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Host and parasite thermal acclimation responses depend on the stage of infection

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Summary

1. Global climate change is expected to alter patterns of temperature variability, which could influence species interactions including parasitism. Species interactions can be difficult to predict in variable-temperature environments because of thermal acclimation responses, i.e. physiological changes that allow organisms to adjust to a new temperature following a temperature shift.

2. The goal of this study was to determine how thermal acclimation influences host resistance to infection and to test for parasite acclimation responses, which might differ from host responses in important ways.

3. We tested predictions of three, non-mutually exclusive hypotheses regarding thermal acclimation effects on infection of green frog tadpoles (*Lithobates clamitans*) by the trematode parasite *Ribeiroia ondatrae* with fully replicated controlled-temperature experiments. Trematodes or tadpoles were independently acclimated to a range of 'acclimation temperatures' prior to shifting them to new 'performance temperatures' for experimental infections.

4. Trematodes that were acclimated to intermediate temperatures (19–22 °C) had greater encystment success across temperatures than either cold- or warm-acclimated trematodes. However, host acclimation responses varied depending on the stage of infection (encystment vs. clearance): warm- (22–28 °C) and cold-acclimated (13–19 °C) tadpoles had fewer parasites encyst at warm and cold performance temperatures, respectively, whereas intermediate-acclimated tadpoles (19–25 °C) cleared the greatest proportion of parasites in the week following exposure.

5. These results suggest that tadpoles use different immune mechanisms to resist different stages of trematode infection, and that each set of mechanisms has unique responses to temperature variability. Our results highlight the importance of considering thermal responses of both parasites and hosts when predicting disease patterns in variable-temperature environments.

Key-words: beneficial acclimation, dormancy, *Helisoma trivolvis*, hibernation, *Rana clamitans*, thermal stress

Introduction

Climate change is expected to alter species interactions with potentially important effects on communities and ecosystems (Parmesan 2006; Tylianakis *et al.* 2008; Lafferty 2009). In addition to changes in mean temperature and precipitation, both the amplitude and frequency of temperature fluctuations are predicted to shift in many regions (Schar *et al.* 2004; Yeh *et al.* 2009; Rohr & Raffel 2010). Changes in mean temperature can strongly influence measures of organismal performance, such as growth rate and swimming speed (Ratkowsky *et al.* 1982; Wilson & Franklin 1999); however, until recently few studies investigated the effects of temperature variability on species interactions, such as predation and parasitism (Paaijmans *et al.* 2010; Duncan, Gonzalez & Kaltz 2013; Raffel *et al.* 2013, 2015; Barbosa, Pestana & Soares 2014; Paull *et al.* 2015).

A core problem in studying variable-temperature effects on organisms is that an organism's recent thermal history can influence its physiological performance at a given temperature, due to thermal acclimation responses and responses to energetic stress (Angilletta 2009; Paull *et al.* 2015). Acclimation responses, or the ability of an

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organism to adjust to environmental changes, have been well-studied in individual organisms, but the potential implications of differential acclimation effects for species interactions in variable-temperature environments remain poorly understood (but see Raffel *et al.* 2013). Prior studies have shown that delays in host acclimation following a temperature shift can make hosts more susceptible to infection (relative to acclimated hosts), leading to increased infection in variable-temperature environments (Raffel *et al.* 2006, 2013, 2015). However, important questions remain unexplored, including the potential for parasite acclimation responses and nonlinear responses of hosts and parasites to acclimation temperature. Here, we address these gaps by testing for linear and nonlinear thermal acclimation effects on a parasite and its ectothermic host.

Parasites of ectotherms are useful for testing how temperature variability influences species interactions because both parasite and host experience the same fluctuations in temperature. Parasites are also generally smaller and should therefore have faster metabolisms than the organisms they infect (Gillooly *et al.* 2001; West, Woodruff & Brown 2002; Brown *et al.* 2004), leading to the general prediction that parasites should acclimate to changing temperatures more rapidly than their hosts (Raffel *et al.* 2013). This effect could give parasites an advantage over their hosts in variable-temperature environments (the 'temperature variability hypothesis', Rohr & Raffel 2010), assuming that thermal acclimation responses lead to increased performance of both the host and the parasite (i.e. 'beneficial acclimation', discussed below). For the purposes of this study, we define an 'acclimation effect' as any effect of acclimation temperature on the shape of an organism's thermal performance curve, which describes physiological performance (e.g. parasite infectivity or host resistance) as a function of temperature (Angilletta 2009).

We considered three hypotheses for mechanisms driving thermal acclimation effects, each of which generates predictions for how an organism's thermal history might influence the performance curve for a given physiological process. (i) The 'beneficial acclimation hypothesis' postulates that an organism acclimated to a particular temperature will have increased physiological performance at that temperature relative to an unacclimated organism due to plastic responses (Leroi, Bennett & Lenski 1994; Fig. 1a). (ii) The 'thermal stress hypothesis' postulates that exposure to energetically stressful (e.g. extreme warm) temperatures could result in depleted energy stores, causing reduced performance across all temperatures (Fig. 1b, Paull *et al.* 2015). (iii) Alternatively, organisms might sometimes advantageously reduce physiological performance following acclimation to a particular temperature, for example to conserve energy by depressing their metabolisms during winter hibernation or summer aestivation (Geiser 2004; Storey & Storey 1990). This 'dormancy hypothesis' suggests that plastic responses to a particular temperature might sometimes involve lowered performance, contrary to predictions of the beneficial acclimation hypothesis (Fig. 1c). These hypotheses are not mutually exclusive, and combinations of responses might lead to nonlinear acclimation responses. For example, a single species might exhibit a dormancy response when acclimated to extreme cold temperatures but respond with beneficial acclimation or thermal stress at warmer temperatures, resulting in intermediate-acclimated organisms performing best overall (Fig. 1d).

We studied the effects of acclimation on host and parasite performance using *Ribeiroia ondatrae*, a trematode parasite that infects tadpoles via cercariae, which are motile infective stages that penetrate tadpole skin and encyst as metacercariae. This parasite is known to cause pathology (e.g. limb deformities) and reduced survival in amphibian larvae (Johnson *et al.* 2012). We were particularly interested in how shifts in temperature affected both parasite infectivity, or the ability of a cercaria to encyst and survive as a metacercaria, and host resistance, or the ability of the tadpole to reduce parasite survival at either of these stages of infection. Infectivity and resistance are difficult to disentangle because both are quantified by measuring parasite infection levels, such that measures of host resistance are typically the inverse of measures of parasite infectivity.

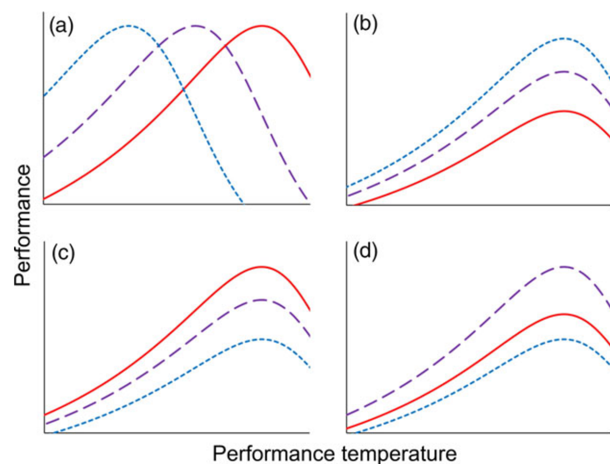

Fig. 1. Hypothetical performance curves describing predictions for three mechanisms by which thermal acclimation might influence organismal performance (cold-acclimated organisms, short-dashed blue; intermediate-acclimated, long-dashed purple; warm-acclimated, solid red). (a) The beneficial acclimation hypothesis (Leroi, Bennett & Lenski 1994) predicts that at cooler temperatures, cold-acclimated organisms will have higher performance relative to warm-acclimated organisms, and vice versa. (b) The thermal stress hypothesis predicts that acclimation to extreme warm temperatures will cause reduced performance across all performance temperatures due to physiological or energetic stress (Paull *et al.* 2015). (c) The dormancy hypothesis predicts that cold-acclimation will result in reduced performance across all temperatures, as an adaptation to conserve energy during adverse winter conditions (i.e. hibernation). (d) It is also possible for an organism to undergo thermal stress when acclimated to high temperatures and dormancy when acclimated to low temperatures, which could lead to intermediate-acclimated organisms performing best across performance temperatures.

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Furthermore, parasites and hosts in this system would likely experience simultaneous temperature fluctuations in a natural setting. However, it is possible to distinguish acclimation effects on infectivity vs. resistance in the lab by subjecting only one focal species at a time (tadpole or trematode) to a temperature shift, while holding the other species at constant temperatures (e.g. Raffel *et al.* 2013). *Ribeiroia ondatrae* has a complex life cycle, which allowed us to subject parasites to various acclimation temperatures while still in their snail intermediate hosts (independent of tadpole acclimation).

We conducted two experiments to quantify changes in parasite or host performance (hereafter referred to as 'infectivity' and 'resistance', respectively) as functions of performance and acclimation temperatures. The first experiment measured effects of parasite thermal acclimation on its ability to encyst in tadpoles at different temperatures. The second experiment measured effects of host acclimation on resistance to different stages of trematode infection: (i) trematode encystment and (ii) persistence as encysted metacercariae (i.e. clearance). We predicted that parasite encystment and clearance would be nonlinear functions of performance temperature, based on the nonlinearity of metabolic performance curves, and that acclimated hosts and parasites would have increased performance at the acclimation temperature relative to unacclimated hosts and parasites (i.e. 'beneficial acclimation').

Materials and methods

ANIMAL WELFARE

The thermal manipulations and experimental infections conducted in this study were in compliance with University of Colorado at Boulder protocol 1106·07 and Oakland University IACUC protocol 12111.

PARASITE LIFE CYCLE COMPLETION AND ANIMAL MAINTENANCE

Ribeiroia ondatrae is a trematode parasite with a complex life cycle involving three hosts. Larval stages of *R. ondatrae* called cercariae leave the snail intermediate host (e.g. *Helisoma trivolvis*) and actively seek a second intermediate host, often a larval amphibian. The cercariae penetrate the amphibian's skin and encyst subcutaneously as metacercariae, often near a developing limb bud (Johnson *et al.* 1999, 2004). They remain encysted until they are cleared by the host immune system or until the amphibian is consumed by the definitive host, which is typically a bird or mammal (LaFonte & Johnson 2013). Adult worms live and reproduce within the definitive hosts, releasing eggs into the host's intestinal tract. The definitive host then excretes faeces containing trematode eggs, from which the free-living miracidium stage emerges. The miracidium infects an aquatic planorbid snail, where it matures into rediae that produce cercariae, thus completing the life cycle. The *R. ondatrae* life cycle was completed in the laboratory by exposing *H. trivolvis* snails (3–5 mm in diameter) to embryonated parasite eggs derived from the faeces of lab-infected rats (Johnson *et al.* 2007; Paull *et al.* 2015).

Three green frog (*Lithobates clamitans*) egg masses were collected by the Charles Sullivan Company in Nashville, TN in May 2012 and May 2013 and shipped to our laboratories in Boulder, CO and Rochester, MI (respectively). Egg masses were placed in shallow pans of aerated, dechlorinated tap water. After hatching, tadpoles were fed a mix of frozen spinach and Tetramin fish flakes until the start of each experiment. We controlled for potential clutch-dependent differences in tadpole susceptibility to *R. ondatrae* infection in both experiments by randomly assigning tadpoles to treatments, and by including clutch as a factor in the tadpole acclimation experiment analyses. *Helisoma trivolvis* snails were initially collected from sites in the East Bay Area of California (trematode acclimation experiment) and Pinchot Lake in Pennsylvania (tadpole acclimation experiment). Snails were maintained in dechlorinated tap or artificial spring water (ASW; Cohen, Neimark & Eveland 1980) and were fed a combination of frozen spinach, fish flakes, leaf litter and natural algae (in outdoor pools). All snails were of similar age and size at the time of cercaria collection. We currently have no evidence for site-specific differences in snails' abilities to support *R. ondatrae* infection.

In both experiments, snails and tadpoles were maintained individually in 0·5-L glass mason jars filled with 300 mL of either local spring water (trematode acclimation experiment) or ASW (tadpole acclimation experiment). To ensure true replication of temperature treatments, incubators were constructed using Styrofoam coolers, heat tape and adjustable thermostats (Raffel *et al.* 2013). Incubators were treated as random effects in statistical analyses to ensure that inferences about temperature treatments were made at the correct levels of replication. Six or eight jars were placed in each incubator (see below), and jars were rotated daily to control for within-incubator temperature variation. Tadpoles were fed *ad libitum* with thawed 1 cm^2 pieces of frozen spinach and snails with an agar gel preparation containing ground Tetramin fish flakes and calcium powder (Paull *et al.* 2015). Twice weekly, soiled water was replaced with clean water of the appropriate temperature for each treatment. All animals were maintained on a 12 : 12 h light:dark cycle.

TEMPERATURE TREATMENTS

Throughout the paper, we refer to the temperature at which an organism was maintained prior to a temperature shift as the 'acclimation temperature', whereas the 'performance temperature' is the second temperature at which we assessed parasite infectivity or host resistance. In both experiments, five or six acclimation temperatures were used and then either parasites or hosts were shifted to one of five or six performance temperatures, yielding 25–36 total treatments per experiment (Fig. 2) and allowing for testing of nonlinear effects of both performance and acclimation temperature. Experimental temperature ranges reflected the range of daily average summer water temperatures (15·5–29·3 °C) observed at 21 California field sites by HOBO data loggers (Onset Computer Corp, Bourne, MA, USA) placed 50 cm below the water surface (Paull *et al.* 2015). HOBO data loggers were also used to verify that temperatures within experimental incubators matched the desired treatment temperatures (Fig. S1).

TREMATODE ACCLIMATION EXPERIMENT

To test whether thermal acclimation affected *R. ondatrae* infectivity, each parasite-infected snail was acclimated for 1 week, and

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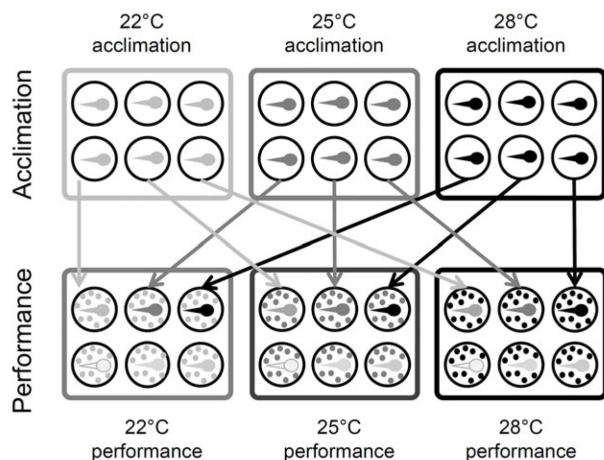

Fig. 2. Design of the tadpole acclimation experiment. We acclimated tadpoles to one of six temperatures (13, 16, 19, 22, 25 and 28 °C; only three are shown here for simplicity) in a series of small incubators (rectangles). Each incubator contained six tadpoles in individual containers (circles). After 3 weeks of acclimation, we moved one tadpole from each incubator to each of six performance temperatures. We then exposed tadpoles to 20 *R. ondatrae* cercariae (dots within the circles) that were acclimated to their respective performance temperatures (cercariae never experienced a temperature shift). Lighter shades of grey represent colder temperatures.

each tadpole for 3 weeks, to one of five temperatures (16, 19, 22, 25, 28 °C). The low-temperature treatment was set at 16 °C to ensure that snails would produce a sufficient number of cercariae for the experiment (Paull & Johnson 2011). A 3-week acclimation period was chosen because work by Bly & Clem (1991) suggests that ectotherm immunity requires a few weeks to acclimate to a temperature drop, but the acclimation period also needed to be short enough to avoid tadpole metamorphosis. The 1-week acclimation period for snails is consistent with that determined by Paull *et al.* (2015). The *R. ondatrae*-infected snails were then moved to their respective performance temperatures (tadpoles did not experience a temperature shift in this experiment). After the water reached the new performance temperature, *R. ondatrae* cercariae were collected and pooled from snails in each acclimation × performance temperature combination, and tadpoles were exposed to 30 *R. ondatrae* cercariae each. All cercariae were collected within 2 h of switching the snails' temperatures to minimize the possibility that parasites could have become acclimated to the new temperature prior to tadpole exposure. One tadpole from each incubator was exposed to cercariae from each of the five acclimation temperatures (five parasite acclimation × five performance temperatures = 25 treatment combinations), in a fully crossed nested design in which tadpoles were nested within incubators (analogous to a split-plot design in which incubators are treated as plots and individual tadpoles as subplots). After allowing 72 h for metacercariae to encyst within tadpoles, tadpoles were euthanized and necropsied to quantify infection. The measure of parasite infectivity for this experiment was the proportion of parasites that encysted in each tadpole.

To control for the potentially confounding effect of temperature on tadpole development, which can influence susceptibility to *R. ondatrae* infection (Rohr, Raffel & Hall 2010; Johnson, Kellermanns & Bowerman 2011), two temporal blocks were used, which differed in how tadpoles were assigned to temperature treatments. In the first block, mean tadpole developmental stage (Gosner 1960) was the same at the beginning of the acclimation period, whereas in the second block, tadpoles were assigned to temperature treatments such that their mean Gosner stage at the *end* of the acclimation period (e.g. at exposure) was projected to be the same across temperature treatments based on published temperature-dependent developmental rates for green frog tadpoles (Berven, Gill & Smith-Gill 1979). Gosner developmental stage of each tadpole at the time of exposure was also recorded for use as a statistical covariate in subsequent analyses. We assumed that all cercariae were at the same developmental stage at the time of their release from snails. Each temporal block contained three incubators at each of the five temperatures, and five tadpoles within each incubator were exposed to parasites, for a total of 150 tadpoles (six total replicates per treatment combination).

TADPOLE ACCLIMATION EXPERIMENT

In the second experiment, tadpoles (but not *R. ondatrae*) were subjected to a temperature shift to determine whether tadpole acclimation influenced their ability to resist trematode infection. Having learned that snails can produce *R. ondatrae* cercariae at 13 °C given a long enough acclimation period (Paull *et al.* 2015), six acclimation and six performance temperatures (13, 16, 19, 22, 25 and 28 °C) were used. Tadpoles were acclimated for 3 weeks and snails for 1 week; then one tadpole per incubator was moved to each of six performance temperatures in a fully crossed, split-plot design (six acclimation × six performance temperatures = 36 treatment combinations, Fig. 2). There were six replicate tadpoles for each temperature treatment combination for a total of 216 tadpoles. Because each tadpole in a particular temperature cross treatment (e.g. 13 °C acclimation × 16 °C performance) experienced a unique incubator combination, each tadpole could be treated as an individual replicate by treating incubators as random effects in our statistical models. Trematode cercariae from each temperature treatment (parasites did not experience a temperature shift) were collected and pooled, and each tadpole was exposed to 20 cercariae at its performance temperature.

Because tadpoles can clear *R. ondatrae* metacercariae post-encystment (LaFonte & Johnson 2013; LaFonte *et al.* 2015), *R. ondatrae* cercariae were tagged with a fluorescent dye to allow for tracking the clearance of trematode metacercariae through time (described below). To provide time for this additional data collection, this experiment was conducted in six temporal blocks, with one replicate per treatment combination in each block. Gosner stage data were collected at the end of the acclimation period for use as a covariate to control for developmental effects on susceptibility, relying on high random variation in green frog tadpole developmental rates to ensure that direct temperature effects on resistance and thermal effects on development could be distinguished.

QUANTIFYING PARASITE INFECTION THROUGH TIME VIA FLUORESCENT LABELLING

To allow *in vivo* tracking of *R. ondatrae* metacercariae, cercariae were labelled with a green fluorescent fatty acid analogue (BODIPY FL C12; Invitrogen, Carlsbad, CA, USA) prior to exposing cercariae to tadpoles (Keeney *et al.* 2008; LaFonte & Johnson 2013; LaFonte *et al.* 2015). Within 5 h of emergence, groups of 30–40 cercariae were exposed to a 100 nM dye solution (made by

adding 10 μL of a dye-dimethyl sulfoxide mixture to 10 mL of water) for 45 min, taking care to maintain cercariae at the appropriate temperature. Then, the dyed cercariae were rinsed in fresh ASW, and each tadpole was exposed to 20 cercariae.

Beginning 12 h after initial *R. ondatrae* exposure, each tadpole was anaesthetized by immersion in a 0·005% (w/v) benzocaine solution for *c.* 10 min. Once immobilized, each tadpole was placed on a Petri dish containing a black clay mold to facilitate tadpole positioning. Tadpoles were viewed under a green fluorescent protein 2 (GFP-2) filter on a fluorescent stereomicroscope (Leica MZ10 F; Leica Microsystems, Wetzlar, Hesse, Germany) equipped with a digital camera (Leica DFC450 C; Leica Microsystems). Using image capture software (Leica Application Suite; Leica Microsystems), six regions of each tadpole were photographed: left, right and ventral posterior (limb bud region) and left, right and ventral anterior (mouth and gill region). These areas accounted for the majority of *R. ondatrae* encystment; however, additional photographs were taken of metacercariae encysted outside these areas. Once photographed, each tadpole was placed in fresh ASW and returned it to its performance incubator. The photograph process was repeated 7 and 14 days post-exposure, and tadpoles were euthanized by immersion in a 0·1% benzocaine solution before taking the final set of photos. The number of cercariae that failed to penetrate each tadpole was recorded after 12 h of exposure by searching the bottom of the tadpole's container for dead or dying cercariae. No cercariae remained actively swimming at the 12 h time point.

QUANTIFYING DIFFERENT STAGES OF PARASITE INFECTIVITY (ENCYSTMENT AND PERSISTENCE)

Metacercariae in tadpoles were quantified at each time point by analysing photographs using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Each metacercaria was counted only once, and photos from 7 and 14 days post-exposure were compared to the 12-h photos to determine how many of the original metacercariae remained at each time point (Fig. S2). *Ribeiroia ondatrae* encystment was measured as the proportion of cercariae that encysted within 12 h of exposure:

$$\text{Proportion encysted} = \frac{\#\text{metacercariae}_{12h}}{20}, \qquad \text{eqn 1}$$

Persistence (survival) of metacercariae through time was also tracked, defined here as the proportion of metacercariae still present as a function of time. However, we suspect that the temperature-dependence of metacercaria persistence is largely mediated by variation in host immune responses rather than parasite thermal responses, given that metacercaria survival is a largely passive process. We therefore chose to view metacercaria persistence from the host's perspective by calculating rates of 'metacercaria clearance', which is the inverse of metacercaria persistence. Thus, metacercaria clearance over the first week was calculated as:

$$\text{Clearance}_{Wk1} = 1 - \frac{\#\text{metacercariae}_{7d}}{\#\text{metacercariae}_{12h}} \qquad \text{eqn 2}$$

and metacercaria clearance over the second week was:

$$\text{Clearance}_{Wk2} = 1 - \frac{\#\text{metacercariae}_{14d}}{\#\text{metacercariae}_{7d}}. \qquad \text{eqn 3}$$

Despite our focus on metacercaria 'clearance' instead of 'persistence', it is important to emphasize that performance temperature effects on metacercaria clearance could have been mediated by either host or parasite thermal responses.

STATISTICAL ANALYSES

Both experiments were analysed using the program R v.3.0.2 (R Development Core Team, 2013), and linear mixed-effects models were used for all analyses (function 'lme' in package 'nlme'; Pinheiro *et al.* 2014). All models included linear effects of acclimation temperature and performance temperature. Quadratic effects of acclimation temperature and performance temperature and the interaction between acclimation and performance temperatures were also tested for. These terms were removed from the final models if they were non-significant, after ensuring that their removal did not alter the significance of any other variables ($P > 0{\cdot}05$). We used type II sums of square errors to obtain F-statistics and P-values for each predictor, treating linear terms as marginal to both interaction and quadratic terms. Using Type II, sums of squares allowed us to avoid problems with predictor collinearity that can arise in statistical models with unbalanced designs (due to tadpole mortality) or polynomial regression with non-centred predictor variables.

All proportional response variables were arcsine-square-root-transformed to improve normality. However, in a few cases, it was found that the tadpole had been exposed to more than 20 cercariae, resulting in an apparent proportion of encysted trematodes greater than one, making this transformation impossible (the number of failed cercariae plus the number of metacercariae 12 h post-exposure was 21 in 4·6% of all tadpoles and 22 in 1·4%). In these cases, the 'proportion encysted' calculations were modified to reflect the higher exposure number prior to transformation (i.e. proportion encysted = 1). Results were unchanged when the unmodified, untransformed data were analysed. Analysing data from both experiments using a logit (instead of arcsine-square-root) transformation, as recommended by Warton & Hui (2011), was also tried, and both methods yielded similar results. However, the arcsine-square root transformation was chosen because it transforms all data in the same manner, while the logit transformation (which is different from the logit link function for binomial analyses) requires adding a small, arbitrary number to zeroes and subtracting a small number from ones.

For the trematode acclimation experiment, the response variable was the proportion of total parasites to encyst within the tadpole. Tadpole developmental stage and temporal block were included as covariates. Performance incubator was also included as a random effect, to ensure that main effects of performance temperature were assessed with the correct unit of replication.

For the tadpole acclimation experiment, encystment and clearance were analysed separately. Performance incubator was included as a random effect in all models except those assessing main or quadratic effects of acclimation temperature. For these model comparisons, acclimation incubator was used as the random effect instead. Temporal block and clutch were included as blocking variables in the tadpole acclimation analysis, and it was tested whether Gosner stage was a significant covariate in all models. Models were simplified by removing the Gosner stage covariate if it was non-significant.

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Results

TREMATODE ACCLIMATION EXPERIMENT

Both acclimation temperature and performance temperature had nonlinear effects on parasite encystment, as indicated by significant quadratic terms (Table S1). Parasite encystment was highest at intermediate performance temperatures, and when parasites had been exposed to intermediate acclimation temperatures (Fig. 3a). For instance, the encystment success of intermediate-acclimated trematodes (19–22 °C) was about 1·5 times greater than that of either cold- (13–16 °C) or warm-acclimated (25–28 °C) trematodes at both extreme low (13 °C) and high (28 °C) temperatures. There was an interactive effect of quadratic acclimation and quadratic performance temperature (Table S1). These temperature effects were consistently observed in both experimental blocks, although parasite encystment proportions were significantly higher in block two than in block one (Table S1). More-developed tadpoles had lower encystment proportions, but this effect did not account for the main effect of block, because there were similar final Gosner stages in the different blocks (mean ± SE: block one = 33·0 ± 0·030, block two = 32·9 ± 0·027).

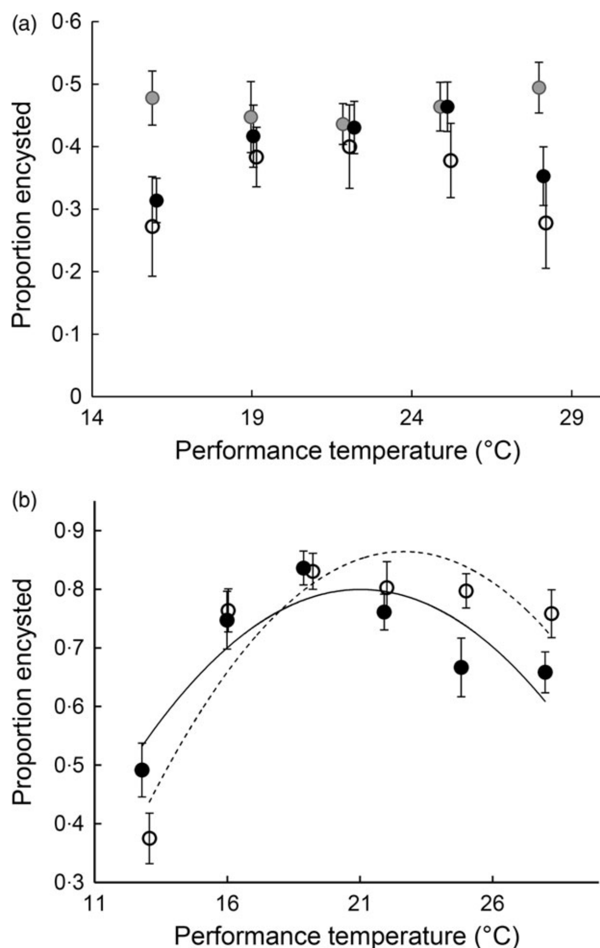

Fig. 3. Proportion of *R. ondatrae* parasites to encyst in tadpoles across performance temperatures in (a) the trematode acclimation experiment and (b) the tadpole acclimation experiment. (a) Trematode acclimation experiment: cold-acclimated trematodes (16 °C, open circles), intermediate-acclimated (19–22 °C, grey circles) and warm-acclimated (25–28 °C, black circles). (b) Tadpole acclimation experiment: cold-acclimated tadpoles (13–19 °C, open circles and dashed lines), warm-acclimated tadpoles (22–28 °C, closed circles and solid lines). Note that we acclimated each organism to a single temperature; however, we grouped acclimation temperatures here, according to similar patterns, for simplicity (See Fig. S4 for separate points for each acclimation temperature). Error bars are ±1 SE.

TADPOLE ACCLIMATION EXPERIMENT

There was a nonlinear relationship between performance temperature and the proportion of encysted trematodes, with the highest encystment success at intermediate temperatures (Fig. 3b, Table S2). However, there was also a significant interaction between acclimation and performance temperatures (Table S2), such that cold-acclimated (13–19 °C) tadpoles were more resistant to parasite establishment at cooler temperatures, and warm-acclimated tadpoles were more resistant at warmer temperatures (Fig. 3b).

Metacercaria clearance was analysed separately for week one vs. week two post-exposure. The proportion of metacercariae cleared in week one increased with both acclimation temperature and performance temperature (Table S3). There was a significant, quadratic effect of acclimation temperature on week 1 metacercaria clearance, with tadpoles acclimated to intermediate temperatures (19–25 °C) clearing a higher proportion of metacercariae than those acclimated to extreme cold (13–16 °C) or warm (28 °C) temperatures (Fig. 4a, Table S3). Clearance for intermediate-acclimated tadpoles was approximately four times greater than that for cold- or warm-acclimated tadpoles at the 16 °C performance temperature, and 2·5 times greater at 19 °C. There was no significant interaction between the linear effects of acclimation and performance temperature, but there was a significant interaction between performance temperature and the quadratic effect of acclimation temperature (Table S3). This result was driven by the nonlinear effect of acclimation temperature being stronger at lower performance temperatures than at higher performance temperatures, at which the acclimation effect seemed to disappear (Fig. 4a).

Unlike for the analysis of clearance in week one, there were no detectable acclimation effects on metacercaria clearance for week two (Fig. 4b, Table S3). There was also a negative quadratic effect of performance temperature on week two metacercaria clearance (Fig. 4b, Table S3). Neither block nor clutch had a significant effect on metacercaria clearance in either analysis of clearance rates (Table S3).

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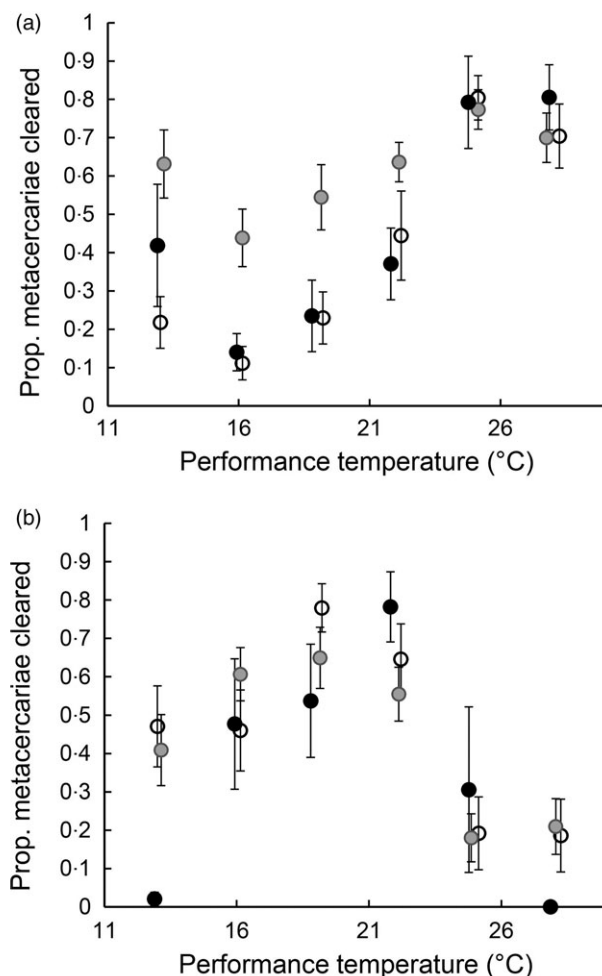

Fig. 4. Proportion of metacercariae cleared by tadpoles (a) from 12 h to 7 days and (b) from 7 to 14 days after exposure to *R. ondatrae*. Although tadpoles were acclimated to six separate temperatures, here we group acclimation temperatures with similar patterns to improve clarity of presentation: 13–16 °C, open circles; 19–25 °C, grey circles; 28 °C, black circles (see Fig. S5 for separate points for each acclimation temperature). Error bars are ±1 SE.

Gosner stage at the time of exposure was not a significant predictor in any of the analyses for the tadpole acclimation experiment, indicating that developmental effects of host acclimation temperature are unlikely to account for observed differences in parasite resistance (Fig. S3).

Discussion

As predicted, both parasite encystment and metacercaria clearance were nonlinear functions of temperature, consistent with the nonlinearity of typical performance curves. However, acclimation effects varied depending on the organism (parasite vs. host) and the stage of infection (parasite penetration, establishment or persistence) examined. The results provided support for all three of our proposed acclimation hypotheses (beneficial acclimation, thermal stress and dormancy). Trematodes acclimated to intermediate temperatures had the highest infectivity overall, consistent with either thermal stress or dormancy effects occurring at extreme high and low temperatures. In contrast, the results for tadpole resistance to parasite encystment supported the beneficial acclimation hypothesis, with warm-acclimated tadpoles being more resistant at warmer temperatures and cold-acclimated tadpoles being more resistant at colder temperatures. Tadpole clearance of encysted trematodes in the week following exposure was greatest in tadpoles acclimated to intermediate temperatures, suggesting either thermal stress or dormancy effects on tadpoles exposed to extreme high or low temperatures. Alternatively, this result might indicate beneficial acclimation at mid- to high temperatures combined with dormancy or thermal stress at cool temperatures. However, there were no significant effects of acclimation temperature on trematode clearance in the second week post-exposure, suggesting that tadpoles had become fully acclimated after 7 days at their new temperatures. Taken together, these results show that thermal acclimation effects on parasite resistance can differ depending on the specific stage of infection the host is responding to, highlighting the likelihood that each type of resistance involves different physiological mechanisms that might respond differently to environmental variation. Each result is discussed in more detail below.

Our experimental design allowed us to disentangle effects of acclimation temperature on parasite infectivity vs. host resistance. However, it is more difficult to determine whether the observed effects of performance temperature were caused by parasite or host thermal responses because both organisms experienced the same thermal environment simultaneously. Finding ways to disentangle effects of performance temperature on parasites vs. hosts is an important outstanding problem for the thermal biology of parasitism.

TREMATODE ACCLIMATION

Trematodes acclimated to intermediate temperatures (19–22 °C) were the most infective across all performance temperatures. Reduced trematode infectivity at warmer temperatures is consistent with the thermal stress hypothesis of Paull *et al.* (2015), who found that *R. ondatrae* cercaria production by infected snails was reduced following exposure to warm temperatures, likely due to reduced energy reserves in thermally stressed snails. In addition, Studer & Poulin (2013) found reduced survival times of trematode cercariae at 30 °C in comparison to those at 20 °C, suggesting that these cercariae had faster metabolisms and exhausted their energy supplies faster at warmer temperatures, consistent with the thermal stress hypothesis. Thermal stress might also explain our observation of reduced performance of cold-acclimated (13–16 °C) cercariae, if the trematodes or their snail hosts are energetically stressed at colder temperatures. However, cool temperatures are less likely to cause energetic stress

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in ectotherms than warm temperatures, due to lower metabolic rates and thus reduced energy usage (Brown *et al*. 2004). Alternatively, this pattern of reduced infectivity of cercariae from cold-acclimated snails might be a side effect of a dormancy response of rediae, the trematode stage that parasitizes snails and produces cercariae. Although there is unlikely a direct fitness benefit of reducing cercaria infectivity, rediae could advantageously reduce their metabolic performance during long-term exposure to cold temperatures. Such a dormancy response could increase trematode fitness in nature by saving energy while host snails are in winter hibernation, or by decreasing cercaria release at times when they are unlikely to be able to find and infect a suitable second intermediate host (i.e. 'cercaria storage': Poulin 2006; Paull *et al*. 2015; Paull, LaFonte & Johnson 2012). This latter hypothesis assumes that cercaria infectivity is limited by some of the same metabolic processes as those limiting metabolic performance of rediae.

TADPOLE ACCLIMATION – RESISTANCE TO PARASITE ENCYSTMENT

Our results supported the beneficial acclimation hypothesis for tadpole resistance to trematode encystment, with cold-acclimated (13–19 °C) tadpoles having fewer encysted trematodes at cooler performance temperatures and vice versa. This suggests that tadpoles undergo shifts in parasite resistance mechanisms in response to temperature variability, as predicted. However, the fact that this was the only measure of trematode resistance to provide unambiguous support for beneficial acclimation suggests that these thermal adjustments are specific to immunological mechanisms involved in resisting this stage of trematode infection (discussed below).

TADPOLE ACCLIMATION – METACERCARIA CLEARANCE

In the first week post-exposure, tadpoles acclimated to intermediate temperatures (19–25 °C) cleared the highest proportion of metacercariae relative to tadpoles acclimated to extreme high (28 °C) or low (13–16 °C) temperatures. However, this pattern was only evident at cooler performance temperatures, with all tadpoles clearing metacercariae at similarly high rates at warm performance temperatures. Beneficial acclimation at intermediate temperatures (19–25 °C) might explain tadpoles' improved performance at cooler temperatures (relative to warm-acclimated tadpoles), if acclimation led to a broadening of the tadpole performance curve rather than a shift in its peak. However, beneficial acclimation cannot explain the relatively poor performance of cold-acclimated tadpoles at cooler temperatures. One possible explanation for this pattern is that tadpoles acclimated to extreme temperatures were subjected to thermal stress. However, as with snails it seems unlikely that tadpoles would become energetically stressed at the cooler temperatures because temperature-induced reductions in metabolism should reduce their energy expenditures (Gillooly *et al*. 2001). In support of this idea, a prior study found evidence of physiological stress in frogs moved to higher (20–30 °C) but not lower (<20 °C) temperatures (Jurani *et al*. 1973). Alternatively, the poor performance of cold-acclimated tadpoles could be explained by a hibernation-like dormancy response. Note that these results are generally consistent with studies on the thermal biology of other free-living organisms, which have often found beneficial acclimation to occur at some but not all temperatures within an organism's normal thermal range (Angilletta 2009).

The similar initial clearance rates for all treatments at the two highest performance temperatures (Fig. 4a) might indicate that increased metabolism at high temperatures can overcome effects of thermal stress or dormancy on metacercaria clearance. However, this lack of acclimation effects at warmer performance temperatures might be simply due to the fact that we were dealing with proportion data, which is bounded at maximum of clearance = 1. Clearance rates increased with performance temperatures for all acclimation treatments and approached this boundary at the two highest performance temperatures, possibly leading to a convergence of all acclimation treatments on similar mean clearance rates (Fig. 4b). The one case where clearance did not increase with performance temperature was for tadpoles at the lowest performance temperature (13 °C), which showed higher than expected proportions of clearance in week one. This might be because parasites had difficulty (or took too much time) encysting at 13 °C (Fig. 3a), which might have made them easier to clear.

In contrast to the first week of parasite clearance, there was no effect of acclimation temperature on parasite clearance in the second week following exposure, despite the fact that clearance continued throughout this time period. This likely indicates that tadpoles had become largely acclimated to their new (performance) temperatures by 1 week after the temperature shift. Relatively little is known about the time required for amphibian immune acclimation, although work by Bly & Clem (1991) indicated that fish lymphocytes require 4–6 weeks to return to base levels following a temperature decrease, and Maniero & Carey (1997) showed that complement levels of cold-acclimated frogs increase over a span of 2–9 days following a temperature increase. Our results suggest that at least some components of amphibian immunity become fully acclimated by 1–2 weeks after a temperature shift.

DIFFERENCES IN HOST RESPONSES AT DIFFERENCE STAGES OF INFECTION

Thermal acclimation had different effects on the temperature-dependence of host resistance, depending on the stage of infection examined (encystment and clearance). This

suggests that each stage of infection is met by different physiological resistance mechanisms that are affected in different ways by thermal acclimation responses. Metacercaria clearance showed evidence of a dormancy response, with reduced performance of cold-acclimated hosts. In contrast, resistance to encystment was greater at lower temperatures in cold-acclimated hosts, consistent with beneficial acclimation. This raises the important question of what might be similar or different about these resistance mechanisms that might account for the observed patterns.

Relatively little is known about which components of the tadpole immune response are involved in each stage of trematode resistance. One potentially important difference is the relative time scales of parasite encystment and cyst clearance. Trematode encystment occurs on a very short timescale (<12 h, as observed in this study) relative to cyst clearance. This means that some components of the immune response are likely involved in cyst clearance but not in preventing encystment. For example, inducible cell-mediated defences like leucocyte migration or proliferation are not induced until at least 6 h post-infection (Taliaferro & Sarles 1939; von Lichtenberg *et al.* 1976), leaving little time for migrating cells to arrive prior to encystment. Therefore, host resistance to trematode encystment is likely accomplished primarily by constitutive defences such as innate humoral immunity (e.g. complement or anti-parasite secretions) and immune cells already residing in the skin or connective tissues (e.g. macrophages; Rollins-Smith *et al.* 2002; Ramsey *et al.* 2010). In contrast, clearance of encysted parasites occurs over days to weeks (Fig. 4, LaFonte & Johnson 2013), and is thought to be accomplished largely by cellular immune responses, including leucocyte migration and proliferation (Patel *et al.* 2009). Determining whether these differences can account for varying acclimation effects on different stages of resistance will require further investigations into how acclimation influences various tadpole immune mechanisms.

CONCLUSIONS AND IMPLICATIONS

The results of this study show that parasite thermal acclimation responses can influence the infection process, and support a small but growing body of evidence that thermal acclimation responses influence parasitic infection in variable-temperature conditions (Raffel *et al.* 2013, 2015). Prior studies demonstrated that host acclimation is important in determining disease transmission under fluctuating temperatures but used only two acclimation temperatures (Raffel *et al.* 2013, 2015), making it impossible to detect nonlinear effects of acclimation temperature on parasite infectivity and host resistance. The temperature variability hypothesis of Rohr & Raffel (2010) predicts that hosts will be more susceptible to parasitic infection following a temperature shift due to more rapid thermal acclimation by the parasite, but this prediction is based on the assumption that both host and parasite acclimation responses will lead to improved physiological performance (i.e. 'beneficial acclimation'). Our results show that parasites and hosts do not always conform to predictions of the beneficial acclimation hypothesis. Instead, we found evidence that thermal acclimation can influence parasite infectivity and host resistance in complex and sometimes nonlinear ways, with effects varying depending on the stage of infection examined. These results show that thermal acclimation effects on host-parasite relationships are more diverse than previously thought.

Acclimation effects on parasite infectivity and host resistance can be expected to vary depending on the specific host-parasite system, as well as between free-living and host-dependent stages of the same parasite. Further studies of additional host-parasite systems will be needed to determine if there are general patterns in parasite and host responses to temperature variability.

Our results highlight the complexities of species interactions under variable temperatures, which could become even more complicated in a natural setting. For instance, our study did not address the potential for thermal habitat selection (i.e. behavioural fever) by infected snails or tadpoles (or both), which could influence *R. ondatrae* transmission in natural ponds (Casterlin & Reynolds 1977; Macnab & Barber 2012). Similarly, thermal habitat selection would be an important consideration when applying our work to predator–prey systems. Furthermore, although the temperature variability hypothesis assumes that parasites will acclimate to shifting temperatures faster than hosts (Rohr & Raffel 2010), the relative acclimation times of organisms have yet to be investigated. In addition, the acclimation responses presented here are short-term adjustments to shifting temperatures; however, our experiments did not address the evolutionary consequences that might arise from increasing temperature variability (i.e. possible broadening of performance curves) and the costs associated with these adaptations. Thus, our study demonstrates that variable temperatures can have complex effects on species interactions; however, many questions remain unexplored.

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Data accessibility

Data are accessible through Dryad Data Repository: http://dx.doi.org/10.5061/dryad.f3k8p (Altman *et al.* 2016).

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Supporting Information

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

Fig. S1. Relationship between treatment temperatures and the average temperatures recorded in incubators throughout (a) the 3-week acclimation period and (b) the 2 weeks following exposure to *R. ondatrae* (open circles).

Fig. S2. Fluorescent metacercariae in the limb bud region of the same tadpole at two time points: (a) 12 h post-exposure to *R. ondatrae* cercariae, and (b) 7 days post-exposure.

Fig. S3. Mean Gosner (1960) stage of tadpoles at time of exposure to *R. ondatrae* cercariae in the tadpole acclimation experiment. Error bars are ±1 SD to describe the data distributions (as opposed to displaying error in estimation of the means).

Fig. S4. Proportion of *R. ondatrae* parasites to encyst across performance temperatures in tadpoles from (a) the trematode acclimation experiment and (b) the tadpole acclimation experiment. Different colors represent different acclimation temperatures (see within-figure legends).

Fig. S5. Proportion of *R. ondatrae* metacercariae cleared by tadpoles across performance temeperatures in the tadpole acclimation experiment (a) from 12 h to 7 days and (b) from 7 to 14 days after exposure to parasites. Each color represents a different acclimation temperature (see within-figure legend in panel a). Error bars are ± 1 SE.

Table S1. Regression statistics from a linear mixed effects model describing the effects of acclimation and performance temperature on *R. ondatrae* encystment in tadpoles (trematode acclimation experiment).

Table S2. Regression statistics from linear mixed effects models describing the effects of acclimation and performance temperature on the proportions of *R. ondatrae* parasites to penetrate and establish in the tadpoles (tadpole acclimation experiment).

Table S3. Regression statistics from a linear mixed effects model describing the effects of acclimation and performance temperature on *R. ondatrae* clearance 1 and 2 weeks post-exposure in tadpoles (tadpole acclimation experiment).

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