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Resistance and tolerance: A hierarchical framework to compare individual versus family-level host contributions in an experimental amphibian-trematode system

William E. Stutz, Dana M. Calhoun*, Pieter T.J. Johnson

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

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ABSTRACT

Hosts have two general strategies for mitigating the fitness costs of parasite exposure and infection: resistance and tolerance. The resistance-tolerance framework has been well developed in plant systems, but only recently has it been applied to animal-parasite interactions. However, difficulties associated with estimating fitness, controlling parasite exposure, and quantifying parasite burden have limited application of this framework to animal systems. Here, we used an experimental approach to quantify the relative influence of variation among host individuals and genetic families in determining resistance and tolerance within an amphibian-trematode system. Importantly, we used multiple, alternative metrics to assess each strategy, and employed a Bayesian analytical framework to compare among responses while incorporating uncertainty. Relative to unexposed hosts, exposure to the pathogenic trematode (*Ribeiroia ondatrae*) reduced the survival and growth of California newts (*Taricha torosa*) (survival: 93% vs. 74%; growth: 0.29 vs. −0.5 vs mm day $^{-1}$). Similarly, parasite infection success (the inverse of resistance) ranged from 8% to 100%. Yet despite this broad variation in host resistance and tolerance among individual newts, we found no evidence for transmissable, among-family variation in any of the resistance or tolerance metrics. This suggests that opportunities for evolution of these traits is limited, likely requiring significant increases in mutation, gene flow, or environmental heterogeneity. Our study provides a quantitative framework for evaluating the importance of alternative metrics of resistance and tolerance across multiple time points in the study of host-parasite interactions in animal systems.

1. Introduction

Parasite infection can be a major cause of fitness variation among individual hosts. Consequently, hosts have evolved two, non-mutually exclusive but fundamentally different general strategies for reducing the fitness costs associated with parasite exposure (Baucom and de Roode, 2011; Restif and Koella, 2004). The first – **resistance** – can be defined as the ability to limit parasite burden following exposure. Highly resistant hosts may therefore exhibit little or even no infection despite substantial exposure to parasites in natural settings. The second – **tolerance** – can be defined as the ability to limit the fitness costs associated with a parasite burden. Plant biologists have long studied the relative importance of both strategies in response to attack by both herbivores and parasites, including their underlying physiological and evolutionary mechanisms (Clarke, 1986; Fineblum and Rausher, 1995; Strauss and Agrawal, 1999). In contrast, this framework has only recently been applied to animal disease systems (Adelman et al., 2013; Johnson et al., 2011; Read et al., 2008). For example, Råberg et al. (2007) illustrated the two strategies in families of laboratory mice exposed to virulent strains of malaria. While some mouse families exhibited relatively higher resistance (as measured by lower peak parasite density during the experiment), others exhibited higher tolerance (as measured by lower rates of red blood-cell and weight loss with increasing parasite burdens) (Råberg et al., 2007). The number of animal-based studies quantifying tolerance, its mechanisms, and its relationship with resistance has steadily grown (Ayres and Schneider, 2009; Gervasi et al., 2014; Hayward et al., 2014a; Lefèvre et al., 2010; Rohr et al., 2010). However, hosts' relative investments in parasite resistance and tolerance depends on parasite exposure frequency, the severity of fitness costs associated with infection, and the mechanisms available for each strategy (Baucom and de Roode, 2011; Reiss et al., 2009). Consequently, a broader understanding of tolerance and its relationship to resistance across natural animal systems requires further investigation in a variety of ecological and organismal settings.

* Corresponding author.
E-mail address: dana.calhoun@colorado.edu (D.M. Calhoun).

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Thus far, the resistance/tolerance framework has been difficult to effectively apply and quantify in animal systems for several reasons. First, tolerance is typically defined in terms of fitness or proxy measurements for fitness (Baucom and de Roode, 2011; Råberg et al., 2009), which can be challenging to measure among animal hosts that are highly mobile or difficult to observe over time under natural conditions (Kruuk, 2004). Second, it can be difficult, if not impossible, to control for exposure differences among animal hosts in free-living hosts. This introduces additional variation in observed parasite loads beyond that due to variation in resistance and tolerance, making it difficult to determine their relative contributions from snapshot measurements of infection in natural systems. While plants can often be exposed to parasites in situations that both mimic natural conditions and allow experimental control over exposure ("common garden" experiments), raising animals and exposing them to parasites in semi-natural or controlled settings may not be feasible in many animal-parasite systems. Third, accurately measuring parasitic loads in animals can present formidable methodological and estimation challenges. In some systems, parasite loads are measured only indirectly (e.g., fecal egg counts, Cattadori et al., 2014), while in other cases parasite burdens change temporally due to parasite growth, parasite reproduction, or host responses (Lefèvre et al., 2010). Both situations may make it difficult to link infection success, clearance, or parasite burdens with different fitness outcomes. Further, all of these challenges may trade off in any particular study system and approach, such that accurate estimates of fitness (or proxies for fitness such as growth) may preclude accurate measures of resistance or tolerance (or vice versa; Baucom and de Roode, 2011). These challenges thus present a non-trivial barrier in animal systems, which must be overcome in part or in whole to further our understanding of the role of both processes in host-parasite relationships.

Concurrently, it is important to recognize that there are multiple pathways or manifestations of resistance and tolerance within a single host-parasite system, each of which may incur differential costs or tradeoffs. Resistance can be broken down into the ability of hosts to resist infection shortly following exposure (hereafter, "resistance") and their ability to eliminate or clear infective stages over time (hereafter, "clearance"; Miller et al., 2005; Råberg et al., 2009). One or both types of resistance may operate in a given population, and appropriate experimental and statistical methods are required to differentiate them when comparing parasite loads at a given time point, age, or age class. In some situations (e.g., with many microparasites), resistance may also manifest as a reduction in parasite growth or reproduction within a host (Miller et al., 2005). Tolerance can similarly be measured in different ways depending on what aspects of fitness are most relevant for the host species and life-stage under investigation. For juvenile and pre-reproductive animals, maintaining growth rates or energy storage are often predominant strategies, while for adults, maintaining reproductive success may matter more. Survival is important for individuals across all life stages. Resistance and tolerance may both come with constitutive costs that are exacted upon the upregulation of defense strategies in response to exposure (Boots and Bowers, 1999; Lochmiller and Deerenberg, 2000; Restif and Koella, 2004), such that individuals or groups with low resistance or tolerance may have higher fitness when parasite exposure is low (Fineblum and Rausher, 1995; Simms and Triplett, 1994). These strategies may be mutually exclusive (Råberg et al., 2009), either because both mechanisms are costly (van der Meijden et al., 1988) but accomplish the same goal (fitness maintenance in the face of exposure), or because physiological limitations and negative genetic correlations prevent the evolution of both strategies simultaneously (Koskela et al., 2002; Råberg et al., 2009). Altogether, future progress in the study of animal systems requires a careful accounting and estimation of multiple measures of resistance and tolerance, their fitness costs in the absence of infection, and any potential trade-offs between them.

Despite these challenges, several recent studies have illustrated how both resistance and tolerance can be investigated in experimental and free-living animal systems. To date, these studies have often taken a quantitative genetic approach, focused on estimating the amount of additive genetic variation and co-variation within animal populations and thus the capacity for evolution by natural selection (Hayward et al., 2014a, 2014b; Lefèvre et al., 2010; Mazé-Guilmo et al., 2014). This is particularly important because selection for increased tolerance can have different evolutionary and epidemiological consequences compared with selection for increased resistance (Miller et al., 2005; Roy and Kirchner, 2000). Theory suggests that selection for resistance will result in a negative feedback between parasite abundance and the selective advantage of resistance; specifically, selection for resistance alleles will result in them becoming more common. This should maintain genetic variation in resistance in populations, allowing hosts to co-evolve in response to changing parasite pressures (Rigby et al., 2002). In contrast, increased tolerance can prolong the lifespan of infected hosts or increase their parasite burdens, generating a positive feedback between parasite abundance and the selective advantage of tolerance and reducing genetic variation in tolerance to near zero. So far, many studies have found evidence for additive genetic variation in resistance in both natural and laboratory systems (Hayward et al., 2014b; Lefèvre et al., 2010; Mazé-Guilmo et al., 2014). However, evidence for additive genetic variation in tolerance (0; Hayward et al., 2014b; Lefèvre et al., 2010; Mazé-Guilmo et al., 2014) as well as correlations between resistance and tolerance (Corby-Harris and Promislow, 2008; Mazé-Guilmo et al., 2014; Råberg et al., 2009) are equivocal, indicating that the evolution of tolerance may be system- and context-dependent.

Genetically similar groups (e.g., siblings) offer a novel opportunity to compare resistance and tolerance metrics. For example, experimental tests involving genetically linked groups can facilitate testing of the relative efficacy of resistance and tolerance mechanisms as a function of environmental drivers, experimental treatments, and population source (Corby-Harris and Promislow, 2008; Stevens et al., 2007). As an illustration, Lefèvre et al. (2010) experimentally exposed 19 different family lines of monarch butterflies (*Danaus plexippus*) to varying doses of a protozoan parasite (*Ophryocystis elektroscirrha*) with the aim of quantifying resistance and tolerance. They concluded that there were significant differences among host families, such that the relationship between exposure dose and the resultant spore load varied by monarch family. Interestingly, there was no genetic variation among families in their tolerance following infection. In contrast, Stevens et al. (2007) found substantial genetic variation in both resistance and tolerance among aspen trees (*Populus tremuloides*) grown under low- and high-nutrient conditions. These results indicated that aspen genotypes differ in their constitutive levels of resistance as well as nutritional quality. Understanding how resistance and tolerance covary across groups that vary in their spatial, environmental, or biological identities will thus be key to future progress developing the resistance-tolerance framework in animals.

Here, we used an amphibian-macroparasite system to experimentally quantify the degree to which multiple measures of both resistance and tolerance vary among host individuals versus families (sibling groups). Macroparasite systems provide a number of advantages for investigating resistance and tolerance (Johnson et al., 2011; Koprivnikar et al., 2012; Rohr et al., 2010). Because exposure dose can be experimentally controlled and, in most cases, parasites do not directly replicate within the host, resistance can be directly quantified as the inverse of the number of observed parasites relative to the number administered (Cattadori et al., 2014; LaFonte et al., 2015). Similarly, clearance of individual parasites – which is an often overlooked component of resistance – can be estimated by measuring parasite loads across time (LaFonte and Johnson, 2013). Lastly, parasite burden often links directly with relevant measures of fitness, such as host survival or growth or even reproduction in some cases; (Johnson et al., 2012). For the current study, we experimentally exposed lab-raised newt (*Taricha torosa*) larvae of different genetic families to varying dosages of the

pathogenic trematode, *Ribeiroia ondatrae*. By quantifying infection across multiple time points and in association with multiple measures of host fitness (e.g., survival and growth), we assessed the relative influence of individual- and family-level host variation in determining resistance and tolerance. To analyze our data, we apply mixed models within a Bayesian framework, thereby allowing for the estimation of among-group variances in resistance, tolerance, general vigor, costs of exposure, and any tradeoffs between these traits. We emphasize how a hierarchical Bayesian approach is conceptually similar to the ANCOVA approach advocated by Råberg et al. (2009), while facilitating explicit quantification of uncertainty in the estimates of resistance and tolerance among populations, hosts, or species.

2. Material and methods

2.1. Study system

We used juvenile (stage T1 − T2; Wong and Liversage, 2004) California newts (*Taricha torosa*) collected as eggs from free-living populations in California as the host species for our experiment and the trematode *Ribeiroia ondatrae* (hereafter "*Ribeiroia*") as the parasite. Infection with *Ribeiroia,* which can use many larval amphibians as intermediate hosts, is associated with decreased host survival and growth and has been linked to high rates of malformation in amphibian populations (Johnson et al., 2013). There currently exists experimental evidence for among-species variation in both initial resistance to *Ribeiroia* infection as well as the ability to clear parasites after exposure (LaFonte and Johnson, 2013), although less is known about tolerance specifically (Johnson et al., 2012). Infective stages of *Ribeiroia* (cercariae) can be administered in exact quantities to individual newts and the resulting level of infection can be quantified at specific time points. We also took advantage of the fact that *T. torosa* mothers lay eggs in discrete clutches, thereby allowing us to isolate and raise sibling families (Kaplan, 1985; Verrell, 1989). This facilitated treating families as groups and estimating the relative contribution of individual- and family-level variation to multiple fitness, resistance and tolerance traits ("transmitted variance", Mazé-Guilmo et al., 2014). Because sibling relationships are only known through mothers, and maternal as well as sib-social effects (e.g., aggressive behavior) may contribute to variation in resistance and tolerance, we refer to among-family variance as "transmitted" rather than strict additive genetic variance. Lastly, because individual newts can be necropsied only once, we can estimate whether newts were clearing infection over the time-course of the experiment by comparing *Ribeiroia* infection within families across successive time points rather than using specific individual hosts.

2.2. Sample collection and animal husbandry

Thirty *T. torosa* egg masses were collected from two neighboring ponds in Santa Clara County, California, and shipped overnight to the University of Colorado in late March 2014. Initial clutch sizes ranged from 12 to 27 eggs. Eggs within a single mass were assumed to come from a single *T. torosa* mother, although the potential for multiple fathers to contribute genetic material make hatching individuals half-siblings (Kaplan, 1985). Egg masses were placed into separate 10 L containers (containing 5 L of dechlorinated, UV-sterilized, and carbon-filtered tapwater [hereafter referred to as 'treated' water]) in a 23 °C environmental chamber and allowed to hatch. Newts were fed brine shrimp nauplii ad libitum and allowed to reach stage 2T (Wong and Liversage, 2004), at which point they were individually transferred to 1.5 L containers containing 1 L of treated water. The identity of each individual host's initial egg mass was carefully tracked and randomized among treatments, meaning that treatments varied such that no treatment only contained one mother's eggs. The experiment was started within two days of transferring the newts to individual containers. Newts were kept at 23 °C and a 12:12 day:night photoperiod during the experiment.

2.3. Experimental design

We utilized a 2 × 4 factorial design with two exposure treatments (exposed vs. sham exposed) along with 4 "endpoint" measurements (36, 96, 168, or 240 h post-exposure). Endpoints were randomized among individuals within families. Exposure treatments were divided randomly among individuals within a family assigned to the same endpoint, such that in the treatment 36 h we had two treatments exposed and sham. The 36 and 240 h treatments were prioritized to ensure large sample sizes for the first and last endpoints.

To obtain *Ribeiroia* cercariae for the experiment, we collected rams horn snails (*Helisoma trivolvis*) from multiple field sites and screened them for infection by isolating individuals in 50-mL centrifuge tubes overnight (Calhoun et al., 2015; Hannon et al., 2017; Paull et al., 2012). *Ribeiroia* cercariae were identified by the presence of esophageal diverticula (Johnson et al., 2004; Schell, 1985). We isolated and pooled cercariae from multiple infected snails and administered 25 newly-emerged (< 6 h) cercariae to each newt host. Sham-exposures were performed using identical procedures but with water only. Experimental animals were randomly exposed to *Ribeiroia* in four, consecutive infection of cercariae over the course of 3.5 weeks. Top-down digital photographs with a visual length reference were taken of all newts to obtain length measurements both before and at the close of the experiment.

Newts that survived to their respective endpoints were euthanized in a 0.5% solution of MS − 222 buffered in sodium phosphate, photographed to measure body size, and necropsied to quantify *Ribeiroia* metacercariae. Animals that died prior to respective endpoints we treated the similarly and were included in resistance model (see below). We used the computer program tpsDig (Rohlf, 2004) to obtain total length measurements for each newt at the beginning and end of the experiment (beginning- and end-length, respectively). Because of the co-habitation among siblings, some newts had damage to the tips of their tails and we therefore noted the presence of tail damage (yes/no) and added the mean length difference between damaged and undamaged newts (0.5 mm) to damaged newts prior to analysis. The adjusted lengths averaged 16.0 mm (range: 12.4–20.7) at initial exposure. We calculated growth rate as the difference in end- and beginning length divided by the number of hours between exposure and endpoint. Growth rates were then back-converted to growth per day.

2.4. Modeling resistance

We defined two types of resistance strategies in this experiment. The first – initial resistance – was defined as the probability of a non-successful infection by a *Ribeiroia* cercariae at 36 h post exposure (or 1 minus infection success). The second measure – clearance – was defined as the rate at which average *Ribeiroia* metacercariae within hosts declined across endpoints. We used our model to estimate the independent contributions of both individual newts and newt family in determining resistance, whereas contributions to clearance could be estimated only at the family level because they depended on comparison of averages among groups over time. We also used the model to asses covariation in the family effects (cf. "relative resistance", Rohr et al., 2010) to determine whether highly resistant families were also those that exhibited high clearance. All newts surviving to their assigned end points were included in the analysis.

To obtain estimates of the individual and family-level contributions to initial resistance and clearance, we modeled the observed *Ribeiroia* counts y for individual newts $i = 1, \ldots, I$ in half-sib family $j = 1, \ldots, J$ using a hierarchical model:

$$y_i \sim Binomial(k, p_i)$$
$$logit(p_i) = \beta_0 + X_i\beta + \gamma_{j[i]}x_{i,time} + \alpha_i + \alpha_{j[i]} + \alpha_{wave[i]} + \alpha_{block[i]} \quad (1)$$

Here p indicates the probability of infection with a single *Ribeiroia* metacercaria and $k = 25$ cercariae added in all cases, where k is the total number of cercariae administered. The design matrix X contains parameters governing the effects of tail damage (mean-centered binary data), family mean beginning-length (centered and scaled among families), clutch size, and individual beginning-length (centered and scaled within families). It also contains a fifth parameter $\beta_{time,}$ which is the effect of time (in hours) post-exposure on the observed *Ribeiroia* counts. If β_{time} is less than one, it indicates that newts (on average) were eliminating *Ribeiroia* metacercariae over time. To simplify interpretation of the model, time post-exposure (t) was centered at $t = 36$ h prior to fitting so that all random intercept terms for resistance are estimated at 36 h post exposure.

The four groups of α effects model the contributions of individual newts (α_i), family ($\alpha_{j[i]}$), infection wave ($\alpha_{wave[i]}$) and the family location in experimental room ('block') prior to separation of individual newts ($\alpha_{block[i]}$) on the probability of successful infection. Each is modeled as a random normal variable with mean = 0 and its own standard deviation σ. The $\alpha_{j[i]}$ effect can be interpreted as the variation in the initial infection success due to family-level variation in resistance. The γ_j parameters model the family-level contributions of the effect of time on *Ribeiroia* counts (i.e. clearance) and are drawn from a normal distribution with mean = β_{time} and standard deviation σ_γ. Note that these γ_j effects are conceptually equivalent to the effects estimated by an interaction between time and family in a traditional ANCOVA, but here the family-specific slope estimates are shrunk toward the overall effect as dictated by the within-family samples sizes and the observed data while also providing explicit measures of uncertainty.

The family-level standard deviations σ_α and σ_γ are jointly modeled by the covariance matrix Σ_j, which contains the element $\rho_{\alpha\gamma}\sigma_\alpha\sigma_\gamma$ on the off-diagonals, where $\rho_{\alpha\gamma}$ is the correlation between the family-level effects on *Ribeiroia* counts at 36 h and on clearance rates. A positive correlation would indicate that families with higher than average resistance also had larger than average clearance rates.

To determine what proportion of the variation in initial resistance (after controlling for the other effects in the model) was due to among- vs. within-family variance, we estimated the repeatability of *Ribeiroia* counts within families at 36 h as:

$$R_j = \sigma_j^2 / (\sigma_i^2 + \sigma_j^2) \quad (2)$$

The repeatability R_j represents an approximate upper bound on the transmittable variance of resistance, taking values between 0 and 1. $R = 1$ means that all of the variation among newts, after marginalizing out the other effects in the model, is captured by family identity. $R = 0$, in turn, means that all of this variation is instead associated with individual host-level variation. Identical repeatability estimates can be made whenever among- and within-family variance in a trait can be estimated.

2.5. Modeling survival-based tolerance

As with resistance, we used two different metrics of tolerance in our experiment. First, we assessed whether increasing *Ribeiroia* loads affected the probability of survival of individual newts (hereafter "survival tolerance"). We also assessed two additional metrics related to survival: the survival probability of unexposed newts and the survival probability of newts exposed to *Ribeiroia* yet independent of infection ("cost of exposure", Rohr et al., 2010). We termed the survival of unexposed newts as "robustness" to distinguish it from the similar estimate for growth rate (for which we used the more standard term "vigor"). As with resistance, we were interested not only in the overall measures of survival tolerance, robustness, and cost of exposure, but also whether these metrics varied among families.

To estimate survival probabilities, we built a hierarchical model to predict the survival (yes/no) of individual newts $i = 1, \ldots, I$ in half-sib family $j = 1, \ldots, J$. The model:

$$y_i \sim Bernoulli(p_i)$$
$$logit(p_i) = \beta_0 + X_i\beta + \delta_{j[i]}x_{i,rib} + \gamma_{j[i]}x_{i,treat} + \phi_{endpoint[i]}x_{i,rib} + \alpha_{j[i]} + \alpha_{endpoint[i]} + \alpha_{wave[i]} + \alpha_{block[i]} \quad (3)$$

includes design matrix X, which contains parameters governing the effects of tail damage, family mean beginning-length, clutch size, individual beginning-length as before. Matrix X also contains parameters β_{rib} and β_{treat}, which indicate the overall effect of *Ribeiroia* metacercariae counts and exposure treatment (independent of infection) on survival probability. Thus β_{treat} is an estimate of the cost (in survival probability) associated with exposure that is independent of infection. Here, the intercept parameter β_0 is the estimated survival of unexposed individuals. *Ribeiroia* counts were scaled by a factor of 0.05, such that β_{rib} was the expected change associated with an increase of 20 *Ribeiroia*.

The model also contained seven effects that varied across different groups. The four α effects modeled the contributions of family J, endpoint, infection wave, and experimental blocking on survival probability, each with mean = 0 and separate standard deviations σ. In this case α_j models family-level contributions to robustness. The δ_j, and γ_j effects indicate the family level contributions to the overall effects of *Ribeiroia* count and exposure treatment, respectively, on survival probability. The $\phi_{endpoint}$ effects of *Ribeiroia* counts on survival probability across different endpoint treatments, accounting for the possibility that increased counts contributed more (or less) to differences in survival as the experiment progressed. The α_j, δ_j, and γ_j effects are draw from a multivariate normal distribution with means equal to zero (α_j) or their associated β parameters (δ_j, and γ_j) and standard deviations σ_α σ_δ and σ_γ jointly modeled by the covariance matrix Σ_j. As in the resistance model, this matrix allows us to determine whether robustness, cost, and survival tolerance are correlated at the family level. The $\alpha_{endpoint}$ and $\phi_{endpoint}$ effects were drawn from a similar multivariate normal distribution, $\Sigma_{endpoint.}$

2.6. Modeling growth-based tolerance

The second metric we used to assess tolerance was the growth rate (in mm day^{-1}) of individual newts. The change in growth rate associated with increased *Ribeiroia* load was designated as "growth tolerance". As with survival, we assessed two additional metrics related to growth rate: the growth rate of unexposed newts (hereafter "vigor") and the change in the expected growth rate due to exposing newts to *Ribeiroia* (i.e., the cost of exposure). To predict the growth rate y in individual $i = 1, \ldots, N$ in family $j = 1, \ldots, J$, we used the model:

$$y_i \sim N(\mu, \sigma_i)$$
$$\mu_i = \beta_0 + X_i\beta + \delta_{j[i]}X_{i,rib} + \gamma_{j[i]}X_{i,treat} + \phi_{endpoint[i]}x_{i,rib} + \alpha_{j[i]} + \alpha_{endpoint[i]} + \alpha_{wave[i]} + \alpha_{block[i]} \quad (4)$$

All the model parameters specified here have the same basic interpretation as described previously, only here we are predicting the expected growth rate rather than the (logit) probability of survival. There is also one additional parameter, σ_i, which is the residual (i.e. newt-level) error. Because we could estimate newt-level variation in growth rate, we were also estimate within-family repeatability of vigor in the same way as repeatability in infection success. All predictors were scaled prior to model fitting as described in the survival model.

2.7. Parameter estimation

Parameters were estimated using Bayesian probability by combining likelihood specifications for each model with prior probability

distributions for all specified parameters. Prior information was available from a similar previous experiments for the average survival (Johnson et al., 2012) and infection success (Johnson et al., unpublished data) in *T. torosa*, which were incorporated with added uncertainty into the intercept priors for those two models. To induce some shrinkage away from biologically implausible parameter values (e.g., variances on the logit scale > 2), we used weakly informative Normal priors for all non-intercept β parameters and half-Normal priors for all family, wave, endpoint, and block-level standard deviations, σ. We applied separate half-Normal hyper-prior distributions with mean = 0 and sd = 1 across all β and across all σ, respectively. LKJ priors with shape = 2 (which induce moderate shrinkage away from extreme correlations) were used for all correlation matrices. Samples from the joint posterior distributions were drawn using the Markov Chain Monte Carlo (MCMC) algorithm in the probabilistic programming language Stan (Stan Development Team, 2014), implemented using the *rstan* package in R (R Core Team, 2014). Parameters that are constrained to be positive are summarized using posterior modes and 95% high probability density intervals (HPDI) intervals. All other parameters are summarized using posterior medians and 95% HPDIs.

2.8. Correlations between resistance and tolerance

To determine whether there was any correlation in the family-level effects on resistance and tolerance, we first randomized the order of the 4000 posterior draws for resistance effects (α_j) and clearance effects (γ_j) from model 1 along with the 4000 tolerance effects (δ_j) obtained from models 3 (survival) and 4 (growth rate). We then calculated the Pearson correlation coefficient r between resistance and tolerance effects and between clearance and tolerance effects at each of the 400 randomized draws, giving us four estimates for the correlations between resistance and tolerance. Note that more precise estimates for these correlations could potentially be obtained by combining the above three models into a single probability model that also included the corresponding correlation parameters. However, our provisional results (described below) using the simulation method gave no indication for correlations across models, and so simplicity of presentation each of the three models was fit separately.

3. Results

3.1. Resistance: initial infection success and subsequent clearance

In total, we used 361 newts from 30 half-sib families, including 193 exposed and 168 unexposed (sham-exposed) individuals. Among surviving exposed newts, observed *Ribeiroia* counts ranged from 2 to 25 with a mean of 10.5 metacercariae, or 42% of the 25 cercariae administered to each individual. Most infections occurred around the hindlimbs and mandible of individual newts (Fig. 1).

The probability of infection for a single *Ribeiroia* cercariae at 36 h was 0.39 [0.19, 0.64 HPDI], indicating that host's initial resistance to infection averaged around 61% at that time point. The estimated resistance was unaffected by factors such as tail damage ($\beta_{tail} = 0.10$, [-0.11, 0.42]), clutch size ($\beta_{clutch} = -0.06$ [-0.23, 0.06]), family mean beginning-length ($\beta = -0.06$ [-0.08, 0.22]), or individual beginning-length ($\beta_{length} = 0.03$ [-0.10, 0.21]). With respect to clearance, observed *Ribeiroia* counts decreased from an average of 11.6 at 36 h post-exposure to 9.1 at 240 h post-exposure (Fig. 1), although the high-probability interval around the effect of time included zero ($\beta_{time} = 0.02$ [-0.19, 0.12]; Fig. 2).

In other words, after accounting for the other effects in the model, there was insufficient evidence that *T. torosa* were clearing infections over the time scale of the experiment (10 days).

Based on comparisons of among-host and among-family variation in the probability of successful infection at 36 h, we found very little support for transmissible heritable variation in resistance (Fig. 3).

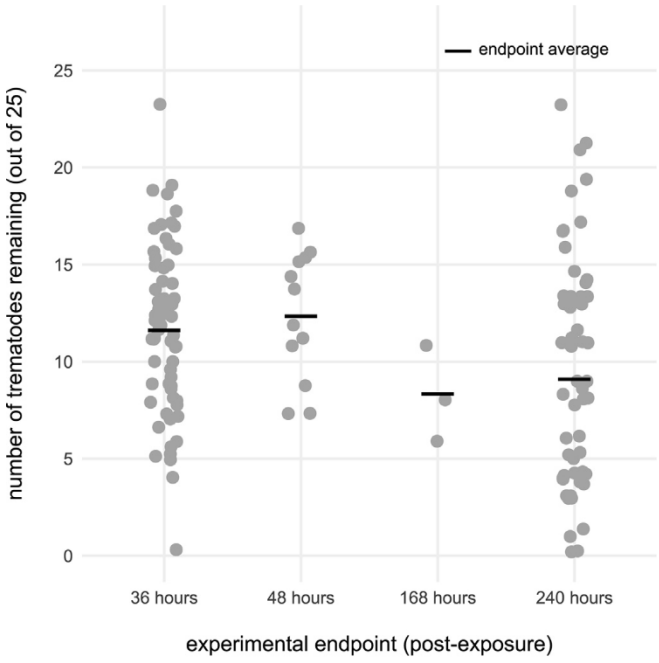

Fig. 1. Observed infection success: Number of *R. ondatrae* trematodes (out of 25 administered) observed in each newt upon dissection. Only newts in the 'infected' treatment are included. Black bars show the mean number of trematodes at each endpoint treatment. Grey dots represent an individual infected newt.

While there was substantial variation among individual newts in the probability of infection ($\sigma_i^2 = 0.44$ [0.29, 0.71]), this was not explained by egg clutch identity (family-level variation), for which the posterior distribution clustered near zero ($\sigma_j^2 = 0.00$ [0.00, 0.09]). The resulting repeatability estimates were similarly close to 0 ($R_j = 0.00$ [0.00, 0.17]), with an absolute upper bound of about 17%. There was also no evidence for among-family variation in clearance ($\sigma_\gamma^2 = 0.00$ [0.00, 0.00]), nor was there any posterior correlation between resistance and clearance among families ($\rho = -0.11$ [-0.85, 0.72]).

3.2. Tolerance: survival

Overall, 76% of newts exposed to *Ribeiroia* survived to the measurement endpoint, compared with 93% of the sham-exposed (control) hosts (estimated robustness of sham-exposed individuals β_0: 0.87 [0.47, 0.98]). Correspondingly, we found an overall negative effect of *Ribeiroia* infection load on the probability of survival ($\beta_{rib} = -2.3$ [-4.3, 0.3]; Fig. 4).

Interestingly, the effect of *Ribeiroia* on survival was greatest among the 96, 168, and 240 h endpoints, but with no effect at 36 hourr post-exposure. At these later endpoints, survival probability decreased by between 0.42 and 0.49 among exposed relative to unexposed individuals (Fig. 5).

There was no discernible effect of parasite exposure independent of infection (i.e., there was no cost of exposure after accounting for parasite load, $\beta_{treat} = -0.05$ [-1.21, 0.85]). Host initial-length had a small, positive effect on survival probability ($\beta_{length} = 0.76$ [0.34, 1.18]), such that survival increased by about 2% for individuals one standard deviation larger than their family average (Fig. 6).

We found no evidence that family initial-length ($\beta = 0.02$ [-0.37, 0.47]), clutch-size ($\beta_{clutch} = -0.04$ [-0.55, 0.32]), or the presence of tail damage ($\beta_{tail} = -0.39$ [-1.20, 0.18]) affected survival.

Although Bernoulli models do not estimate individual (i.e. residual) level variance, we nonetheless were able to estimate the among-family variance in robustness, costs of exposure, and the effect of *Ribeiroia* load on survival (tolerance), as well as any correlations among these

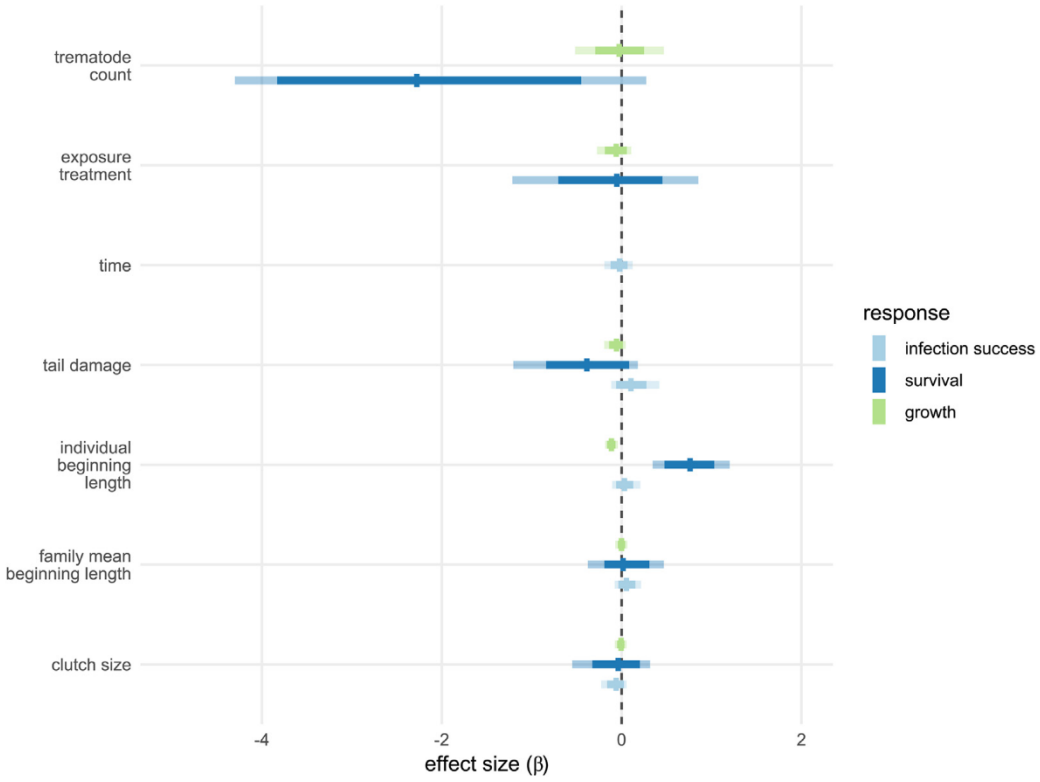

Fig. 2. Effect size (β) estimates: estimated effect sizes across all three models (infection success, growth, and survival). Short vertical lines indicate posterior means; darker bars indicate 80% HDPI; lighter bars indicate 95% HDPI. 'Missing' bars indicate cases where a particular effect was not included in the model. To better compare across models, effect sizes have *not* been back-transformed to the raw data scales.

responses. Posterior family-level variances were clustered around zero, suggesting that family-level variation for all three traits had a low probability of being biologically meaningful (e.g., $\sigma^2 > 0.1$). We also found no evidence for correlations between these traits among families (Fig. 7).

3.3. Tolerance: growth rate

Observed growth rates differed substantially between exposure treatments over the 10 day observation period, with a mean growth rate of -0.05 mm day^{-1} in unexposed newts compared with -0.29 mm day^{-1} among exposed newts (a nearly 6-fold difference). The effect of *Ribeiroia* load on growth rate was only notable at 36 h ($\beta_{treat} = -0.11$ [-0.20, -0.01]), with effects leveling off to near-zero at the 96, 168, and 240 h endpoints (Fig. 5). Of the other individual- and family-level predictors in the model, only host initial-length affected subsequent growth rate: newts that were 1 standard deviation larger at the beginning of the experiment had a growth rate that was 0.11 [0.04, 0.18] mm day^{-1} lower than average-sized individuals (Fig. 6). Consistent with the low observed growth rate in sham-exposed newts, the model-estimated vigor was near zero ($\beta_0 = -0.07$ [-0.27, 0.14] mm day^{-1}). There were also no discernible differences in vigor estimates across waves and endpoints, suggesting that growth rate (independent of infection) was not affected by the timing of the experiment nor the time intervals between exposure and endpoint.

As with the previous two models, family identity (based on the egg mass source) accounted for relatively little of the observed variation in newt growth rates, costs of exposure, or growth-based tolerance. While there was appreciable variation in growth rates among individual newts ($\sigma_i^2 = 0.48$ [0.44, 0.53]), estimates of among-family variation (the α_j, δ_j, and γ_j terms) all had posterior distributions clustered near zero-variance. Further, because of the large disparity between residual (i.e. individual) and among-family level variances, the resulting intra-family repeatability (see equation [2]) estimate for vigor was also clustered near zero ($R_j = 0.00$ [0.00, 0.10]; Fig. 7).

3.4. Correlation of resistance and tolerance

Resistance and tolerance were relatively uncorrelated among families. The simulated correlations of resistance with survival-based tolerance ($r = 0.04$ [-0.36, 0.38]) and growth-based tolerance ($r = -0.03$ [-0.38, 0.33]) were both centered around zero. Correlations between clearance and survival-based tolerance ($r = 0.06$ [-0.32, 0.41]) and growth-based tolerance ($r = -0.01$ [-0.36, 0.34]) were similarly uncertain. Given the lack of posterior variance in these traits, covariances between these traits is likely small and not biologically meaningful.

4. Discussion

Applications of the resistance-tolerance framework in animal-parasite systems are lacking relative to studies in plants, and only recently has work in animals systems expanded beyond model systems to more broadly include wild mammal (Hayward et al., 2014b), fish (Mazé-Guilmo et al., 2014), amphibian (Rohr et al., 2010), and insect (Ayres and Schneider, 2009; Lefèvre et al., 2010) host populations. Nonetheless, several challenges persist in the effective application of this paradigm to non-model systems. For instance, both resistance and tolerance can be measured in multiple, alternative forms, which for animal hosts can include initial resistance or post-infection clearance (both measures of resistance) and survival- or growth-mediated changes associated with infection (different measures of tolerance). Because such effects may also vary with the time-point at which infection is measured as well as the environmental or genetic conditions of the host, it is important to use an experimental approach to explicitly quantify the relative and interactive influences of each component.

Here, we exposed newt hosts (*T. torosa*) to a fixed dose of the virulent trematode, *R. ondatrae*, in a controlled laboratory experiment. By dividing hosts from the specific clutches into alternative treatments, we tested the relative influence of host-vs. family-level variation on the observed responses in both resistance and tolerance. Using a hierarchical Bayesian approach to estimation, we found substantial, among-

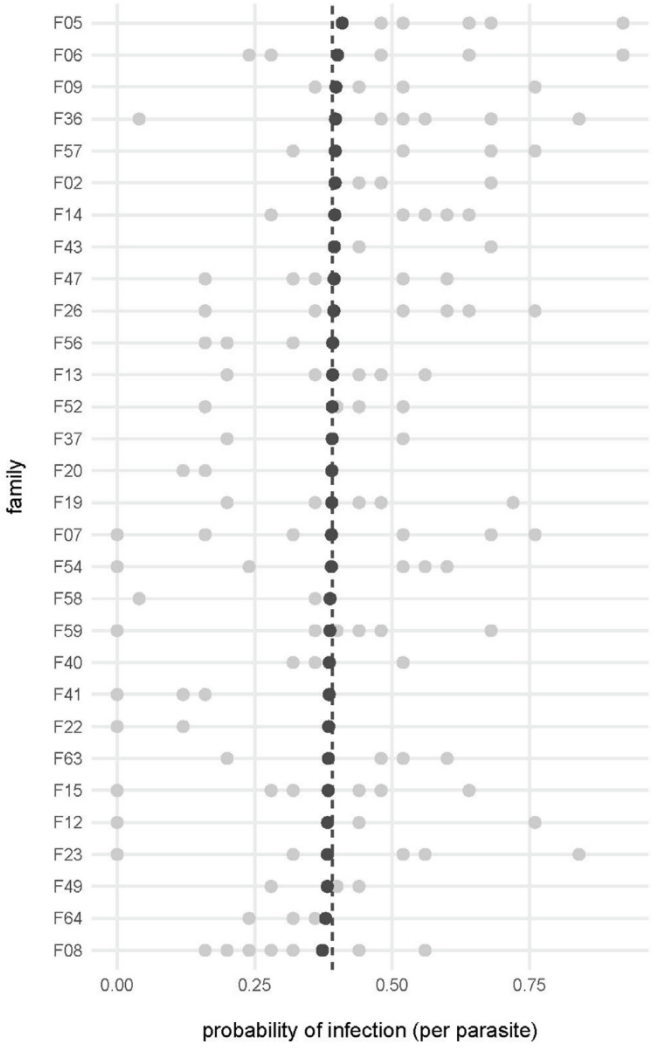

Fig. 3. Infection success by family: model-estimated, family-level effects (dark grey circles) on the probability of infection (per parasite). Observed probabilities of infection (per parasite) for individual newts are shown as light grey circles. The dashed vertical line indicates the experiment-wide probability of infection.

host variation in resistance to *Ribeiroia* after 36 h. Host survival and growth rate both decreased with increasing infection load, although these responses depended strongly on the time point of observation, with growth effects manifesting early (within 36 h) and survival effects more prominent later in the experiment. Despite considerable heterogeneity in the responses of individual newts to parasite exposure, the specific egg mass from which a host was obtained had little to no influence on the observed resistance, clearance, tolerance, or fitness costs. Overall, these results indicate that while *Ribeiroia* exposure has significant effects on host growth and survival in as little as 36 h, the influence of genetically transmittable or family-level drivers in dictating host resistance or tolerance over the studied time period (10 days) was relatively small. Future studies incorporating these measures of resistance and tolerance over longer time periods, particularly up to and through metamorphosis, may yet reveal additive genetic variance for these traits.

Results of the current experiment indicate that newt hosts were able to resist, on average, about 61% of the attempted infections of *Ribeiroia* cercariae by 36 h post-exposure. This value is consistent with estimates from other *Taricha torosa*, which typically range from 20–65% over the same time interval (Johnson unpublished data), which is less than bluegill (92% resistant; Calhoun et al., 2015). Given that trematode cercariae have a relatively short time-frame within which to find a suitable host (Hannon et al., 2017), some fraction of these parasites likely fail to locate the target host within the experimental arena before expiring. Other parasites may be prevented from successful establishment through host defensive strategies, including anti-parasite behaviors (Daly and Johnson, 2011; Hart and Hart, 2018; Koprivnikar et al., 2006) as well as immunological forms of resistance (LaFonte et al., 2015; McMahon et al., 2014). Previous studies have suggested that amphibian host resistance (or inversely, their suitability for infection or competence) varies considerably among different species (Johnson et al., 2012; Rohr et al., 2010; Stockwell et al., 2016). The current experiment builds upon these past findings by explicitly quantifying the influence of within-species variation (i.e., among different families) on initial resistance as well as subsequent clearance. More specifically, the egg mass from which newt hosts were derived had little effect on either form of resistance, despite significant among-individual variation. Indeed, we found no evidence for any clearance-based resistance, either overall or within families, over the 10 days of the experiment. In part, this may stem from the short time-span of the study; a previous experiment in which *Ribeiroia*-exposed newts were raised to metamorphosis reported an average infection success of 22% (Johnson et al., 2012), suggesting this species may clear metacercariae over longer time periods. Relating observed infection levels to measures of fitness can be challenging when hosts are clearing infection across time, as it can be difficult to estimate the change in fitness associated with dynamic (as opposed to static) infections. Given the lack of clearance in this study, observed *Ribeiroia* counts at successive endpoints likely did not differ systematically from the initial infection success at 36 h, making our tolerance estimates less challenging to estimate. Relatively little is known about how newts and other amphibians clear metacercariae; exposure to exogenous corticosterone tends to lessen the rate of parasite clearance (LaFonte and Johnson, 2013), whereas variation in water temperature has little effect (Altman et al., 2016) nor does antimicrobial peptides (Calhoun et al., 2016).

With respect to newts specifically, recent evidence has suggested that the concentration of tetrodotoxin in the skin of species of *Taricha* can inhibit the success of invading micro- and macroparasites. This neurotoxin interferes with the propagation of signals through sodium channels, ultimately leading to paralysis or even death among exposed organisms. While often studied in the context of its effects on potential predators, tetrodotoxin has been shown to reduce the survival of the infectious stages for 5 different species of trematode parasites (Calhoun et al., 2017). Correspondingly, adult newts (*T. torosa* and *T. granulosa*) with higher tetrodotoxin (TTX) concentrations in their skin tend to support lower parasite richness and reduced probabilities of microparasite infection (Johnson et al., 2018). Maternal investment of TTX into deposited egg masses is hypothesized to help protect embryonic and larval newts against natural enemies, which could parasitic infections. Because TTX concentrations ought to vary among egg masses as a function of maternal identity, it is somewhat surprising that we did not detect more of an influence of egg mass origin on infection success. However, TTX levels were not explicitly measured in this study, and the short timespan of the experiment (10 days) and the small number of ponds from which egg masses were collected (n = 2) may have precluded our ability to detect such effects. Subsequent studies that are able to measure level of TTX prior to exposures and include multiple pond locations will be of large value as they would provide the ability to test effects of mother influenced TTX level on natural enemies and whether those effects vary with pond attributes.

Higher loads of infection also caused significant reductions in host survival and growth rate. However, these effects depended critically on the time point of observation. In the first 36 h post-exposure, less-resistant individuals survived equally well as more resistant individuals, but tended to have lower growth rates. In contrast, after 36 h, less-resistant individuals had equivalent growth rates but were more likely to die. This interesting pattern suggests that *Ribeiroia* infection initially

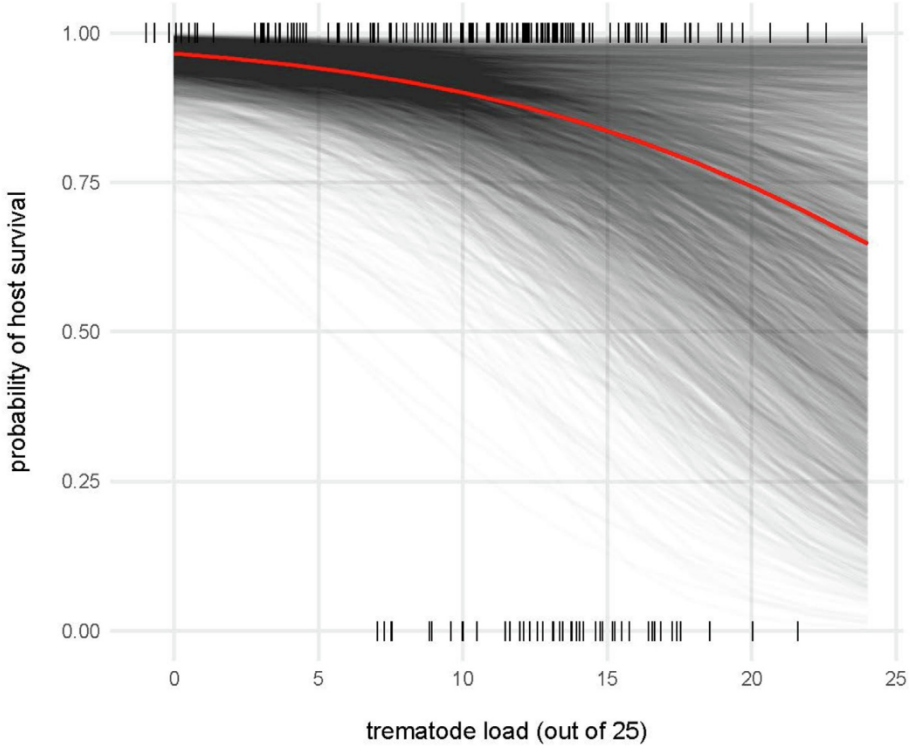

Fig. 4. Effect of trematode load on probability of host survival: Lines shows the model-estimated change in the probability of host survival of increasing trematode load (out of 25). The red line shows the posterior median; grey lines show estimates from all individual draws for the joint posterior. Rugs along the x-axis at 0 and 1 indicate survival of individual hosts.

reduces growth, which affects subsequent survival, but that newts able to maintain growth rates over the first 36 h suffered no later consequences in terms of mortality. The difference in survival as a function of newt resistance over time most likely led to the observed decrease in infection load (about 2.5 metacercariae) over the course of the experiment, rather than any variation in clearance. A similar, 7-day experiment using *R. clamitans* and *B. americanus* showed that *Ribeiroia* exposure (20 cercariae) increased time-to-death by ~2–3 days relative to controls (Rohr et al., 2010). Our observed decrease in survival among exposed *T. torosa* is also consistent with a previous study showing that infected *T. torosa* were more likely to survive before metamorphosis relative to unexposed newts (Johnson et al., 2012). The same experiment also showed reductions in host mass and length at metamorphosis in association with *Ribeiroia* exposure. The current study reveals that this survival effect arises as early as ten days post-exposure, if not earlier, and that effects on host growth are more pronounced among heavily infected individuals at early time points (e.g., 36 h post exposure).

In recent years, researchers have focused studies of resistance-tolerance in animal systems on estimating the degree to which both resistance and tolerance show additive genetic variation. These estimates are important because theoretical work suggests these strategies should generate different population dynamics and evolutionary outcomes in host-parasite systems (Best et al., 2009; Roy and Kirchner, 2000). While the presence of additive genetic variation in resistance is well-supported in the limited number of systems for which it has been investigated (Hedrick, 2002; Boon et al., 2009; Råberg et al., 2009), evidence for genetic variation in tolerance and genetic covariation

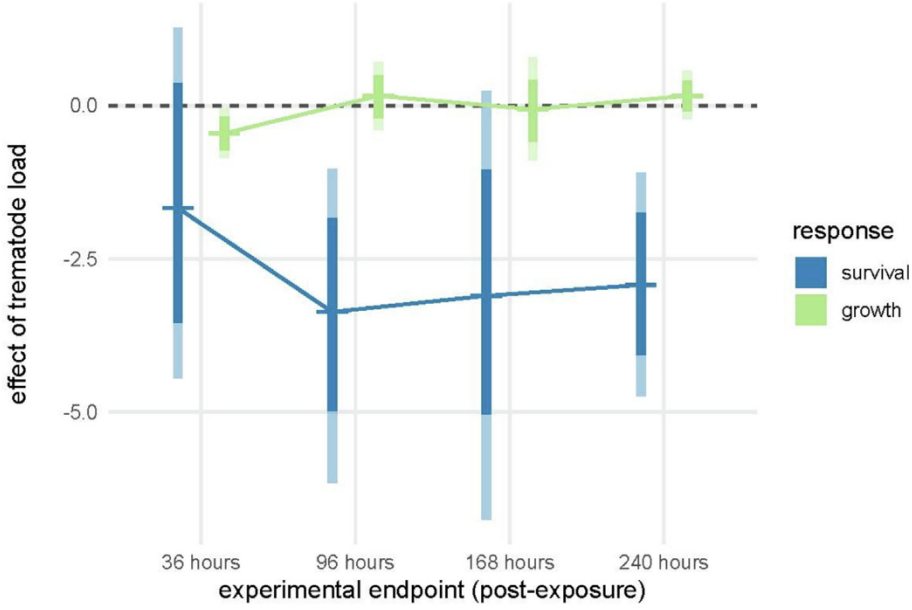

Fig. 5. Effects of trematode load on survival and growth: estimated effect sizes (β) of trematode load on survival and growth across the four different experimental endpoints. Short horizontal lines indicate posterior means; darker bars indicate 80% HDPI; lighter bars indicate 95% HDPI. To better compare across models, effect sizes have *not* been back-transformed to the raw data scales.

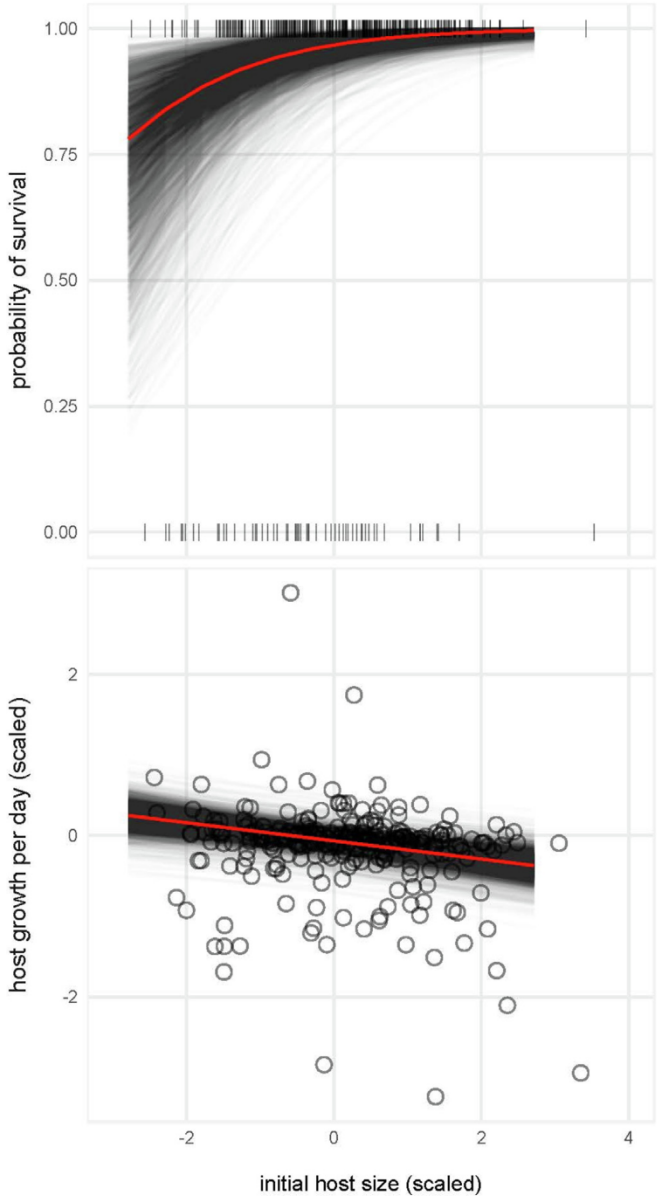

Fig. 6. Effects of initial host size on survival (top) and growth (bottom): Lines shows the model-estimated change in the probability of host survival (top) or the change in growth per day (bottom) as a function of the scaled host length at the beginning of the experiment. The red line shows the posterior median; grey lines show estimates from all individual draws for the joint posterior. Rugs along the x-axis at 0 and 1 in the top figure indicate survival of individual hosts. Points in the bottom figure indicate the lengths and growth rates of individual newts.

between tolerance and resistance have been mixed. In contrast to other animals systems, we found little support for transmittable (genetic + maternal) variation in resistance; after accounting for other sources of variation (e.g., host length, clutch size, infection wave), almost all of the remaining variance in infection success was among individual newts as opposed to among families. We found a similar pattern of individual- and family-level variation in growth, and of family-level variation in both clearance and survival, indicating a lack of transmittable variation in these traits as well. However, for tolerance traits these results are consistent with theory, which suggests that selection for increased tolerance would reduce genetic variation for tolerance in the host population to near-zero (Best et al., 2009; Roy and Kirchner, 2000).

It is important to keep in mind that excessive environmental variation can swamp estimates of transmittable variance (Lloyd-Smith et al., 2005). In our experiment, newt hosts were raised from eggs in a common environment and we accounted for many of the additional factors that could have generated residual variance in our analysis. Although we used field-collected snails for conducting experimental infections, which could introduce additional genotypic variation in the parasites, we took care to aggregate cercariae from different infected snails and randomized the exposure of amphibian hosts. Previous research also suggests that *R. ondatrae* genetic variation is small over the spatial scale of our study (Davies et al., 2015; Tkach unpublished) likely supporting the apparent lack of transmissible (and thus likely heritable) variation in both resistance to *Ribeiroia* and tolerance of infection (either growth or survival) among *T. torosa* within ten days of exposure. Given the short time-period of this study, it is possible that there could be measurable genetic variation in clearance over longer time-periods. Similarly, our experiment may not have persisted long enough to document among-family variation in survival or growth if these differences only become apparent over the entire course of juvenile development. However, our experiment does reveal that, barring extensive immigration or mutation in the host gene pool, there is likely little opportunity for adaptation to increase initial resistance or tolerance within the first ten days of exposure.

In addition to estimating resistance and tolerance, our analytical approach shows how the same models can be used to investigate variation in other fitness-associated phenotypic traits at the individual and family levels. In both the survival and resistance models, the global intercept terms were interpretable as the expected values for unexposed individuals, which we differentiated as robustness (for survival) and vigor (for growth). We detected substantial among-newt variation in vigor that was unexplained by newt size, tail damage, average family size, or clutch size (which is likely correlated with maternal investment; Kaplan, 1985), but no appreciable variation among families. In addition to vigor and robustness, we also modeled the costs of exposure as the regression parameter associated with exposure treatment (which is the difference in the survival/growth estimate between treatments when *Ribeiroia* count equals zero). Consequently, the direction and magnitude of the treatment effect indicates the degree to which newts suffered a fitness cost associated with exposure that was independent of subsequent infection (Rohr et al., 2010). Based on these results, there was no evidence of an overall cost of exposure for survival or growth. There was also no indication that more resistant families had lower vigor or robustness (posterior correlations between these traits were all near zero with high uncertainty), indicating a probable lack of constitutive costs. These findings stand in contrast to the results of Rohr et al. (2010), although looked at averages among exposed animals, found that *R. clamitans* had significantly lower survival costs from trematode infection relative to *B. americanus* when measured through metamorphosis. Consequently, it may be possible that such costs could impact survival (or growth) over longer time periods in newts.

Hierarchical models offer a powerful analytical approach for investigating tolerance (and resistance), consistent with previous quantitative genetic studies (Leinonen et al., 2013; Mazé-Guilmo et al., 2014). Unlike the more typical ANCOVA approach (Råberg et al., 2009), hierarchical modeling allows for estimates of variation at different levels of the analysis, some of which correspond to heritable variation while others link to aspects of environmental or experimental variation. Bayesian hierarchical models are particularly advantageous as they facilitate not only point estimates of variation, but also estimations of uncertainty, propagation of uncertainty across levels, and correlations between resistance and tolerance within a united framework (e.g. Mazé-Guilmo et al., 2014). More importantly, Bayesian hierarchical models allow ecologists to directly measure how and to what degree resistance and tolerance vary across other groups as well, including variation among populations, among host and parasite species, or among experimental treatments. For instance, the current results showed how the effects of parasite load varied on host growth and

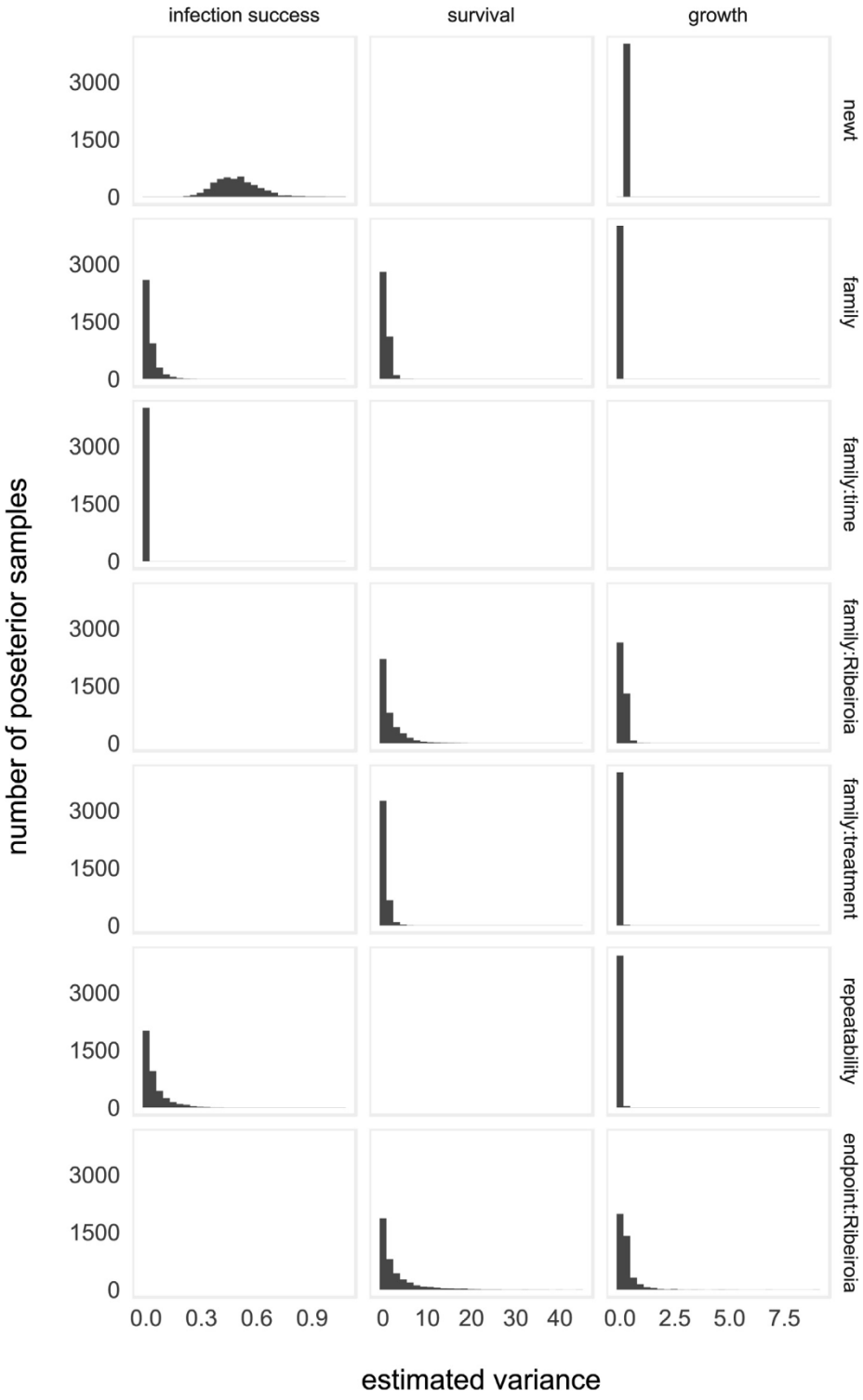

Fig. 7. Variance component estimates: Histograms show the distribution of draws from the posterior for each of the estimated variance components for each of the three fitted models (infection success, growth, and survival). Single vertical bars at the left of each panel indicate cases where the posterior variance is very small (i.e. provides no support for any variance at that level). Panels without histograms indicate that the particular variance component was not estimated for a given model.

survival varied at different time points in our experiment. Hierarchical models allowed for more precise estimates of these effects relative to standard ANCOVA through techniques such as partial pooling, which differentially weight treatments as a function of sample size. Such approaches will prove especially beneficial in investigating how measures such as robustness and tolerance vary across environmental gradients (e.g., resource availability) or among different host species within the same ecological communities. Recent advances in probabilistic programming (e.g., Stan Development Team, 2014) coupled with interfaces for standard statistical analysis such as the *rstan* package for R (R Core Team, 2014) make Bayesian hierarchical modeling more accessible for ecologists interested in these (and other) important empirical and theoretical questions.

Conflicts of interest

No competing interests declared.

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