeed, research on mice unveiled a negative correlation between tolerance and resistance across mouse strains (Raberg, Sim & Read 2007).

The resistance-tolerance paradigm might have no greater importance than for amphibians. Amphibians are arguably the most threatened vertebrate taxon on the globe (Stuart *et al*. 2004) and many of their declines have been associated with emerging infectious diseases (Daszak, Cunningham & Hyatt 2003). For example, the amphibian chytrid fungus increased in prevalence in the 1980s and has subsequently decimated amphibian species worldwide (Lips *et al*. 2006; Rohr *et al*. 2008a). The global loss of amphibians has prompted captive breeding and artificial selection and release efforts (Mendelson *et al*. 2006), which could greatly benefit from knowledge of resistance and tolerance. For instance, artificial selection (and release to the environment) for amphibian tolerance to a deadly parasite might be more likely to successfully re-establish the amphibian in nature than selection for resistance. This is because parasites often evolve more quickly than their hosts and tolerance, unlike resistance, is not expected to strongly select for parasite countermeasures (Boots 2008). Given the number of threatened amphibian species, their complex lifecycles and the myriad of parasites with which they are challenged, knowing which amphibian species and ontogenetic stages are least resistant or least tolerant to which parasites would help target future research and management efforts.

We studied amphibian resistance and tolerance to larval trematode infections for several reasons. First, in contrast to microparasites, larval trematodes do not replicate in their hosts and are seldom cleared by their hosts once established (but see Holland 2009), allowing for a more straightforward quantification of parasite burden. Secondly, populations of frogs can average over 1000 larval trematodes per individual (Rohr *et al*. 2008c), suggesting substantial tolerance to these infections. Thirdly, larval trematode infections can cause substantial amphibian mortality and morbidity (e.g. limb malformations, kidney damage), and have been linked to amphibian mortality events (Fried, Pane & Reddy 1997; Johnson *et al*. 1999; Schotthoefer *et al*. 2003b; Holland *et al*. 2007). Finally, two of three of the amphibian trematode taxa we studied, *Ribeiroia ondatrae* and *Echinostoma* spp., are considered emerging parasites (Johnson & Sutherland 2003; Beasley *et al*. 2004; Skelly *et al*. 2006). We therefore designed an experiment to quantify resistance and tolerance, through development (i.e. across a context), of two amphibian species, *Bufo americanus* (American toad) and *Rana clamitans* (green frog), to three trematode taxa – *Echinostoma trivolvis*, *Ribeiroia ondatrae* and an undetermined species of plagiorchid.

## TERMINOLOGY, HYPOTHESES AND PREDICTIONS

We define resistance as the inverse of the proportion of cercariae that successfully encysted. While previous studies have focused on disentangling tolerance from resistance, there are also at least two components of the fitness effects of parasitism which have not been disentangled previously (Fig. 1). Other researchers have assumed that organisms exposed to parasites but not infected, represented by the *y*-intercept of the relationship between burden and fitness, will have the same fitness as control organisms not exposed to parasites (Fig. 1a,c; Raberg, Graham & Read 2009). However, exposure to parasites could have fitness consequences even for hosts that do not get infected (Fig 1b,d), either due to direct damage from parasites at the time of exposure or due to the cost of parasite resistance (Lochmiller & Deerenberg 2000), which might be considerable for hosts with 100% resistance (i.e. zero parasite burden). Hence, there can be a difference in fitness between organisms not exposed to parasites and the *y*-intercept of the relationship between parasite burden and fitness (Fig. 1b,d). We refer to this difference (or slope; see Fig. 1) as 'cost of parasite exposure'; a greater difference (or more negative slope) indicates a greater cost of exposure. In contrast, the subsequent loss of fitness due to infections, measured by the slope of the relationship between parasite burden and fitness, is 'tolerance' (Fig. 1). Throughout this study, time to death is used as our fitness proxy in our tolerance and cost of parasite exposure estimates.

Hereafter, we will use the terminology 'relative resistance' and 'relative tolerance' to refer to investment in one defence

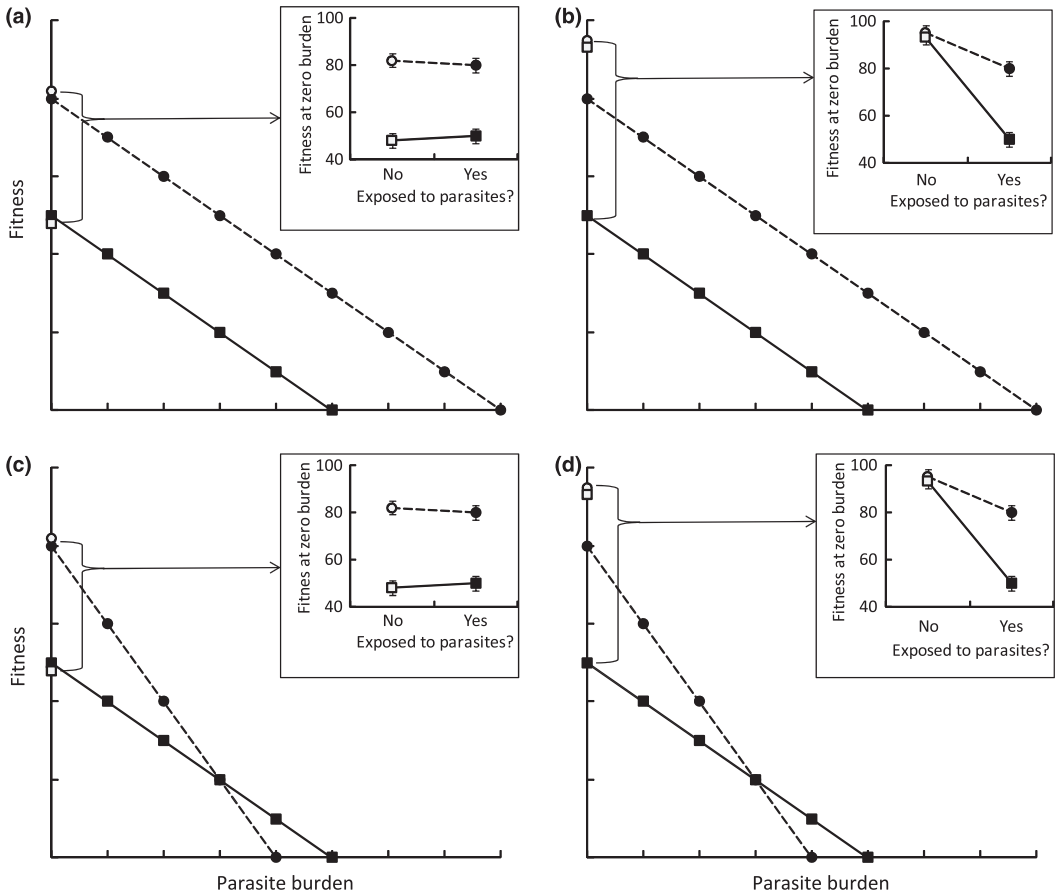


**Fig. 1.** A heuristic model comparing fitness of species A (circles) and B (squares) when exposed (solid symbols) and not exposed to parasites (open symbols). (a) A difference in vigour between the species (because their fitness differs when they are not exposed to parasites), but no difference in cost of parasite exposure (because the slopes in the inset do not differ) or tolerance (because the slopes in the main graph do not differ). (b) A difference in cost of parasite exposure, but no differences in vigour or tolerance. (c) A difference in vigour and tolerance, but no difference in cost of parasite exposure. (d) Differences in both cost of parasite exposure and tolerance, but no difference in vigour. Error bars are only shown in insets for simplicity. Only a subset of possible combinations of vigour, cost of parasite exposure and tolerance of infection are shown.

strategy relative to the other, whereas 'resistance' or 'absolute resistance' and 'tolerance' or 'absolute tolerance' will refer to resistance and tolerance independent of investment in the alternate defence strategy. We distinguish between relative and absolute measures for three reasons. First, many theoretical models focus on relative rather than absolute measures (Restif & Koella 2004). Secondly, it is theoretically possible that two species could differ in their absolute measures of resistance and tolerance but have identical relative investments in each. Thirdly, scientists have hypothesized negative relationships between virulence and tolerance (Restif & Koella 2004), but a hypothesis that low virulence selects for high absolute tolerance, rather than high relative tolerance, would be tautological because, by definition, low virulence means that the host has high tolerance. Thus the emphasis for this hypothesis is on relative tolerance and relative resistance. Importantly, relative resistance and relative tolerance do not imply that these two defence types must trade off. Hosts could increase both absolute resistance and absolute tolerance by diverting resources from other activities. Hence the trade-off could be between resistance or tolerance and other activities rather than between resistance and tolerance themselves.

We hypothesized that host size would have little effect on resistance but would affect absolute tolerance because a parasite should cause proportionally greater harm in a small than in a large host (Rohr, Raffel & Sessions 2009a). We also predicted that, after controlling for host size, the amphibian species would differ in their relative resistance because of different life-history traits. Given that green frogs overwinter in ponds but American toads leave ponds just 3–5 weeks after hatching, we expected that green frogs would have higher average larval trematode exposure and thus would experience greater selection for relative tolerance to larval trematodes than American toads (when controlling for size differences).

In our comparison of different trematode taxa, we hypothesized that trematode size and mode of entry would influence virulence (how deadly a parasite is to a host) and thus affect selection for tolerance or resistance in a given host (Rohr, Raffel & Sessions 2009a). Specifically, we predicted that *R. ondatrae* would have the highest virulence of the three trematodes tested because it has the largest cercariae and burrows through amphibian skin. Hence, it was predicted to be most likely to select for host resistance. However, cercarial

size and mode of entry lead to conflicting predictions regarding the virulence of *E. trivolvis* vs. the plagiorchid. *Echinostoma trivolvis* cercariae are about two times larger than the plagiorchid cercariae, but they appear to cause less tissue damage during infection because they enter through the cloaca of amphibians (Fried, Pane & Reddy 1997; Schotthoefer, Cole & Beasley 2003a), whereas the plagiorchid penetrates amphibian skin using a stylet and proteolytic enzymes (Rohr *et al*. 2008c).

Additionally, we formed predictions about the context-dependency of resistance vs. tolerance investments. Larval trematode infections tend to be more virulent to younger tadpoles (Schotthoefer, Cole & Beasley 2003a) and there is no known consistent change in the rate of exposure with age. Hence, we predicted that younger tadpoles would invest more in relative resistance mechanisms than older tadpoles, which would invest more in relative tolerance mechanisms. Identifying developmental stages that are particularly susceptible to infections might facilitate effectively timing management strategies and targeting future research efforts.

## Materials and methods

*Bufo americanus* Holbrook (American toad) and *Rana clamitans* Latrielle (green frog) egg masses were collected from the neighbouring 'Parking lot' (40°45′51·4″N, 78°0′58·6″W) and 'Beaver 1' ponds (40°45′52·6″N, 78°0′43·6″W), respectively, in State College, Pennsylvania, USA, during the summer of 2006. Both species were reared in a common garden environment from the embryo stage. That is, each was placed in 37·8 L tanks containing aerated, dechlorinated tap water maintained at room temperature (21–22 °C) on a 12 : 12 h photoperiod. The tadpoles were fed fish flakes and rabbit chow (ground alfalfa pellets) *ad libitum*.

To obtain the trematodes *Echinostoma trivolvis* and a plagiorchid species, we collected several hundred *Planorbella trivolvis* snails from a pond in Harrisburg, Pennsylvania, USA (40°4′56·0″N, 76°46′2·0″W). *Ribeiroia ondatrae* appears to be rare in Pennsylvania and thus *P. trivolvis* infected with *R. ondatrae* were shipped from Illinois. The snails were maintained in aerated, artificial spring water (ASW, as described by Cohen, Neimark & Eveland 1980) at room temperature (21–22 °C) on a 12:12 h photoperiod and were fed boiled lettuce and fish flakes *ad libitum*.

A total of 240 tadpoles were used in this experiment. The experiment was organized into three temporal blocks with 40 American toad and 40 green frog tadpoles in each block. Of the 40 tadpoles per amphibian species, 10 served as controls (i.e. not exposed to cercariae), 10 were exposed to *E. trivolvis* cercariae, 10 were exposed to plagiorchid cercariae, and 10 were exposed to *R. ondatrae* cercariae. Each one of these 240 tadpoles was placed individually into a plastic cup containing 75 mL of ASW and treatments were assigned randomly to the tadpoles in each block. With the exception of the control tadpoles, each tadpole was exposed to 20 cercariae of the assigned trematode species. Exposure to this number of cercariae is reasonable considering that certain populations of recently metamorphosed northern leopard frogs (*Rana pipiens*) can average over 1000 larval trematodes per individual (Rohr *et al*. 2008c). To obtain cercariae, at least five snails per trematode species per block were placed in 75 mL of ASW and positioned under a light source to stimulate cercarial shedding. Within 1 h of shedding, we used a pipette to transfer the cercariae to the cups with the tadpoles.

For 7 days after initial cercarial exposure, the tadpoles were maintained at room temperature (21–22 °C) under natural light and were fed fish flakes *ad libitum*. Mortality was assessed daily and dead tadpoles were immediately preserved in 70% ethanol (30% water). At the end of 7 days, the surviving tadpoles were euthanized by immersion in 1% benzocaine and preserved in 70% ethanol. We quantified the snout-vent length and Gosner stage (Gosner 1960) of each preserved tadpole. Thus, Gosner stage was not manipulated but varied naturally among individuals. All tadpoles were between Gosner stage (Gosner 1960) 24 and 37 when they were exposed to cercariae. The preserved tadpoles were cleared and stained using the protocols of Hanken & Wassersug (1981) and their trematode cysts were counted under a compound scope.

### STATISTICAL ANALYSES

We used General Linear Models and/or survival analysis for all statistical tests. Predictors were removed from each model by backward selection if $P > 0{\cdot}05$. *P*-values were calculated with *F*-ratio tests (general linear models) or submodel deviance tests (survival analyses), using the procedure for Type II sum of square errors (Langsrud 2003). Non-significant two-way interactions were removed by backward selection (if $P > 0{\cdot}05$) unless they were components of a significant three-way interaction. The temporal block effect was included in all statistical models. Because toad tadpoles developed more rapidly than green frog tadpoles and represented a wider range of developmental stages, we removed toad tadpoles greater than Gosner (1960) stage 30 from all analyses, to provide a fair comparison of stage-dependent resistance and tolerance in the two tadpole species.

If there was a significant interaction with tadpole spp., we tested for effects on each tadpole species individually to elucidate the interaction. When there were significant effects of trematode spp., we tested for effects of each trematode relative to the control. Given the controversy regarding which multiple comparison tests are most appropriate, we chose not to make any alpha adjustment for multiple comparisons and leave determinations of significance to the reader (Gotelli & Ellison 2004). Statistics were calculated using R statistical software (R Development Core Team 2006).

We analysed tadpole survival as a proxy for host fitness using a survival analysis with Cox proportional hazards, testing for interactive effects of tadpole spp., trematode spp. and developmental stage (package 'survival' in R). Tadpoles surviving to the end of the experiment were censored to account for our lack of information about their true times to death. Censoring is a standard technique that down-weights the influence of these individuals in the survival analysis.

Tadpole survival after trematode exposure can be broken down into two components: host ability to resist or prevent infections and host ability to tolerate infections. As an inverse measure of host resistance to infection, we used the (arc-sine transformed) proportion of trematodes encysted (out of 20 cercariae). To test for effects of tolerance, we re-conducted our survival analysis but we included main effects and interactions with parasite burden in our statistical model. Nonlinear relationships between fitness and parasite burden can lead to spurious variation in tolerance (Tiffin & Inouye 2000); thus we also tested for nonlinear relationships by adding a quadratic effect of parasite burden to our models of tolerance.

Tolerance analyses often first entail subtracting fitness estimates in the presence of the parasites from fitness in the absence of parasites, to control for differences in overall vigour (fitness in the absence of

parasite exposure; Fig. 1). Although some studies have used the *y*-intercept of burden vs. fitness as a measure of vigour, survival of hosts that are not exposed to parasites is a better indicator of vigour because exposure to parasites could have fitness consequences even for hosts that do not get infected. Mortality in our control animals was negligible, with only one control green frog and two control toads dying during the experiment. We therefore used the raw mortality data for parasite-exposed tadpoles (excluding the controls) in our analysis of tolerance. Given that we had no differences in vigour between our two species, significant main effects of host or trematode species in our tolerance analyses represent differences in their costs of parasite exposure, whereas a significant host or trematode species × burden interaction represents differences in tolerance of infection (Fig. 1 b, d).

To test many of our hypotheses, it was necessary to quantify relative investment in tolerance vs. resistance, but these two variables are quantified in different units. To facilitate comparison between the two variables, we transformed each to standard deviation units (i.e. *z*-scores; difference from the grand mean divided by the standard deviation). Hence, this allows us to compare the number of standard deviations resistance and tolerance are from their grand mean. For tolerance, we obtained the residuals from the final censored survival analysis (as shown in Table S2) and then transformed these residuals into *z*-scores. For resistance, we obtained the residuals from the final model on the arc-sine proportion of encysted trematodes and transformed them into *z*-scores. We multiplied each *z*-score by −1 so that positive values indicate higher resistance or tolerance. We then subtracted the resistance score for each individual from its tolerance score to obtain an estimate of relative investment in tolerance. Hence, negative difference scores indicate greater estimated investment in resistance relative to tolerance, whereas positive difference scores indicate greater estimated investment in tolerance relative to resistance. We subtracted the values rather than using a ratio because the properties of ratios are not ideal for statistical analyses. If the numerator in a ratio is equal to or greater than the denominator, the value can be between 1 and ∞, whereas if the denominator is greater than or equal to the numerator, the value can only be between 0 and 1. Hence, both the variance and weight in a statistical analysis will be larger when the numerator is greater than the denominator than when the denominator is greater than the numerator. For the resistance, tolerance and relative tolerance analyses, we tested for interactive effects of trematode spp., tadpole spp., and developmental stage, including significant two- and three-way interactions with parasite burden in the tolerance analysis. We repeated these analyses with tadpole length as a covariate to test whether size differences between the host species could eliminate the significance of any effect containing host species.

## Results

### TREMATODE VIRULENCE – EFFECTS OF TREMATODE EXPOSURE ON HOST SURVIVAL

Tadpole survival was significantly affected by treatment (trematode spp. and controls), tadpole species, and tadpole developmental stage (Treatment: $\chi^2 = 28{\cdot}1$, d.f. = 3, $P < 0{\cdot}001$; Tadpole spp.; $\chi^2 = 37{\cdot}1$, d.f. = 1, $P < 0{\cdot}001$; Stage: $\chi^2 = 15{\cdot}7$, d.f. = 1, $P < 0{\cdot}001$; Treatment × tadpole spp.: $\chi^2 = 2{\cdot}2$, d.f. = 3, $P = 0{\cdot}536$). Overall, *R. ondatrae* was more deadly than *E. trivolvis* or plagiorchid cercariae (Fig. 2), but the only significant difference in survival was between *R. ondatrae*- and plagiorchid-exposed tadpoles ($\chi^2 = 5{\cdot}7$, d.f. = 1, $P = 0{\cdot}017$; $P > 0{\cdot}2$ for the other two comparisons). Green frogs had higher overall survival than toads (coef. = 2·54 for Toad = 1; Fig. 2), apparently because toads had higher mortality due to infection (Fig. 2). Control tadpoles had negligible rates of mortality, with no effects of tadpole spp. or developmental stage (both $P > 0{\cdot}5$, Fig. 2). Toads experienced significant mortality due to infection by all three trematode species, whereas green frogs only experienced significant mortality due to *R. ondatrae* (Fig. 2). More highly developed tadpoles had significantly higher survival (coef. = −0·53), but developmental stage did not significantly influence the effect of Treatment or Tadpole spp. on survival (interactions including Stage all $P > 0{\cdot}2$).

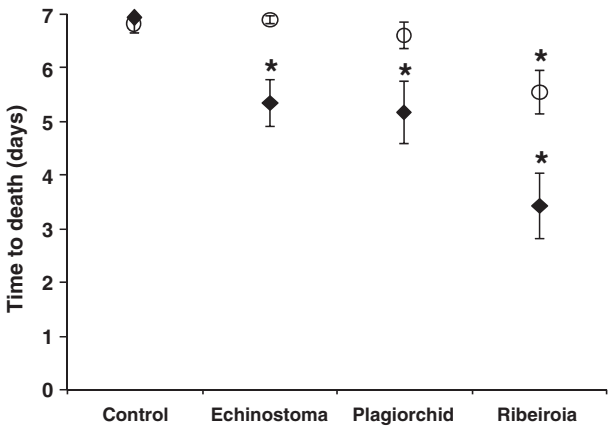


**Fig. 2.** Effects of trematode infection on survival of green frog tadpoles (open circles) and toad tadpoles (filled diamonds). Averages were based on both dead and euthanized animals (time to euthanasia = 7 days); euthanized animals were censored in the survival analysis. Error bars indicate standard errors and an asterisk indicates a significant difference from the control treatment.

### ABSOLUTE RESISTANCE TO TREMATODE INFECTIONS

Resistance was significantly affected by tadpole species and tadpole developmental stage, with a nearly significant main effect of trematode species (Table S1). *Ribeiroia ondatrae* had higher proportions of cercariae successfully encyst than did *E. trivolvis* or the plagiorchid (*R. ondatrae* vs. *E. trivolvis*: $F_{1,81} = 3{\cdot}5$, $P = 0{\cdot}039$; *R. ondatrae* vs. plagiorchid: $F_{1,84} = 5{\cdot}9$, $P = 0{\cdot}018$), whereas *E. trivolvis* and the plagiorchid had similar encystment rates ($F_{1,88} = 0{\cdot}003$, $P = 0{\cdot}955$; Fig. 3a). However, the effect of trematode spp. on resistance depended significantly on tadpole spp. (Table S1). This interaction was driven by green frogs having higher average resistance than toads to *R. ondatrae* and the plagiorchid (controlling for the main effect of developmental stage) but similar resistance to *E. trivolvis* (Fig. 3a).

More highly developed tadpoles had generally lower encystment rates (Table S1), but this effect depended on

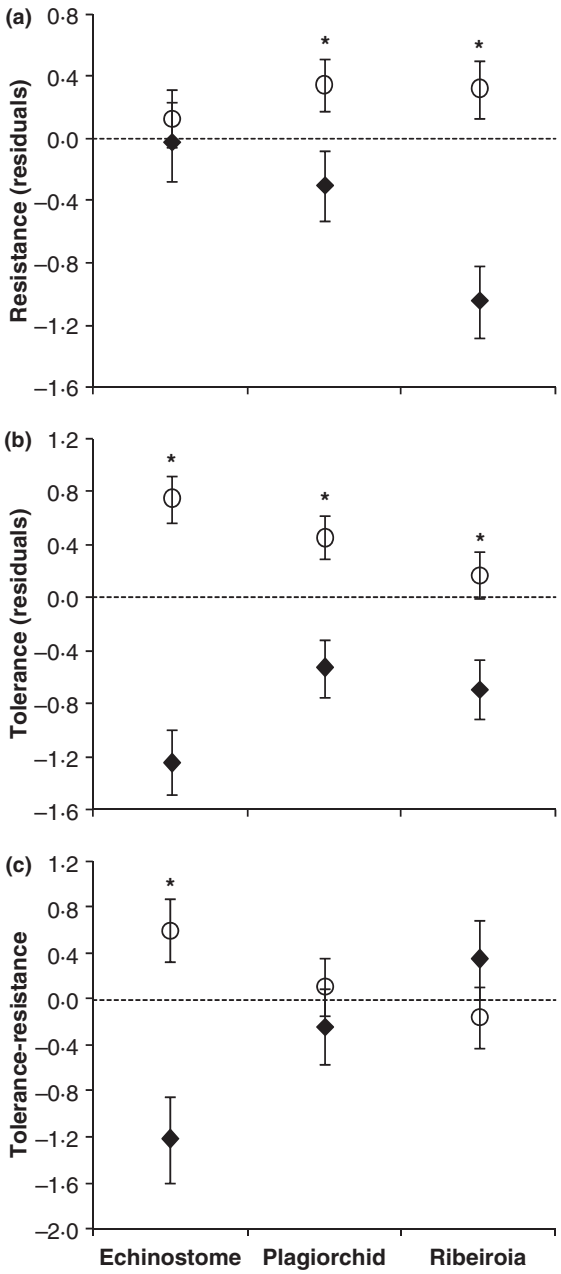


**Fig. 3.** Effects of tadpole species (open circles = green frogs; filled diamonds = toads) on (a) resistance to trematode infection, (b) tolerance to trematode infections and (c) the difference between tolerance and resistance (relative tolerance). For tolerance, we obtained the residuals from the final censored survival analysis (as shown in Table S2) and then transformed these residuals into *z*-scores. For resistance, we obtained the residuals from the final model on the arc-sine proportion of encysted trematodes and transformed them into *z*-scores. We multiplied each *z*-score by −1, so that positive values indicate higher resistance or tolerance. Least squares means from the statistical model are presented to control for the effects of developmental stage. Error bars indicate standard errors; an asterisk indicates a significant difference between tadpole species. A horizontal dashed line through (0, 0) indicates the null model of no effect.

trematode and tadpole species (Table S1). Both toads and green frogs became less resistant to *E. trivolvis* infections through development at approximately the same rate (Fig. 4a; Stage × tadpole spp.: $F_{1,40} = 1{\cdot}31$, $P = 0{\cdot}259$). Toads developed resistance to the plagiorchid more rapidly through development than green frogs (Fig. 4b; Stage × tadpole spp.: $F_{1,43} = 20{\cdot}2$, $P < 0{\cdot}001$), but green frogs developed resistance to *R. ondatrae* more rapidly through development than toads (Fig. 4c; Stage × tadpole spp.: $F_{1,36} = 8{\cdot}7$, $P = 0{\cdot}006$).

### COST OF PARASITE EXPOSURE AND ABSOLUTE TOLERANCE

Over the range of parasite burdens we studied, we found no evidence that the relationship between burden and host survival was nonlinear (parasite burden$^2$: $\chi^2 = 1{\cdot}157$, d.f. = 1, $P = 0{\cdot}282$; Fig. 5). Green frogs had significantly lower cost of trematode exposure than toads, as indicated by the lower *y*-intercept of toads than green frogs in Fig. 5 and the significant main effect of tadpole spp. (Table S2, Fig. 3b). This difference in cost of trematode exposure was independent of trematode species (non-significant tadpole × trematode spp. interaction; Table S2). The two amphibian species, however, did not significantly differ in their tolerance because we did not detect any tadpole spp. × parasite burden interaction (Table S2). Tolerance increased with tadpole developmental stage (Table S2), an effect that was particularly pronounced for *R. ondatrae* (*E. trivolvis*: $P = 0{\cdot}089$; plagiorchid: $P = 0{\cdot}015$; *R. ondatrae*; $P = 0{\cdot}004$; Table S3, Fig. 4d–f). However, green frogs had a more rapid increase in tolerance through development than toads (Tadpole spp. × stage; coef. = 1·58, $\chi^2 = 12{\cdot}2$, d.f. = 1, $P < 0{\cdot}001$; Table S2, Fig. 4d–f). The significant and non-significant results in the survival analysis on tolerance match those for a logistic regression analysis on survival until the end of the experiment (same model but with binomial response), indicating that the findings are robust.

### RELATIVE INVESTMENT IN TOLERANCE VS. RESISTANCE

Although there were no main effects on relative tolerance (all $P > 0{\cdot}05$), trematode spp. significantly interacted with both developmental stage and tadpole spp. (Trematode spp. × stage: $F_{2,123} = 12{\cdot}6$, $P < 0{\cdot}001$; Trematode spp. × tadpole spp.: $F_{2,123} = 18{\cdot}7$, $P < 0{\cdot}001$; Stage × tadpole spp.: $F_{2,123} = 0{\cdot}6$, $P = 0{\cdot}435$; Trematode spp. × stage × tadpole spp.: $F_{2,123} = 5{\cdot}8$, $P = 0{\cdot}004$). The trematode spp. × tadpole spp. interaction was driven by green frogs having higher relative tolerance to *E. trivolvis* than toads ($F_{2,40} = 26{\cdot}2$, $P < 0{\cdot}001$), but similar relative tolerance to *R. ondatrae* ($F_{1,36} < 0{\cdot}1$, $P = 0{\cdot}960$) and the plagiorchid ($F_{1,43} = 0{\cdot}9$, $P = 0{\cdot}356$; Fig. 3c). This effect was driven mostly by differences in resistance and cost of parasite exposure, given that tolerance did not differ between tadpole species (Table S2, Fig. 5). Interactions including stage can be explained by more developed tadpoles having higher relative tolerance to *E. trivolvis* ($F_{1,40} = 15{\cdot}6$, $P < 0{\cdot}001$, coef. = 0·52), but not to *R. ondatrae* ($F_{1,36} = 1{\cdot}7$, $P = 0{\cdot}197$; $P > 0{\cdot}05$ for

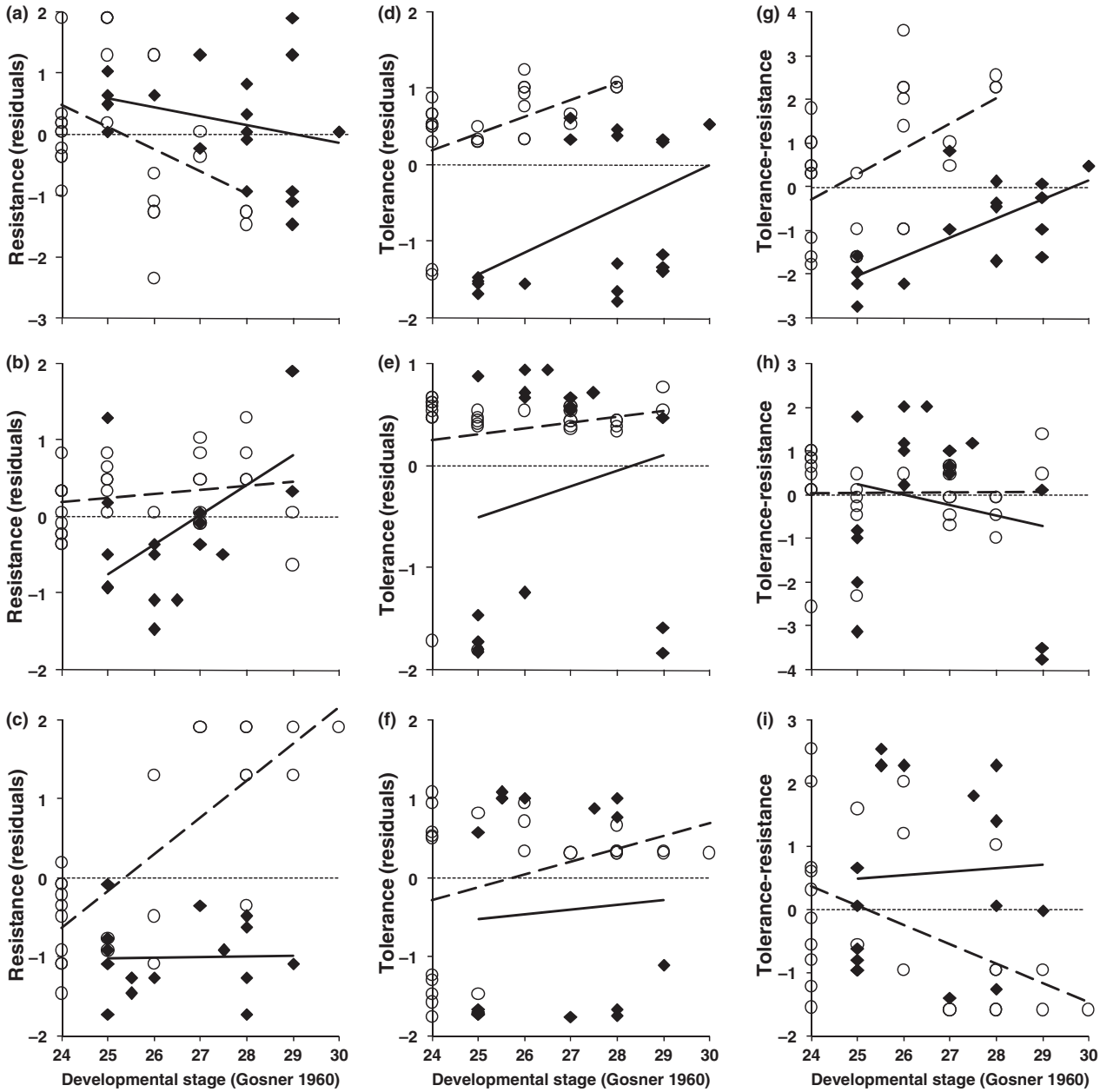


**Fig. 4.** Effects of tadpole development on (a–c) resistance to trematode infection, (d–f) tolerance to trematode infection and (g–i) the difference between tolerance and resistance (relative tolerance). For tolerance, we obtained the residuals from the final censored survival analysis (as shown in Table S2) and then transformed these residuals into *z*-scores. For resistance, we obtained the residuals from the final model on the arc-sine proportion of encysted trematodes and transformed them into *z*-scores. We multiplied each *z*-score by −1 so that positive values indicate higher resistance or tolerance. Separate panels indicate responses to (a, d, g) *Echinostoma trivolvis*, (b, e, h) the plagiorchid and (C, F, I) *Ribeiroia ondatrae*. Open circles and dashed trendlines represent green frogs, whereas filled diamonds and solid trendlines represent toads. A horizontal dashed line through (0, 0) indicates the null model of no effect.

both Tadpole spp. × stage interactions). There was no significant main effect of development on relative tolerance to plagiorchids ($F_{1,43} = 0{\cdot}3$, $P = 0{\cdot}559$), but there was a significant difference in this relationship between the tadpole species, with toads decreasing their relative tolerance more rapidly through development than green frogs (Tadpole spp. × stage: $F_{1,43} = 13{\cdot}8$, $P < 0{\cdot}001$, coef. = −1·09).

### EFFECT OF HOST SIZE ON ABSOLUTE RESISTANCE AND TOLERANCE AND RELATIVE TOLERANCE

Tadpole length was not a significant predictor of resistance ($F_{1,122} < 0{\cdot}1$, $P = 0{\cdot}967$) but was a significant positive predictor of tolerance ($\chi^2 = 29{\cdot}4$, d.f. = 1, $P < 0{\cdot}001$) and relative tolerance ($F_{1,122} = 24{\cdot}3$, $P < 0{\cdot}001$). However, adding length to the statistical models did not eliminate the

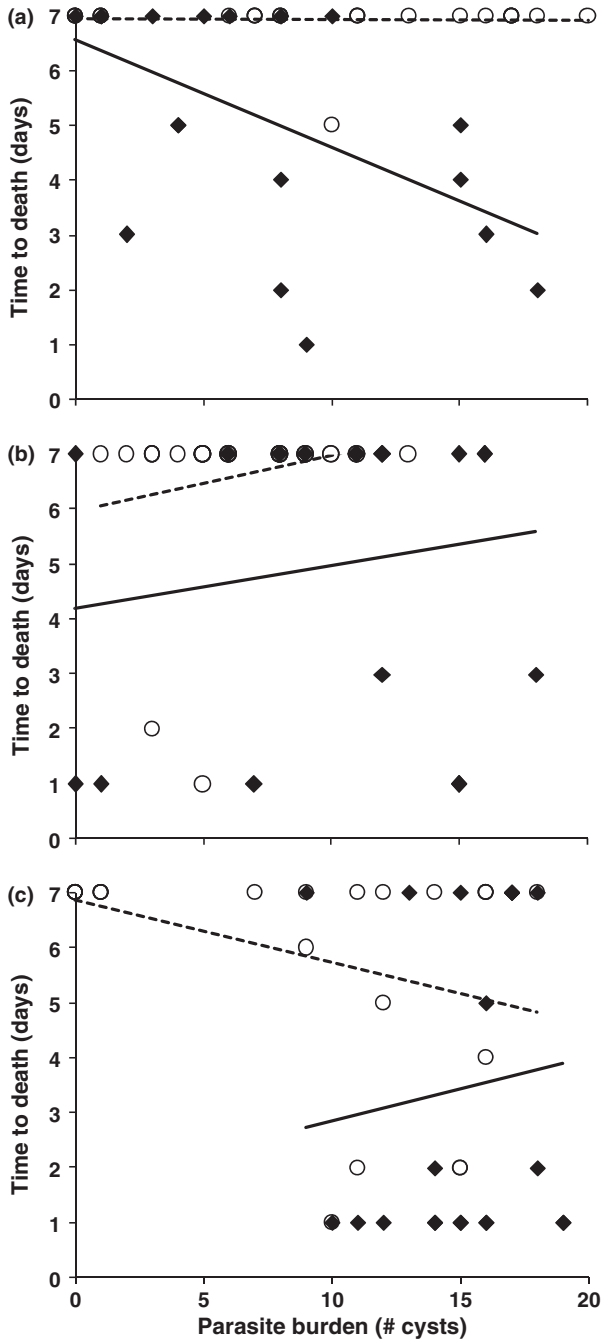


**Fig. 5.** Relationship between parasite burden and tadpole time to death (up to the end of the experiment at 7 days) for exposure to (a) *Echinostoma trivolvis*, (b) the plagiorchid and (c) *Ribeiroia ondatrae*. Open circles and dashed trendlines are for green frogs, whereas filled diamonds and solid trendlines are for toads. Linear trendlines were added to only qualitatively represent the patterns; survival analyses were conducted on the data and the residuals from this analysis were normally distributed around the mean. There are cases where multiple points are on top of one another. All graphed points represent tadpoles that were exposed to cercariae; nearly all tadpoles not exposed to cercariae lived for 7 days [Control toads = 6·95 ± 0·05 (SE); Control green frogs = 6·83 ± 0·17].

significance of any effect containing host species for any given parasite (one-tailed test), nor did it change the direction of any effects. Thus, although host size accounted for a considerable amount of variation in tolerance, size differences between the host species alone could not fully account for their differences in tolerance or relative tolerance.

## Discussion

### SURVIVAL AND ABSOLUTE RESISTANCE AND TOLERANCE

By rearing *B. americanus* and *R. clamitans* in a controlled environment and quantifying their survival after a standardized exposure to three trematode taxa, we showed that parasite-associated mortality was higher for *B. americanus* than for *R. clamitans*, for younger than for older tadpoles, and for tadpoles exposed to *R. ondatrae* cercariae than to *E. trivolvis* or plagiorchid cercariae. These patterns in parasite-associated mortality can be partitioned into resistance and tolerance defences of the hosts to the specific parasites. *Bufo americanus* should incur a smaller cumulative cost of aquatic trematode exposure because its aquatic tadpole stage is shorter. Consequently, it was expected to have lower absolute resistance and tolerance to infection than *R. clamitans*, and the results supported this prediction, even after controlling for size differences between the host species. Johnson & Hartson (2009) also revealed that *B. americanus* have a relatively low resistance and high mortality risk associated with *R. ondatrae* exposure. Within each amphibian species, more developed and larger tadpoles appeared to be more tolerant than less developed and smaller tadpoles, also as expected.

Smaller tadpoles of a given developmental stage might have been more likely to succumb to trematodes because the cercariae were relatively larger and thus might have caused relatively more damage. Similarly, the large, skin-penetrating *R. ondatrae* was more deadly to both host species than was either *E. trivolvis* or the plagiorchid. For toads, of the trematodes tested, only *E. trivolvis* showed a negative slope for tolerance and a *y*-intercept for the tolerance relationship matching the survival of toads not exposed to cercariae (Fig. 5). This was also the only trematode tested that does not infect toads via skin penetration. All of the toad tadpoles exposed to *R. ondatrae* and plagiorchid cercariae likely had 20 trematode-induced holes in their bodies (because all the cercariae seemed to enter the tadpole), regardless of the number that successfully encysted. These 20 puncture wounds are likely why the number of encysted *R. ondatrae* and plagiorchid cercariae were not negatively associated with toad health, and why toads that were exposed to these cercariae but had zero successfully encyst (*y*-intercept for the burden–fitness relationship) had significantly lower survival than toads that were not exposed to trematodes (Fig. 5). These results are consistent with the notions that larger size makes trematodes more virulent, that penetration of amphibian skin by cercariae is more immediately harmful to the amphibian than the trematode infection itself and that responses to trematode-induced skin injuries drive host differences in their costs of trematode exposure. However, other factors such as

the location of encystment, parasitic growth within the host and toxins produced by the parasite might also be important determinants of trematode virulence, and many more species of trematodes must be tested before we can confidently identify trematode traits that predict virulence.

Unlike past studies, we partitioned changes in tolerance into that which was putatively caused by exposure to parasites and that caused by subsequent infections (Fig. 1). Interestingly, the tadpole species significantly differed in their cost of parasite exposure, because they had similar vigours (survival in the absence of trematode exposure) but different *y*-intercepts for the relationship between parasite burden and host survival. However, they did not differ in their tolerance because the slopes of their relationships between survival and parasite burden were not significantly different. These findings suggest that the cost of parasite exposure might be an important component of the overall parasite tolerance of hosts and that researchers should not assume that the *y*-intercept of the relationship between host health and parasite burden will always match the average health of hosts not exposed to parasites.

### RELATIVE RESISTANCE AND TOLERANCE AND TRADE-OFFS

We hypothesized that high rates of parasite exposure and/or low pathogen virulence should select for host tolerance relative to resistance, whereas low rates of parasite exposure and/or high pathogen virulence should favour resistance relative to tolerance. This hypothesis was supported by a recent mathematical model (Restif & Koella 2004, p. E97). *Rana clamitans* was predicted to have higher relative tolerance to trematodes than *B. americanus* because *R. clamitans* tadpoles have higher exposure to trematodes (due to a longer larval period). The data for *E. trivolvis* corroborated this prediction, but there was no indication of a difference in relative tolerance for *R. ondatrae* or the plagiorchid. We also predicted that tadpoles should invest in relative resistance early in life but relative tolerance later in life, because trematodes were more deadly to younger than to older tadpoles. This prediction was corroborated for both host species in response to *E. trivolvis*, but was not supported for *R. ondatrae* or the plagiorchid. We suspect that these predicted responses to *E. trivolvis* but not to the other trematode species reflect local adaptation of frog species to a locally abundant trematode species. *Echinostoma trivolvis* is the only trematode species to have been observed naturally occurring in the two ponds where the frogs were collected (J. Rohr and T. Raffel, pers. obs.), and thus was the only parasite species tested for which host selection has certainly occurred. Although the responses of both tadpole species to this locally abundant trematode are consistent with the hypothesis that high rates of parasite exposure and/or low pathogen virulence should select for host tolerance relative to resistance, measurements of resistance and tolerance of many more host and parasite species, controlling for phylogenetic relationships, will be necessary to provide a rigorous test.

Several researchers have reported a trade-off between resistance and tolerance (Fineblum & Rausher 1995; Stowe 1998; Kover & Schaal 2002; Raberg, Sim & Read 2007), but others found no correlation between the two defence strategies (Simms & Triplett 1994; Mauricio, Rausher & Burdick 1997). Both negative and positive associations between resistance and tolerance are possible in mathematical models, depending on the environmental context (Restif & Koella 2004). Researchers have also speculated or demonstrated that hosts trade off defences against multiple natural enemies (Ayres & Schneider 2008; Ayres, Freitag & Schneider 2008; Raffel, Martin & Rohr 2008). Indeed, evidence suggests that polymorphisms increasing defence against one parasite might reduce defences against other parasites, leading authors to advocate conducting resistance-tolerance studies on multiple parasite species (Ayres & Schneider 2008; Read, Graham & Raberg 2008). However, demonstrations of trade-offs in many empirical studies might not be valid because resistance and tolerance are not independent of one another given that tolerance has parasite burden as the denominator. This is why we did not test for a correlation between resistance and tolerance in this study.

### IMPORTANT CAVEATS

It is important to note that the results of resistance-tolerance studies can depend on many factors, such as the length of the study, the quantified fitness or health parameter, and whether both behavioural and physiological resistance are quantified. For some factors and species, there might be a latency for their effects on fitness to become apparent (e.g. Rohr *et al.* 2006). Hence, the duration of the study can affect the conclusions, but few, if any, tolerance studies last long enough for every organism to have opportunities to reproduce and to die, often because of logistical constraints. At the southern ends of their ranges, *B. americanus* and *R. clamitans clamitans* can have larval periods of 3–4 and 7–9 weeks respectively (Skelly & Werner 1990). Hence, this 1-week study could account for up to a third and a seventh of the larval periods of these two species. Although this study could represent a considerable portion of their larval periods, 1 week is not likely to be a considerable portion of the larval periods at the northern ends of their ranges or a considerable portion of their entire life span. Hence, we cannot discount the possibility that a longer experiment would have produced different results. However, we believe this is unlikely for two reasons. First, 60% of the tadpoles that died did so within 2 days of exposure (23·5% daily mortality rate), with far fewer dying later in the experiment (average mortality rate of 11·8% for days 3–7), and species differences in the fitness effects of parasite exposure tended to persist or diverge through the experiment (Fig. S1). These patterns of mortality through time suggest that, if the experiment had been longer, the observed differences in the fitness effects of parasite exposure would have increased. Secondly, several studies document the persistent effects of larval environments on estimates of amphibian fitness (e.g. Smith 1987; Scott 1994; Carey, Cohen & Rollins-

Smith 1999; Relyea & Hoverman 2003; Rohr & Palmer 2005; Rohr *et al*. 2006).

Conclusions in tolerance studies can also depend on the chosen fitness proxy. Certain fitness proxies, such as growth, might not be as reliable as others, such as survival or reproduction. Often, the chosen fitness proxy is, again, a function of logistical constraints.

Hosts can use various strategies to defend against parasites and this too might affect conclusions about investments in resistance and tolerance (Boots & Bowers 1999; Restif & Koella 2003, 2004). We exposed our tadpoles to cercariae in small cups, isolating physiologically based mechanisms for resistance by preventing behavioural avoidance of cercariae. However, cercariae can induce tadpole avoidance and activity changes that can alter parasite infection rates (Koprivnikar, Forbes & Baker 2006; Rohr *et al*. 2009b), and these defence mechanisms might also influence overall levels of tadpole resistance and tolerance. Hence, inferences for every tolerance study should be limited to the study conditions.

Finally, it is also important to keep in mind that tolerance studies are inherently correlational because we cannot control the number of parasites with which a host is infected. That is, both burden and host health are response variables with error. Consequently, it is difficult to determine whether burden is the cause of the change in host health or whether host health is the cause of the change in burden.

### A COMMUNITY AND PARASITE PERSPECTIVE

Much of the emphasis on resistance and tolerance has focused on host–parasite interactions, but resistance and tolerance might also affect host–host interactions and thus species coexistence patterns (Raffel, Martin & Rohr 2008). For example, by having an adverse effect on the fitness of the parasite, resistant hosts could facilitate invasions and establishment of less resistant or less tolerant hosts (Raffel, Martin & Rohr 2008). Hence, hosts with high resistance might represent keystone species in communities, facilitating the persistence of hosts with inferior defences against given parasites (Raffel, Martin & Rohr 2008).

Resistance and tolerance should also have considerable effects on decisions made by parasites. For example, parasites might choose among hosts, or induce behavioural manipulations in vectors to facilitate host choice, based on fitness consequences of infecting particular host species (Raffel, Martin & Rohr 2008). Much of the work on host–parasite interactions has focused on resistance (Read, Graham & Raberg 2008; Raberg, Graham & Read 2009), but parasites likely select hosts based on both resistance and tolerance. Parasite fitness should be maximized by choosing hosts with low resistance and high tolerance. Indeed, supershedders and perhaps superspreaders, at both the individual and species level, are more likely to materialize when parasites differentially select hosts that have low resistance and high tolerance. Superspreaders are a considerable focus of disease management because of their disproportional impact on disease outbreaks (Lloyd-Smith *et al*. 2005), and thus understanding resistance and tolerance might have important management implications.

### APPLICATIONS FOR MANAGEMENT

Two out of three of the trematodes we studied are considered emerging infections of amphibians that appear to be driven by human activities, such as increases in pesticide and fertilizer use (Johnson & Sutherland 2003; Beasley *et al*. 2004; Skelly *et al*. 2006; Johnson *et al*. 2007; Rohr *et al*. 2008b,c). This suggests that amphibian trematodiasis might be controlled through landscape management. However, to effectively manage the health of any host population or community, we must first understand which parasites pose the greatest risk to which hosts and under what contexts. Otherwise, management efforts might be directed at host species that are relatively tolerant to infections, towards parasite species that are relatively benign to hosts or towards environmental contexts that pose little disease threat. By identifying particularly sensitive hosts and developmental stages, deadly parasites and inauspicious disease contexts, this study should serve to improve the prospects for effectively managing amphibian trematodiasis. Additionally, amphibian artificial selection and reintroduction efforts might benefit from selecting for amphibian tolerance to virulent pathogens because tolerance, unlike resistance, should limit the selection of parasite countermeasures. Given the importance of resistance and tolerance strategies to animal health, disease management and co-evolution, we encourage further research on resistance-tolerance investments in wild animals across multiple hosts, parasites and contexts.

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## Author contributions

J.R.R. was responsible for hypothesis and question development, experimental design and implementation, and oversight of the project. J.R.R. wrote most of the paper, but all authors contributed to the writing. T.R.R. oversaw the clearing and staining and cyst counting, conducted the statistical analyses and was integral to data interpretation. C.A.H. counted the cysts.

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## Supporting information

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Cumulative host mortality as a function of parasite exposure and experimental day.

**Table S1.** Statistical results for the interactive effects of tadpole and species and tadpole developmental stage on the proportion of cercariae encysted per tadpole.

**Table S2.** Statistical results for the interactive effects of tadpole and species and tadpole developmental stage on tadpole tolerance to metacercariae.

**Table S3.** Statistical results for the interactive effects of tadpole and trematode and tadpole developmental stage on tadpole tolerance to metacercariae.

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