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Temperature-driven shifts in a host-parasite interaction drive nonlinear changes in disease risk

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Abstract

Climate change may shift the timing and consequences of interspecific interactions, including those important to disease spread. Because hosts and pathogens may respond differentially to climate shifts, however, predicting the net effects on disease patterns remains challenging. Here, we used field data to guide a series of laboratory experiments that systematically evaluated the effects of temperature on the full infection process, including survival, penetration, establishment, persistence, and virulence of a highly pathogenic trematode (*Ribeiroia ondatrae*), and the development and survival of its amphibian host. Our results revealed nonlinearities in pathology as a function of temperature, which likely resulted from changes in both host and parasite processes. Both hosts and parasites responded strongly to temperature; hosts accelerated development while parasites showed enhanced host penetration but reduced establishment (encystment) and survival outside the host. While there were no differences in host survival among treatments, we observed a mid-temperature peak in parasite-induced deformities (63% at 20 °C), with the lowest frequency of deformities (12%) occurring at the highest temperature (26 °C). This nonlinear effect could result from temperature-driven changes in parasite burden owing to shifts in host penetration and/or clearance, reductions in host vulnerability owing to faster development, or both. Furthermore, despite strong temperature-driven changes in parasite penetration, survival, and establishment, the opposing nature of these effects lead to no difference in tadpole parasite burdens shortly after infection. These findings suggest that temperature-driven changes to the disease process may not be easily observable from comparison of parasite burdens alone, but multi-tiered experiments quantifying the responses of hosts, parasites and their interactions can enhance our ability to predict temperature-driven changes to disease risk. Climate-driven changes to disease patterns will therefore depend on underlying shifts in host and parasite development rates and the timing of their interactions.

Keywords: amphibian decline, amphibian malformations, climate change, emerging disease, freshwater, global warming, mismatch hypothesis, *Pseudacris regilla*, species interactions

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Introduction

A fundamental challenge in climate research is to understand how current and forecasted shifts in temperature will affect species interactions. Climate change has been linked to poleward range-shifts, earlier phenologies, and declining body sizes of individual species (Parmesan & Yohe, 2003; Menzel *et al*., 2006; Gilman *et al*., 2010; Walther, 2010; Gardner *et al*., 2011). Not all species are responding equivalently, however, leading to growing interest in the implications of such changes for interspecific interactions. Differences among species in their individual responses to climate change can scale up to alter interspecific interactions by creating novel communities, shifting the timing of interactions, and altering competitive dominance (Gilman *et al*., 2010; Traill *et al*., 2010; Walther, 2010; Woodward *et al*., 2010). Such changes will have broad-reaching implications for the resilience of communities and the ecosystem services that they provide (Folke *et al*., 2004; Hoegh-Guldberg & Bruno, 2010; Thackeray *et al*., 2010).

Despite our growing understanding of these ecological responses to climate change, their effects on disease risk remain a pressing and controversial topic (Wilson, 2009; Rohr *et al*., 2011; Hoverman *et al*., in revision). Some of this controversy stems from the complex effects of temperature on hosts and parasites, leading to difficulties predicting how such changes will alter their interactions and the disease outcome (Paull & Johnson, in press). Temperature can directly alter both parasite virulence and host immunity, each of which affect parasite burdens and host pathology (Braid *et al*., 2005; Studer *et al*., 2010; Møller, 2010). For example, warmer temperatures enhanced both the growth rate of the fungal pathogen, *Aspergillus sydowii*, as well as the anti-fungal activity of its coral host, although the pathology associated with infection (measured as change in zooxanthellae abundance), was still greatest at high temperatures (Ward *et al*., 2007). Similarly,

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temperature often elevates both the development and mortality rates of individual hosts or parasites and changes the seasonal windows of transmission such that the balance between these factors becomes important for determining the net effect of elevated temperatures on parasite encounter rates by hosts (Kutz *et al*., 2005; Lafferty, 2009; Paull & Johnson, in press). For instance, development rates of the dengue vector, *Aedes aegypti*, increased over the temperature range of 10–35 °C, while survival rates declined at temperatures greater than 30 °C (Tun-Lin *et al*., 2000). Increased temperatures could thus decrease transmission, even in the face of elevated pathogen replication, if parasite or vector survival rates decline simultaneously. A further complication to predicting the response of host-parasite interactions to climate change is that host and parasite responses to temperature are not generally linear, with the performance of organisms increasing up to a particular temperature, beyond which it declines (Thieltges & Rick, 2006; Angilletta, 2009; Lafferty, 2009). This suggests that the range of temperatures experienced under future climate change and local thermal evolutionary contexts will be an important determinant of the net response of particular diseases to climatic shifts.

Understanding the effects of temperature on parasite and host responses is further complicated by the potential for indirect climate-effects on the timing of host-parasite interactions. Increasingly, disruptions of interspecific interactions such as predator-prey and plant-herbivore dynamics have been observed because seasonal events such as breeding, migration, and flowering, are advancing earlier in the season in some species but not in others (Memmott *et al*., 2007; Post *et al*., 2008; Primack *et al*., 2009). Yang & Rudolf (2010) recently noted that the types and consequences of many interspecific interactions are mediated by the developmental stages of the interactors, suggesting that the inclusion of ontogeny in phenological models could enhance our predictions of climate-driven changes to these interactions. This could be particularly important for host-parasite dynamics, given that host developmental stage can be a critical determinant of both host resistance and tolerance to disease (Kelly *et al*., 2010; Johnson *et al*., 2011). For instance, if hosts and parasites differ in the sensitivities of their growth rates to temperature, or in the phenological cues (e.g., temperature, precipitation, or daylength) used for reproduction, hosts could be exposed to parasites earlier or later in their development with climate change, with potential consequences for host infection rates and pathology (Paull & Johnson, 2011).

The complexities inherent to understanding climate-driven changes to host-parasite interactions highlight the need for mechanistic approaches to studying temperature-driven changes in disease dynamics. Here, we used an interaction between an amphibian host and a highly virulent trematode parasite to systematically evaluate the effects of temperature on multiple stages of the infection process. We assessed how responses of the host and parasite to temperature combine to influence this host-parasite interaction and host pathology. The goals of the experiment were to (1) determine how temperature influences both lethal and non-lethal forms of host pathology (e.g., mortality, developmental malformations, and growth) and (2) assess whether differences in pathology were the result of changes to parasite or host responses to temperature, or a combination of factors at each step of the infection process. Our study offers a detailed look at this issue by considering both short- and long-term effects of temperature on host infection and pathology and by differentiating between effects on parasite establishment and persistence within hosts.

Materials and methods

Study system

Ribeiroia ondatrae (Trematoda: Digenea) is a complex life cycle parasite that must be transmitted sequentially from a first intermediate host (snail) to a second intermediate host (amphibian or fish) to a definitive host (bird or mammal) to complete its life cycle (Johnson *et al*., 2004). Parasites emerge from snails as mobile cercariae that burrow into the skin of developing larval amphibians. Once the cercariae have penetrated amphibian skin, they become subcutaneously encysted as metacercariae, usually in a region localized near the larval amphibian's developing hind limbs (Sessions & Ruth, 1990; Johnson *et al*., 1999). The pathology associated with infection (mortality and severe limb deformities) depends on when larval amphibians become infected (earlier infection leads to more severe pathology) and the number of parasites with which they are infected (Schotthoefer *et al*., 2003a,b; Johnson *et al*., 2011). The malformations resulting from infection may benefit the parasite by increasing the likelihood that the amphibian host is consumed by the definitive host (Johnson *et al*., 2004; Goodman & Johnson, 2011). Throughout the manuscript, we refer to parasite survival as survival in the absence of the host, parasite penetration as the success of host skin penetration, parasite establishment as the success of cyst formation, parasite infectivity as the net result of penetration and establishment (e.g., burden shortly after infection), and virulence as the level of pathology caused by the parasite. Infection in this system is a two-part process involving skin penetration followed by cyst formation, each of which could disrupt development and influence host pathology. Although traditional definitions of infectivity tend to focus on the ability of the pathogen to establish, we break this into its components to enhance mechanistic understanding.

Overview of approach

We recorded the temperature at 19 *Ribeiroia*-positive wetlands in the East Bay area of California during the amphibian-growing season and used these measurements to define a realistic temperature range for experiments, including current as well as forecasted changes in temperature. We conducted two short-term experiments: one to study the effects of temperature on parasite survival in the absence of hosts, and another to study parasite penetration, establishment, and infectivity in hosts. To test how temperature influenced parasite persistence and virulence, and host pathology, we performed a long-term experiment, exposing amphibians at different temperatures and raising them to metamorphosis. Both tadpole exposure experiments were conducted at the same time, in the same location, with tadpoles and parasites from the same population to ensure that conditions were as similar as possible and to strengthen inferences made between experiments. While *Ribeiroia* infects a wide variety of amphibian host species, we focused on *Pseudacris regilla* because of its sensitivity to developing pathology as a result of infections and its ubiquity in California wetlands where we focused our field survey efforts (Johnson *et al*., 2002).

Selection of temperature range for experiments

To determine a realistic temperature range for our experiments, we used field data from the same region where we collected *P. regilla* eggs and *Ribeiroia*-infected *Helisoma trivolvis*. We placed Hobo underwater dataloggers (Onset Computer Corp., Bourne, MA, USA) 50 cm below the water surface from May to August 2010 at 19 California ponds. Based on these data, we selected a temperature range of 17–26 °C to be comparable with the current mean summer (May–August) temperature range (coldest site mean = 17.7 °C, SD = 1.6 °C; overall sites mean = 20.8 °C, SD = 2.3 °C; warmest site mean = 24.1, SD = 2.3). Given that the temperature of North American small lakes is forecasted to change between 5 and 10 °C by 2100 (Sharma *et al*., 2007; Fang & Stefan, 2009), the use of an upper temperature limit (26 °C) that was five degrees above mean May–August pond temperatures (yet below the maximum temperature of 27.9 °C recorded at the warmest site) is likely a conservative test of climate-driven changes to pond temperatures in the region.

Temperature effects on parasite activity and survival

We tested the effect of temperature (17, 20, and 26 °C, SD = 1 °C for all) on cercarial survival in the laboratory. We collected *Ribeiroia*-infected rams-horn snails (*H. trivolvis*) from wetlands in the East Bay area of California. *Ribeiroia ondatrae* cercariae pooled from three of these snails were isolated into individual wells of 96-well-plates containing 5 mL of water and twelve parasites per plate. Well plates were placed inside 1 L waterproof containers and submerged into one of nine water baths (33 L), with one well plate per water bath, and three water baths per temperature treatment. A Hobo datalogger (Onset Computer Corp.) was also placed alongside the well plate in each water bath. We controlled temperature in the heated water baths (20 and 26 °C) using 250 Watt Jager heaters (Eheim, Deizisau, Germany), while temperature in the remaining baths were maintained at the level of the temperature-control chamber (17 °C). It should be noted that pooling cercariae from three snails may have limited the genetic variation of cercariae in the study. The experiment began at 0:00 hours, at which point cercariae were a maximum of 3 h old. We recorded cercarial survival 5 h later (and at 2 h intervals thereafter) by briefly examining each well under a microscope. We rotated trays 90° when they were returned to their temperature treatments to limit any positional effects with respect to the heaters. The trials were concluded after 33 h when all cercariae were no longer moving.

Short-term effects of temperature on parasite penetration and establishment

We tested the effects of temperature (17, 20, and 26 °C, SD = 1 °C for all) on parasite penetration and establishment (e.g., metacercarial cyst formation) in tadpoles after 48 h. For tadpole experiments, clutches of Pacific chorus frogs (*P. regilla*) eggs were collected from wetlands in California, hatched in the laboratory, and raised communally until tadpoles reached Gosner (1960) stages 28–29. We fed tadpoles *ad libitum* a water-based slurry containing equal parts ground TetraMin and *Spirulina* fish flakes on a daily basis, and changed the water every 3 days using aged and treated tapwater. We acclimated cercariae (15 min) and tadpoles (36 h) to the appropriate temperature treatment prior to exposing tadpoles in 800 mL glass jars held in a total of nine total water baths of the appropriate temperature (three baths per temperature). We exposed each tadpole (n = 21 per temperature) to 25 cercariae (<4 h old at time of exposure) for 45 min. To quantify the number of cercariae that failed to infect the tadpole, we filtered the 800 mL of exposure water using a vacuum pump (Doerr, model LR-22132) with fiberglass 0.7 micron filters. Filters were stained with a solution composed of 88% water, 11.7% acetic acid, and 0.3% Light Green® (Fisher Scientific, Pittsburgh, PA, USA) to dye the cercariae, which were subsequently quantified on a stereodissecting microscope (Daly & Johnson, 2011). To test filtration reliability, we counted cercariae on filters from control jars that contained no tadpoles (n = 5 per temperature). After metacercarial cysts had a chance to form within the tadpoles (48 h), all tadpoles were preserved in formalin after being euthanized in a solution of tricaine methanesulfonate (MS-222) buffered with sodium bicarbonate. Tadpoles were subsequently necropsied to quantify the number of metacercarial cysts.

Long-term effects of temperature on parasite burden and host pathology

We conducted a longer-term experiment with multiple tadpole exposures over time to assess the influence of temperature (17, 20, and 26 °C, SD = 1 °C for all) on host pathology (growth, survival and limb malformations). We maintained tadpoles individually in 2.5 L containers, with eight containers positioned in

each water bath, and a total of 18 water baths (six per temperature). Each temperature treatment consisted of 16 uninfected control tadpoles, and 32 *Ribeiroia*-infected tadpoles. We randomly assigned tadpoles between Gosner stages 27–28 to each treatment and acclimated them for 24 h prior to the first exposure. Tadpoles were exposed four times to a dose of seven cercariae every 3 days (28 parasites per tadpole over 9 days). *Ribeiroia* cercariae were temperature-acclimated for 15 min prior to each exposure. Because developmental stage at infection can influence host pathology, we randomly staged five tadpoles from each treatment several hours prior to infection to determine how temperature was influencing tadpole development rates. To limit positional effects, we rotated container positions within baths every 3 days, and water baths every 2 weeks. When tadpoles metamorphosed, they were euthanized, weighed, measured (snout-vent-length), checked for deformities, preserved, and later necropsied to quantify *Ribeiroia* infection.

Analyses

We statistically controlled for pseudoreplication within our studies by nesting subjects within water baths and including bath as a random effect using linear mixed-effects models (Crawley, 2007; Zuur *et al*., 2009; Rohr *et al*., 2011). This analysis recognizes and accounts for the fact that tadpoles from the same water bath, even though maintained in separate individual containers, could nonetheless have correlated responses if there is a bath-level effect. For the cercarial survival experiment, we used a parametric survival analysis to test the effects of temperature on parasite mortality, including water bath as a random effect. For the short-term tadpole infection experiment, we used generalized linear mixed effects models (GLMMs) for split-plot designs to analyze the effect of temperature (fixed effect) and bath (random effect) on the percentage of parasites penetrating and forming metacercarial cysts within hosts (coded as number of 'successes' and 'failures' with a binomial distribution). In the long-term tadpole pathology experiment, we tested the effects of parasite exposure, temperature, and their interaction (fixed effects) and bath (random effect) on host mortality using a GLMM with the binomial distribution, and on time-to- and size-at metamorphosis using linear mixed effects models. Among animals that survived to metamorphosis, we further tested the effects of temperature (fixed effect) and bath (random effect) on malformations (0 vs. 1) and the percentage of cercariae recovered as metacercarial cysts using a binomial distribution. We used Akaike Information Criterion corrected for small sample size (AICc) to assess the relative support for either a linear or quadratic relationship between temperature and deformities, as well as between temperature and parasite burden at metamorphosis (including bath as a random effect in all models). To determine whether a temperature-driven acceleration in tadpole development occurred during the infection process, we used a linear mixed effects model to test the effects of time, temperature, and their interaction (fixed effects) and bath (random effect) on tadpole developmental stage. Tadpoles that died prior to metamorphosis were excluded from analyses. As a post-hoc analysis, we used a GLMM with a binomial distribution to test whether changes in host time-to-metamorphosis or parasite burden at metamorphosis explained the temperature driven shifts in malformations (with bath included as a random effect). The survival analysis was performed using the function survreg in R. Following Bolker *et al*. (2009), GLMMs were fit using the Laplace approximation method using the lme4 package (lmer function) while the linear mixed effects analyses were performed using the nlme package (lme function) in R (R Development Core Team 2008).

Results

Temperature effects on cercarial survival

Increases in temperature caused a marked and monotonic decrease in cercarial survival. Cercarial survival time correlated negatively with temperature, such that cercariae survived for a mean ± standard error (SE) of 22.0 ± 0.8 h at 17 °C, 14.6 ± 0.6 h at 20 °C, and 9.3 ± 0.4 h at 26 °C (Survival analysis $\chi^2 = 755.3$, df = 5, $P < 0.01$, Fig. 1).

Short-term effects of temperature on parasite penetration and establishment

Temperature had opposite effects on host skin penetration and parasite cyst formation (establishment). Higher temperature increased the percentage of parasites that successfully penetrated tadpole skin (GLMM $Z = 2.5$, $P = 0.01$), which was estimated as the number of cercariae not recovered through filtering (Fig. 2a). By contrast, the percentage of cercariae that successfully encysted following skin penetration declined with temperature (GLMM $Z = -2.1$, $P = 0.04$, Fig. 2b). These conflicting effects of temperature on parasite penetration and establishment lead to no significant difference in the number of metacercariae detected among tadpoles examined 48 h after parasite exposure (GLMM $Z = -0.3$, $P = 0.79$, Fig. 2c). Among control filters (without hosts), we recovered a mean percentage of 98.4%, 99.2%, and 96.8% parasites per filter at 17, 20, and 26 °C, respectively (out of a total of 25 parasites), indicating the efficacy of the filtration process for recovering parasites.

Long-term effects of temperature on parasite burden and host pathology

Host growth. Warming temperatures caused a strong increase in tadpole development rate (time × temperature: $t = 7.8$, df = 3, $P < 0.01$). By the end of the parasite exposures (day 10), tadpoles raised at 17 °C were, on average, at Gosner stage 31, compared to an average stage of 37 among tadpoles raised at 26 °C (Fig. 3). Correspondingly, temperature reduced time-to-meta-

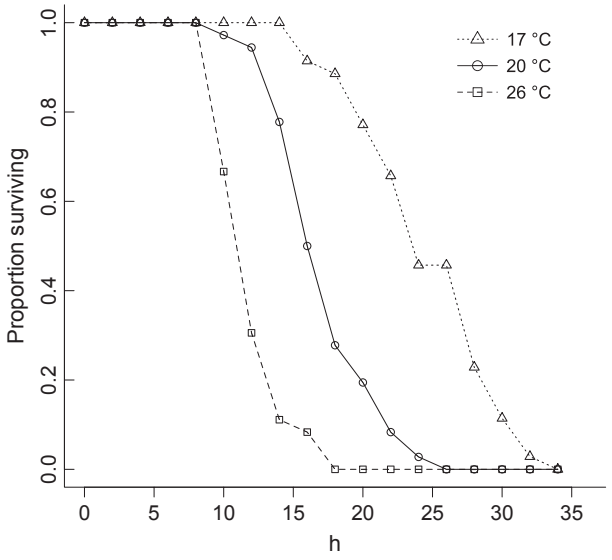

Fig. 1 Proportion of *Ribeiroia ondatrae* cercariae surviving in each treatment over time.

morphosis as well as length and mass of hosts at metamorphosis (time: $t = -6.7$, df = 14, $P < 0.01$, length: $t = -3.7$, df = 14, $P < 0.01$; mass: $t = -4.15$, df = 14, $P < 0.01$). In the warmest treatment, hosts developed nearly three times faster than at the coldest treatment.

Parasite burden. In contrast to the short-term infection study, hosts raised to metamorphosis in the long-term exposure study exhibited fewer metacercariae at 26 °C compared to the other temperature treatments (GLMM $Z = -2.6$, $P = 0.01$). While metamorphosing frogs in the 17 and 20 °C treatments had a mean ± SE of 11.29 ± 0.77 and 12.79 ± 0.73 metacercariae, respectively, only 8.93 ± 0.83 metacercariae were recovered in hosts from the 26 °C treatment (Fig. 2d). The overall relationship between temperature and parasite burden was slightly better characterized by a quadratic rather than linear model (ΔAICc = 2.6, Table 1).

Host mortality and malformations. The prevalence of severe limb deformities varied significantly with temperature (GLMM $Z = -2.0$, $P = 0.04$), such that frogs in the 20 °C treatment exhibited the highest frequency of malformations (63%) compared to 38% in the 17 °C treatment and 12% in the 26 °C treatment (Fig. 4). A Tukey HSD test confirmed that the difference was only

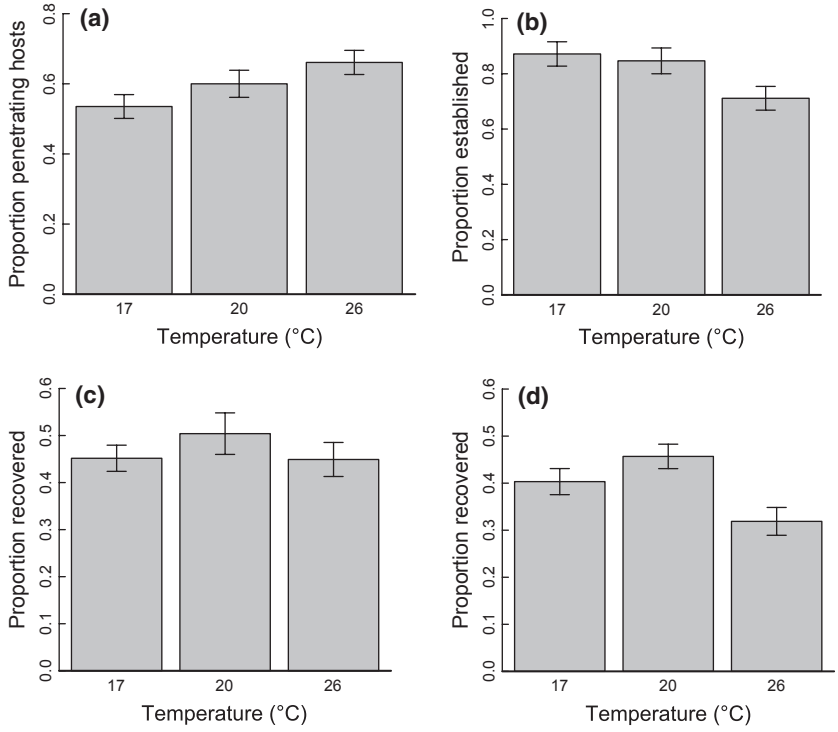

Fig. 2 Effect of temperature on different stages of the *Ribeiroia* infection process, including penetration (a), establishment (b), burden 48 h post-infection (c), and burden at metamorphosis (d). The proportion of parasites penetrating tadpole skin was measured as the proportion of parasites not recovered by filtration after the exposure period. The proportion of parasites that established once inside the host was calculated as the number of metacercariae divided by the number of parasites that penetrated the hosts. Parasite burden 48 h post-infection was recorded from the short-term trials, while burden at metamorphosis was recorded from the long-term trials. All bars show mean ± SE.

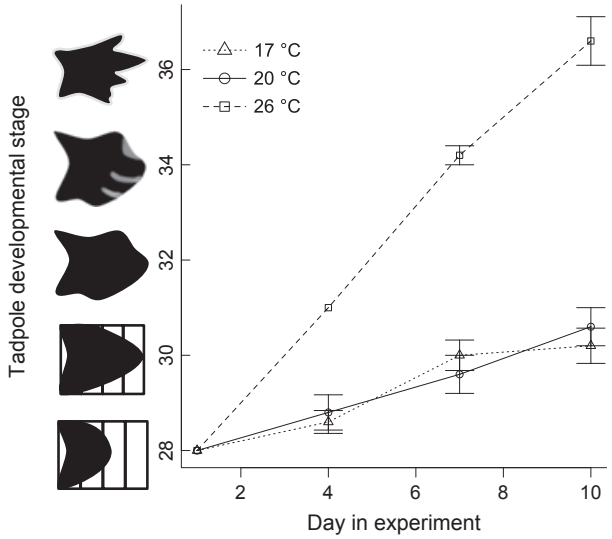

Fig. 3 Mean ± SE developmental stage (Gosner, 1960) of tadpoles just prior to each of the four *Ribeiroia* exposure events at each temperature. Pictures along the *y*-axis show development of the limb buds for each corresponding stage.

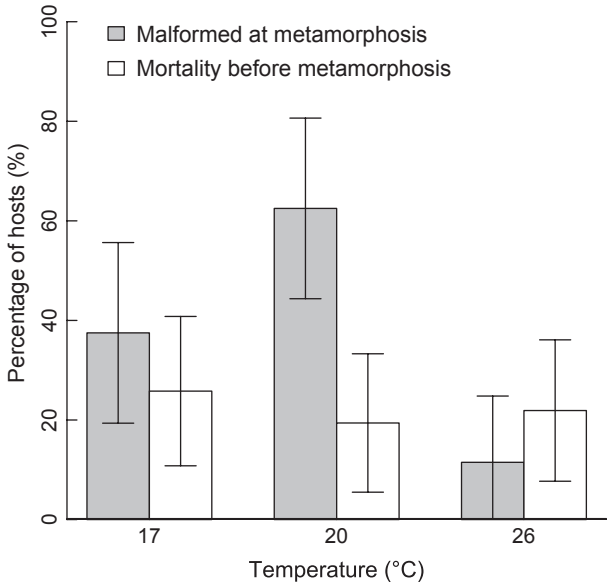

Fig. 4 Percentage of *Ribeiroia*-exposed *Pseudacris regilla* that survived to metamorphosis and emerged deformed (shaded bars), or died before metamorphosis (white bars) at each temperature ± 95% CI.

Table 1 Comparison of models of the percentage of metacercariae recovered from metamorphosing frogs using linear and quadratic relationships with temperature

Model*	K†	AICc‡	AICc§	*w*¶
Quadratic	4	148.5	0	0.79
Linear	3	151.1	2.6	0.21

*These describe the modeled relationship with temperature.
†Number of parameters (bath was included as a random effect).
‡Corrected Akaike's Information Criteria for small sample sizes.
§Difference in AIC values from top model.
¶Model weight.

Table 2 Comparison of models of the proportion of infected tadpoles emerging deformed using linear and quadratic relationships with temperature

Model*	K†	AICc‡	AICc§	*w*¶
Quadratic	4	90.6	0	0.87
Linear	3	94.4	3.8	0.13

*These describe the modeled relationship with temperature.
†Number of parameters (bath was included as a random effect).
‡Corrected Akaike's Information Criteria for small sample sizes.
§Difference in AIC values from top model.
¶Model weight.

significant ($\alpha = 0.05$) between the 20 and 26 °C treatments. Observed deformities included polymelia (extra limbs), taumelia (folds in the leg bones forming a bony triangle), cutaneous fusion (skin webbing connecting the upper and lower leg bones) and femoral projections (small extension of bone or skin from the hind limb). The relationship between deformities and temperature was best supported by a nonlinear, second-order polynomial model (ΔAICc = 3.8 relative to linear model, Table 2). Neither exposure to *Ribeiroia* nor temperature affected tadpole mortality (exposure: $Z = -0.2$, $P = 0.82$, temperature: $Z = -0.6$, $P = 0.58$, Fig. 4). Survival to metamorphosis was 77%, 85%, and 81% in the 17, 20, and 26 °C treatments, respectively, and none of the control tadpoles exhibited malformations at metamorphosis.

Factors explaining malformations. As a *post-hoc* analysis, we considered potential factors responsible for the observed change in malformations, particularly the significant decrease in malformation frequency at the warmest temperature. Although neither host development time, parasite burden at metamorphosis, nor their combination yielded a significant model to explain malformations when all data were analyzed concurrently (Time: $Z = 1.6$, $P = 0.12$; Burden: $Z = 1.5$, $P = 0.14$), host time to metamorphosis ($Z = 2.3$, $P = 0.02$), positively predicted the change in malformations

between the 20 and 26 °C treatments (i.e., excluding the 17 °C), while parasite burden had no effect on malformation risk ($Z = 1.39$, $P = 0.17$).

Discussion

Mechanistically assessing the effects of climate change on disease requires an understanding of the influence of temperature on the parasite, the host, and the product of their interactions. In the current study, parasites showed strong responses to temperature both inside and outside of the host. Although parasite penetration of hosts was enhanced at warmer temperatures, the survival of parasites outside of the host (as free-swimming cercariae) responded negatively to the range of temperatures used in this study, and the ability of parasites to establish (encyst) within hosts was reduced at the warmest temperature. For hosts, larval development was accelerated in the warmest treatment, which likely reduced the time period in which hosts were highly sensitive to infection and pathology (Schotthoefer *et al*., 2003a; Johnson *et al*., 2011). These contrasting effects of temperature on host and parasite processes likely contributed to the nonlinear relationships that we observed. For instance, temperature had a nonlinear relationship with parasite burdens in hosts at metamorphosis. We also observed a mid-temperature peak in the frequency of severe limb malformations, which are expected to be lethal in nature (Goodman & Johnson, 2011). Because each component of the infection process (penetration, establishment, and persistence) may respond differently to temperature, simple measures of parasite burden alone may not be adequate to predict or understand host pathology in response to climate shifts, underscoring the value of considering temperature-driven changes to each component of the infection process.

Nonlinearities in temperature-driven changes to host pathology are likely to result when rising temperatures influence both parasite and host processes. Here, the prevalence of limb deformities peaked in the mid-temperature treatment, with 5 × more deformities among metamorphosing frogs raised at 20 °C relative to those at 26 °C, and almost twice the prevalence of deformities compared to those at 17 °C. Two potential factors could contribute to this pattern, either individually or in combination. First, by altering host development rates, temperature likely altered the vulnerability of hosts to the pathogenic effects of *Ribeiroia*. Malformation frequency declines steadily in tadpoles exposed to *Ribeiroia* at Gosner stages 30 and beyond (Schotthoefer *et al*., 2003a; Johnson *et al*., 2011) owing to the occurrence of 'critical windows' of susceptibility to infection and/or malformations. In the current study, temperature caused a substantial acceleration of host development, such that ~75% of the parasites administered to hosts at 26 °C were added after hosts surpassed Gosner stage 30 (Gosner, 1960), the stage after which deformity risk declines sharply (Johnson *et al*., 2011). In contrast, the mean developmental stage of tadpoles in the cooler treatments did not surpass this point until the final exposure (Fig. 3). Correspondingly, individual hosts within the 20 and 26 °C treatments were significantly more likely to be deformed when they took longer to reach metamorphosis based on the post-hoc analyses. Thus, the observed reduction in the prevalence of deformities at 26 °C is consistent with the hypothesis that the sharp increase in host developmental rate in the 26 °C treatment effectively moved hosts outside of the 'critical window' of sensitivity. Based on this hypothesis, however, we would have expected a further increase in malformations from 20 to 17 °C where hosts developed most slowly, which was not observed. This trend may have been counteracted by the reduced parasite penetration at 17 °C, which could have reduced the disruption of developing cells in the limb buds of larval amphibians (see Stopper *et al*., 2002).

Alternatively or additionally, the decrease in malformations between the intermediate and warmest temperatures may have resulted from changes in parasite burden. Based on necropsy data at metamorphosis, hosts from the warmest treatment supported, on average, 30% fewer parasites than those at the intermediate temperature, and the overall relationship between temperature and infection was similarly nonlinear to that characterizing the relationship between temperature and malformations. In our *post-hoc* analyses, parasite burden at metamorphosis had no significant relationships with malformation prevalence. However, temperature is well known to influence ectotherm immunity (Maniero & Carey, 1997; Raffel *et al*., 2006), and we therefore exercise caution in assessing the role of burden on deformities until more information is available on the contributing effects of temperature on amphibian infection at metamorphosis.

Temperature could influence patterns of amphibian infection at metamorphosis through either (1) differences in infection success as a function of host stage or temperature, or (2) temperature-driven differences in parasite persistence (e.g., clearance). Previous studies involving trematode and/or amphibian host species have reported nonlinear or negative relationships between tadpole stage at infection and parasite burden (Schotthoefer *et al*., 2003b; Holland *et al*., 2007; Rohr *et al*., 2010; Raffel *et al*., 2011), particularly for echinostome parasites. However, previous work using the same species of amphibian and parasite indicated that host resistance differed little across the developmental

stages (Gosner 28–37) used in this study, despite large differences in pathology (Johnson *et al*., 2011). Additionally, results from the short-term experiment showed no effect of temperature on parasite burden 48 h after infection (Fig. 2a). The second hypothesis that parasite persistence (or the ability of cysts to maintain themselves after establishment) decreased with temperature is consistent with previous literature on the temperature-dependence of ectothermic immunity (Maniero & Carey, 1997; Raffel *et al*., 2006). Despite the rapid growth of hosts at warm temperatures, which afforded them less time for parasite clearance, observed infections at metamorphosis were lowest at the highest temperature. Field and laboratory studies in amphibian systems have demonstrated that lymphocytes and eosinophils are higher in hosts living in warmer conditions (Maniero & Carey, 1997; Raffel *et al*., 2006), and eosinophils are considered to be an important component of the tadpole response to *R. ondatrae* metacercariae (Kiesecker, 2002). Further work using blood smears along with a recently developed technique for fluorescently dyeing cercariae to track their fate in live animals (Keeney *et al*., 2008) will help to clarify how temperature affects amphibian immunity and resistance, and parasite clearance, and how these factors influence temperature-driven changes in deformity risk.

Trade-offs in the responses of parasites to increased temperature are likely common in natural systems and have the potential to cause unanticipated effects of climate-change on disease. We observed an apparent trade-off between the ability of the parasite to penetrate the host skin and both its survival outside the host and ability to encyst within the host. The trade-off with survival is consistent with the 'energy limitation hypothesis', which states that the finite energy stores of this free-living stage of trematodes are used up more quickly at high temperatures as a result of higher activity levels (Pechenik & Fried, 1995; Thieltges & Rick, 2006; Studer *et al*., 2010). Thus, the enhanced cercarial penetration observed here likely resulted from greater parasite activity levels at warmer temperatures, which increased the likelihood that parasites encountered and penetrated hosts. However, increases in activity might come at the cost of establishment: only 71% of the parasites in the warmest treatment successfully established metacercarial cysts after penetration, compared to 87% in the coldest treatment. Similar tradeoffs have been reported in other disease systems; for instance, Woodhams *et al.* (2008) found that lower growth rates and establishment success of the pathogen *Batracochytrium dendrobatidis* at low temperatures were counteracted by longer survival and greater release of infectious stages, allowing the pathogen to maintain high performance over a wide range of temperatures. Such contrasting effects of temperature on development and mortality rates of parasites, vectors and hosts are common in many disease systems, highlighting the important potential for nonlinearities in disease responses to temperature that will likely over-ride more simplistic predictions of increased disease due to faster parasite development rates (Lafferty, 2009; Paull & Johnson, 2011).

Ecologically relevant laboratory experiments testing temperature-driven changes to the full infection process can provide insight into how future climate change will influence disease in host populations. Because we used temperature and infection levels guided by field data, we can combine our mechanistic understanding of how temperature influences host pathology with inferences about potential changes to the density and seasonal abundance of hosts and parasites in this system to better understand how climate change may influence pathology risk. Here, we found that temperature hastened tadpole development and shortened the window during which hosts were susceptible to deformities. Under field conditions, however, temperature will also accelerate parasite development within upstream hosts such as snails, leading to earlier cercarial emergence and often greater cercarial production (Poulin, 2006; Paull & Johnson, 2011). If temperature-driven increases in cercarial production occur, this could override the observed reduction in malformation risk at warmer temperatures. Additionally, because amphibian breeding is jointly constrained by precipitation and temperature (Duellman & Trueb, 1986), climate change may not always lead to earlier amphibian breeding (Blaustein *et al*., 2001; Gibbs & Breisch, 2001; Todd *et al*., 2011), particularly in systems where precipitation is projected to decline. Thus, the timing of precipitation events and the magnitude of temperature-driven increases in parasite and host development rates will be central in governing the timing of host-parasite interactions and host pathology. Importantly, however elevated mortality of snail hosts with increasing temperature, or evaporation could counter-act this temperature-driven increase in cercarial production. This emphasizes the value of extending the approach used here to simultaneously evaluate the effects of climate-driven changes to the population dynamics of the snail, amphibian and avian hosts to predict climate-driven changes in this system.

Conclusion

Predicting how diseases will respond to climate change requires a mechanistic understanding of how temperature will influence hosts, parasites, and their interactions. Because hosts and parasites experience

trade-offs between life history characteristics such as development rates and survival in response to temperature increases, nonlinear relationships between temperature and infection or pathology are likely to be common. Taken together, our results suggest that predictions of climate-driven changes to disease risk should incorporate an understanding of how climate affects the ontogeny and phenology of hosts and parasites, and the predicted range of temperatures most likely to be experienced at the time of infection. Given that stage-dependent parasite virulence and host tolerance are common to a variety of disease systems (Kelly *et al*., 2010; Patankar *et al*., 2011), incorporating ontogeny into studies of climate-driven changes to host-parasite interactions will enhance our understanding of how host pathology may respond to climate change (Ryce *et al*., 2004; Yang & Rudolf, 2010), including the potential for nonlinear effects of temperature on host pathology. Our results also indicate that contrasting effects of temperature on the infection process may not be apparent from simple comparisons of parasite burdens across temperatures, requiring instead a more thorough investigation of the temperature-dependence of different stages of the infection process. Temperature-driven changes to host immunity and parasite penetration may become particularly important when considering the impact of other environmental changes on hosts and parasites.

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