The Journal of Experimental Biology 216, 4204-4211

doi:10.1242/jeb.089896

RESEARCH ARTICLE

Cryptic impacts of temperature variability on amphibian immune function

Kimberly A. Terrell[1,*], Richard P. Quintero[2], Suzan Murray[2], John D. Kleopfer[3], James B. Murphy[2], Matthew J. Evans[2], Bradley D. Nissen[2] and Brian Gratwicke[1]

[1]Center for Species Survival and [2]Center for Animal Care Sciences, Smithsonian Conservation Biology Institute, National Zoological Park, 3001 Connecticut Avenue NW, Washington, DC 20008, USA and [3]Virginia Department of Game and Inland Fisheries, 3801 John Tyler Highway, Charles City, VA 23030, USA

*Author for correspondence (terrellk@si.edu)

SUMMARY

Ectothermic species living in temperate regions can experience rapid and potentially stressful changes in body temperature driven by abrupt weather changes. Yet, among amphibians, the physiological impacts of short-term temperature variation are largely unknown. Using an *ex situ* population of *Cryptobranchus alleganiensis*, an aquatic North American salamander, we tested the hypothesis that naturally occurring periods of temperature variation negatively impact amphibian health, either through direct effects on immune function or by increasing physiological stress. We exposed captive salamanders to repeated cycles of temperature fluctuations recorded in the population's natal stream and evaluated behavioral and physiological responses, including plasma complement activity (i.e. bacteria killing) against *Pseudomonas aeruginosa*, *Escherichia coli* and *Aeromonas hydrophila*. The best-fit model (ΔAIC_c=0, w_i=0.9992) revealed 70% greater *P. aeruginosa* killing after exposure to variable temperatures and no evidence of thermal acclimation. The same model predicted 50% increased *E. coli* killing, but had weaker support (ΔAIC_c=1.8, w_i=0.2882). In contrast, plasma defenses were ineffective against *A. hydrophila*, and other health indicators (leukocyte ratios, growth rates and behavioral patterns) were maintained at baseline values. Our data suggest that amphibians can tolerate, and even benefit from, natural patterns of rapid warming/cooling. Specifically, temperature variation can elicit increased activity of the innate immune system. This immune response may be adaptive in an unpredictable environment, and is undetectable by conventional health indicators (and hence considered cryptic). Our findings highlight the need to consider naturalistic patterns of temperature variation when predicting species' susceptibility to climate change.

Key words: thermal physiology, salamander, protein complement, *Cryptobranchus*, climate change.

Received 19 April 2013; Accepted 6 August 2013

INTRODUCTION

Temperate-region ectotherms experience broad thermal ranges and are therefore expected to tolerate, and in some cases benefit from, a warmer climate (Addo-Bediako et al., 2000; Deutsch et al., 2008; Kellermann et al., 2012; Snyder and Weathers, 1975; van Berkum, 1988). By contrast, tropical and polar ectotherms are adapted to a narrower and more stable range of temperatures (Janzen, 1967; Sunday et al., 2011), and are considered more vulnerable to anthropogenic climate change (Clusella-Trullas et al., 2011; Deutsch et al., 2008; Stillman, 2003; Tewksbury et al., 2008). While latitudinal patterns of thermal tolerance have received less attention among freshwater species, there is some evidence that this relationship extends to freshwater fish (Naya and Bozinovic, 2012) and amphibians (Duarte et al., 2012). Given that air and water temperatures are strongly correlated in freshwater systems (Morrill et al., 2005), it seems logical that aquatic species at low latitudes also would be particularly susceptible to climate change. Yet none of these predictions consider the potential for thermal variation itself to negatively impact ectothermic physiology. These potential impacts would be greatest in temperate regions, where climate is relatively less stable. Although freshwater habitats are somewhat buffered from rapid changes in air temperature, these systems can undergo substantial warming/cooling over relatively short time scales (see Isaak et al., 2012).

The role of short-term (i.e. daily, weekly or monthly) temperature variation in predicting species' responses to climate change has been largely overlooked, but is an area of growing research interest, particularly in amphibians (Niehaus et al., 2006; Niehaus et al., 2011; Niehaus et al., 2012; Raffel et al., 2006; Raffel et al., 2012). Exposure to naturalistic patterns of thermal variation accelerates maturation and improves jumping performance in the striped marsh frog (*Limnodynastes peronii*) (Niehaus et al., 2006), yet does not influence the metabolic performance of larvae (Niehaus et al., 2011). Rapid temperature shifts (from 25 to 15°C) increase susceptibility to *Batrachochytrium dendrobatidis* in the Cuban tree frog (*Osteopilus septentrionalis*), but the physiological basis of this relationship remains unclear (Raffel et al., 2012). Intriguingly, *O. septentionalis* can acclimate to a consistent (but not randomized) series of temperature shifts, suggesting that the pattern of thermal variation itself can influence amphibian disease susceptibility (Raffel et al., 2012).

Although thermal acclimation is well studied among amphibians (reviewed in Angilletta, 2009a), this research generally focuses on acclimation to constant temperatures. Given that most ectotherms live in thermally dynamic habitats, there is a need to better understand how short-term temperature variation influences both physiology and acclimation. To our knowledge, the only previous study of the influence of naturalistic patterns of temperature

variation on amphibian immune function is a field-based investigation (Raffel et al., 2006). These investigators correlated short-term and seasonal weather patterns to changes in leukocyte counts (i.e. reduced numbers of circulating lymphocytes, neutrophils and eosinophils) in the aquatic salamander *Notophthalmus viridescens* (Raffel et al., 2006). Yet the functional consequences of these changes are unclear, particularly as corticosteroid hormones can influence the distribution of leukocytes in blood and tissue (Davis et al., 2008).

Understanding the environmental determinants of amphibian physiological function is important because this taxon is experiencing declines on an unprecedented, global scale (Hof et al., 2011; Stuart et al., 2004; Young et al., 2004). Identifying potential sources of immune suppression is essential (Blaustein et al., 2012; Kiesecker, 2011), as extirpations have been linked to pathogenic disease, most notably chytridiomycosis (Cheng et al., 2011; Lips et al., 2006) and *Ranavirus* (Miller et al., 2011). Yet for many amphibians, particularly North American salamanders, the causes of dwindling population numbers are enigmatic (Caruso and Lips, 2013; Highton, 2005; Stuart et al., 2004). Climate change is hypothesized to be a contributing factor to ongoing declines (Stuart et al., 2004) and a major driver of future extinctions among Appalachian salamanders (Milanovich et al., 2010). Understanding how temperature variation influences amphibian physiology may help elucidate the causes of ongoing declines and will be important in accurately predicting extinction risk from climate change.

The hellbender [*Cryptobranchus alleganiensis* (Daudin 1803)] is a fully aquatic salamander inhabiting rivers throughout the Appalachian region of the eastern USA and in parts of the Ozarks (Nickerson and Mays, 1973). Both the Ozark subspecies (*C. a. bishopi*) and its eastern counterpart (*C. a. alleganiensis*) have experienced widespread extirpations and population declines, likely due in part to habitat degradation and poaching (Furniss, 2003; Mayasich et al., 2003; Wheeler et al., 2003). Lack of recruitment has been observed in declining populations, suggesting impaired reproduction or increased juvenile mortality (Wheeler et al., 2003). Intriguingly, *C. alleganiensis* is known to experience persistent, idiopathic skin lesions that can progress to tissue degeneration and, in severe cases, loss of all four feet (Nickerson et al., 2011). Although the cause remains unknown, combinations of opportunistic pathogens have been identified on afflicted salamanders, leading to speculation that these individuals may suffer from environmentally based immune suppression (Nickerson et al., 2011). A better understanding of the relationship between thermal variation and immune function in *C. alleganiensis* could perhaps provide clues to the etiology of this enigmatic disease.

We empirically tested the physiological and behavioral impacts of naturalistic, short-term temperature variation on *C. a. alleganiensis* under controlled laboratory conditions. We measured indicators of innate immune function, physiological stress and overall health in juvenile salamanders before, during and after a period of temperature changes that mimicked naturally occurring thermal variation in the population's natal stream (Fig. 1). Response variables included the ability of plasma complement proteins to kill bacterial pathogens (*Escherichia coli*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*) found on diseased *C. alleganiensis* (described above) (Nickerson et al., 2011), as well as proportions of circulating leukocytes, growth rates and behavioral activity patterns. We focused on leukocytes that are known to increase (neutrophils and eosinophils) or decrease (lymphocytes) in circulation when corticosteroids are elevated (Davis et al., 2008).

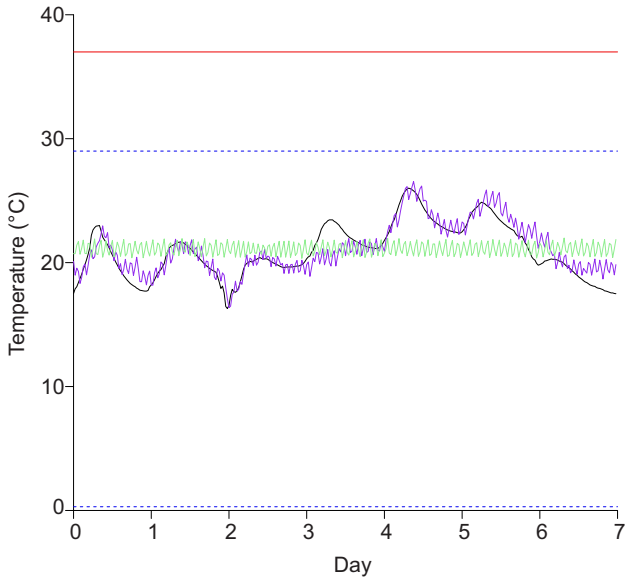

Fig. 1. Representative 7 day thermal cycle for *Cryptobranchus alleganiensis* salamanders (*N*=9 per group) maintained at constant temperatures (green line) *versus* variable temperatures (purple line) mimicking fluctuations in the population's natal stream (black line). Temperatures were within the species' normal thermal range (dashed blue lines) and below its critical thermal maximum (red line). The lower limit of the thermal range (0°C) has been offset for clarity.

Given the previously reported link between thermal variation and amphibian disease susceptibility (Raffel et al., 2012), our hypotheses were that (1) naturally occurring periods of relatively unstable temperatures cause physiological stress and compromise salamander immune function, and (2) individuals become acclimated to temperature patterns and regain physiological function after 3 to 5 weeks of continued exposure.

MATERIALS AND METHODS

Animal husbandry

Eighteen captive-reared juvenile *C. alleganiensis alleganiensis* (aged 3 years, sex unknown) were maintained in groups of three among six independent 151 liter aquaria, each with a 114 liter sump tank on a closed-loop pumping system. Each aquarium was equipped with pebble substrate and natural rock hides, and independent heating, cooling and filtration systems. Aquaria were illuminated by fluorescent ceiling lights on an astronomic timer that was programmed to the dates when stream temperature data were collected (i.e. August–September 2011). Hellbenders were fed (15 g per tank) a mixed, commercially produced diet of chopped earthworms, live ghost shrimp and frozen/thawed crayfish or krill twice a week. Tank water was partially (30%) changed ~1 h after feeding using gravel siphoning to remove detritus and uneaten food items (live shrimp remained until eaten). Tanks were replenished with municipal water of the same temperature that had been passed through a two-stage carbon filter. Water chemistry (ammonia, free chlorine, total chlorine and pH) was tested once a week in a randomly selected tank using commercially available kits. Ammonia ranged from 0 to 0.04 ppm (mean ± s.d.=0.014±0.012 ppm) and total chlorine ranged from 0 to 0.01 ppm (0.002±0.004 ppm). Free chlorine was not detected. Water pH ranged from 7.5 to 7.8 (7.76±0.10) and was within the range of values recorded in hellbender streams during recent field surveys (6.52–8.36).

Table 1. Magnitude of thermal variation of *C. alleganiensis* stream habitat in southwestern New York from August 2011 to August 2012

	Absolute temperature change (°C)		
Time scale	Minimum	Mean ± s.d.	Maximum
Hourly	0	0.14±0.16	3.9
Daily	<0.1	1.9±1.3	7.4
Weekly	0.9	5.3±2.3	11.1
Monthly	4.0	9.5±3.3	17.7

Temperature experiment

Water temperatures were recorded every 30 min over 12 months using a HOBO Water Temperature Pro v2 Data Logger (U22-001, Onset Computer Corporation, Bourne, MA, USA) that was secured on the stream bed, adjacent to the 'nest rock' in southern New York where the hellbenders were obtained as embryos. Temperatures ranged from 0 to 29°C, and varied significantly on hourly, daily, weekly and monthly time scales (Table 1). Most thermal variation occurred during the spring and summer, whereas winter temperatures were stable across all time scales (Table 2). We identified five periods where rapid warming/cooling occurred (Δ10–11°C in 7 days) that began in mid-March, late April, late May, late August and late September. We selected a 7 day period (30 August–5 September 2011) where temperatures ranged from 16 to 26°C, because we could maintain the laboratory within this range and thus minimize changes in body temperature during blood collection. Although there is little historical stream temperature data for this region (as is the case in most regions), we consider these temperatures to be within the population's normal thermal range. Mean air temperatures in this region were higher than normal during August and September 2011, but still lower than average July temperatures (National Climatic Data Center, 2013).

We reproduced the above pattern of temperature variation at an hourly scale (Fig. 1) using a custom-engineered system and Siemens Insight Workstation (Siemens Corporation, Washington, DC, USA). We exposed captive hellbenders (N=9) to six consecutive 7 day temperature cycles (42 days total). This number of cycles allowed us to test for evidence of thermal acclimation through longitudinal sampling, while providing individuals with 14 days to recover from each blood collection. Physiological data collection, feeding and water changes were carried out at times in the temperature cycle when all aquaria were at 21°C. The laboratory was maintained at 21°C to minimize changes in hellbender body temperature during physiological assessments. Overall, our automated system was highly effective at recreating the 7 day pattern of warming/cooling, with our experimental temperatures differing from corresponding *in situ* values by a mean of 0.3±1.9°C (Fig. 1). This modest variation was likely due to the system's requirement to continually provide power to either the heater or the chiller, which we partially compensated for by allowing temperatures of the control group (N=9, maintained at 21°C) to fluctuate by ±1°C (Fig. 1).

Table 2. Seasonal patterns of thermal variation of *C. alleganiensis* stream habitat in southwestern New York from August 2011 to August 2012

	Seasons representing the most[a]:	
Time scale	Variable temperatures	Stable temperatures
Hourly	Summer	Year-round
Daily	Summer	Winter, Fall, Spring
Weekly	Spring, Fall	Winter
Monthly	Spring	Winter, Fall

Spring: March–May; Summer: June–August; Fall: September–November; Winter: December–February.
[a]Represented within the corresponding 90th percentiles.

Blood collection

We collected blood from each salamander every 2 weeks during the experimental period, i.e. 1, 3 and 5 weeks after the onset of temperature fluctuations. To determine baseline values, we also collected blood 6 and 3 weeks before the experimental period, and 1 and 3 weeks after the experimental period. Blood (≤0.4 ml) was collected from the caudal tail vein using a 22 gauge needle and a 1 ml syringe within 4 min of capture. Each animal was weighed in a separate sterile container after blood draw. Towels and gloves were changed between successive animals. A small volume (5 µl) of blood was immediately smeared onto a glass microscope slide in duplicate for leukocyte counts. After drying, the slides were fixed in 100% methanol for 5 min, stained with DipQuick (MWI Veterinary Supply, Boise, ID, USA), and examined at 1000× magnification using a standard light microscope. For each smear, 100 leukocytes were counted and identified as neutrophils, lymphocytes, eosinophils or monocytes (Heatley and Johnson, 2009). Proportions of each cell type were averaged between duplicate smears. The remainder of each blood sample was transferred to a heparinized tube and centrifuged (15 min, 3200 ***g***) within 2 h to isolate plasma for bacterial killing assays. Plasma samples were frozen at −80°C until analysis.

Bacteria-killing assay

Bacteria-killing ability of *C. alleganiensis* plasma was tested separately against *E. coli* (ATCC8739), *P. aeruginosa* (ATCC 9027) and *A. hydrophila* (ATCC 35654) using a modification of the absorbance-based protocol described previously (Liebl and Martin, 2009). Bacteria were obtained as lyophilized pellets (Fisher Scientific, Waltham, MA, USA) and rehydrated in 10 ml amphibian phosphate buffered saline (PBS). An isolated colony of each bacterium was obtained by streaking onto a 5% blood agar plate (Fisher Scientific) and used to inoculate 10 ml of tryptic soy broth. The broth was incubated for 24 h at 30°C to yield a working stock solution. Bacterial concentration of each stock solution was determined by plating serial dilutions (10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}) onto 5% blood agar. Plates containing 30–300 isolated colonies were counted to determine colony-forming units (CFUs) per milliliter. For each sample, 5 µl of thawed plasma was combined with 20 µl of bacteria (diluted to 10^6 CFUs ml^{-1}) and 75 µl of amphibian PBS in duplicate in a 96-well plate. A blank (plasma and PBS only) was included for each sample. Positive (plasma-free) and negative (bacteria-free) controls were included in each plate in triplicate. Plates were shaken and subsequently incubated (21°C, 1 h) to allow bacteria killing to occur. Tryptic soy broth (200 µl) was then added to each well (except for blanks), and plates were incubated (30°C) for 10 h (*A. hydrophila*), 12 h (*E. coli*) or 16 h (*P. aeruginosa*) to allow bacterial growth to occur. Length of incubation corresponded to the amount of time needed for the bacterium to reach its exponential growth phase, which we previously determined by measuring changes in sample absorbance over time. Absorbance was read at 405 nm immediately after incubation. Bacteria-killing ability was calculated as the difference in absorbance between the sample and the blank, divided by the positive control.

Behavioral observations

We observed diurnal (11:00–14:00 h) and feeding (14:00–16:00 h) behaviors once a week throughout the experimental period. Nocturnal (22:00–02:00 h) behaviors were observed twice a week during the same period. For each tank, salamander behavioral state (active *versus*

Table 3. Linear mixed effects models of physiological responses to temperature change

Model	Fixed effects
Null	Date + Study period + Treatment group[a]
Rapid and sustained response	Date + (Study period × Treatment group)
Delayed and sustained response	Date + (Offset study period × Treatment group)[b]
Rapid response followed by acclimation	(Date × Study period) + (Study period × Treatment group)
Delayed response followed by acclimation	(Date × Offset study period) + (Offset study period × Treatment group)

Individual was included as a random effect in each model.
[a]Treatment effect was represented by an interaction between treatment group and study period, since there could be no effect of temperature change during baseline data collection.
[b]Study period was offset by 2 weeks to represent a delayed treatment effect.

inactive) was recorded as a point observation every 30 s over 3 min. Active behaviors included swimming, crawling and floating. Animals that were stationary and in contact with the gravel substrate were considered inactive. Side-to-side rocking (a potential sign of stress) was recorded as a behavioral event if it was observed between point observations. Each 3 min observation period was repeated twice, for a total of 9 min per tank. Because individuals could not be reliably identified during observations, we recorded the total number of salamanders in the tank engaging in each behavior. This number was summed and averaged among replicate sets of observations.

Statistical analyses

All data were analyzed using R statistical software (R Development Core Team, 2008). We compared linear mixed models within each response variable using the lmer function in the lme4 package (Bates et al., 2012) and calculated Akaike weights (w_i) for each model from corrected Akaike's information criterion (AIC_c) values. We used an AIC_c model comparison approach in order to investigate the potential for a delayed response to temperature changes as well as an acclimation effect. Model terms are provided in Table 3. A rapid response assumed that physiology was altered within 1 week of the onset/cessation of temperature fluctuations (i.e. before the subsequent blood collection). A delayed response assumed that this impact occurred between 1 and 3 weeks (i.e. after the subsequent blood collection). To compare all models using a single data set, study period was included as two distinct predictor variables, representing either a rapid or a delayed response. Tank was included in the initial analysis as a random effect, but did not influence AIC values of any treatment model. Because few tanks (N=3) were nested within each treatment, we omitted tank from the final analysis to avoid representing a treatment effect in the null model. We plotted estimates and standard errors for treatment effects models that had an Akaike weight (w_i) of >0.01. If there was no support for an effect of temperature treatment on a given response variable (i.e. $w_i \leq 0.01$), we plotted estimates and standard errors of the null model.

We analyzed behavioral data in a hypothesis-testing framework because observations were made during the experimental period only (and hence we directly compared treatment and control groups). We tested for an effect of temperature fluctuations using a linear mixed model with treatment group included as a fixed effect. Tank was included as a random effect because individuals housed together frequently interacted with one another (e.g. through direct contact), likely influencing behavioral activity. Results were considered significant at $P<0.05$.

RESULTS

Comparison of linear mixed effects (LME) models based on weights calculated from AIC_c values revealed a single supported model for all physiological response variables except *E. coli* killing (Table 4). We found strong support ($\Delta AIC_c=0$, $w_i>0.99$) for an effect of temperature variation on *P. aeruginosa* killing. This model included a 1–3 week delay in physiological response, but no thermal acclimation effect, and predicted a 70% increase in killing ability after exposure to variable temperatures (Fig. 2A). The same model predicted a 53% increase in *E. coli* killing (Fig. 2B) but had less support ($\Delta AIC_c=1.8$, $w_i=0.29$). *Escherichia coli* killing data better supported the null model, which represented no effect of temperature variation ($\Delta AIC_c=0$, $w_i=0.71$). Paired *t*-test comparison of all control group samples (N=53) revealed that plasma proteins were more effective ($P<0.0001$) at killing *E. coli* compared with *P. aeruginosa*, with a mean of 56±39% and 37±14% of bacterial cells killed, respectively. In contrast, plasma proteins were completely ineffective against *A. hydrophila* (i.e. 0% of bacterial cells were killed in every sample).

Across all samples (N=122), lymphocytes were by far the predominant leukocyte (mean ± s.d., 94.7±3.5%), with lower percentages of neutrophils (2.5±2.6%) and eosinophils (1.0±1.1%) present in circulation (Fig. 3). Monocytes also were observed infrequently (1.8±1.7%) and basophils were not present. Model comparisons (Table 4) revealed strong support for the null model

Table 4. Akaike weights (w_i) for linear mixed effects models of physiological responses to temperature change

	Model			
Response variable	Null[a]	Rapid and sustained	Delayed and sustained	Acclimation[b]
P. aeruginosa killing	0	0	**>0.99**	<0.01
E. coli killing	0.71	0	0.29	0
Lymphocytes	**>0.99**	<0.01	<0.01	0
Neutrophils	**>0.99**	<0.01	<0.01	0
Eosinophils	**>0.99**	<0.01	<0.01	0
Growth rates	**>0.99**	<0.01	<0.01	0

[a]No effect of temperature fluctuations.
[b]Includes a delayed response term. No support ($w_i=0$) was observed for the acclimation model with a rapid response term.
Bold values indicate strong support ($w_i>0.99$) for the corresponding mixed effects model.

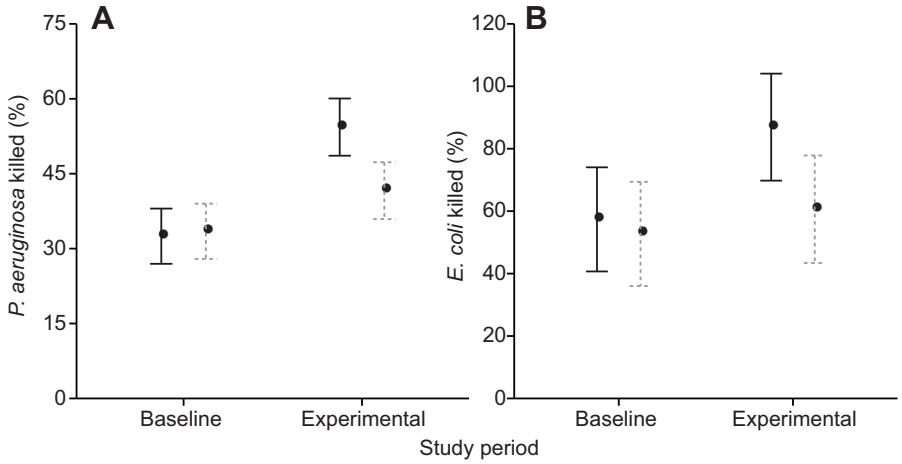

Fig. 2. Linear mixed model estimates and standard errors for (A) *Pseudomonas aeruginosa* (ΔAIC_c=0, w_i>0.99) and (B) *Escherichia coli* (ΔAIC_c=1.8, w_i=0.29) killing ability in *Cryptobranchus alleganiensis* salamanders exposed to variable temperatures (*N*=9, solid error bars) during the experimental period compared with a control group maintained at constant temperatures (*N*=9, dashed error bars). Model terms included a 1–3 week delayed treatment response but no acclimation effect.

(i.e. no effect of temperature fluctuations) in leukocyte data (ΔAIC_c=0, w_i>0.99; Fig. 3) and growth rates (ΔAIC_c=0, w_i>0.99; Fig. 4).

As expected, salamanders were more active (*P*<0.0001) during nocturnal *versus* diurnal hours and most active (*P*=0.002) during feeding periods (Fig. 5A). Temperature fluctuations did not influence the frequency (*P*=0.355) or temporal pattern (*P*=0.319) of active behaviors. Rocking behavior was observed infrequently (<5% of observations) and was not influenced by temperature fluctuations (*P*=0.207; Fig. 5B).

DISCUSSION

Current understanding of amphibian thermal physiology is based largely on the influence of absolute temperatures, especially the limits of heat/cold tolerance (Angilletta, 2009b). To our knowledge, this was the first study to empirically test the influence of temperature variation on amphibian immune function by mimicking natural, fine-scale patterns of warming/cooling. Our study yielded four major findings. First, and contrary to our expectations, variable temperatures enhanced (rather than suppressed) innate immune function in salamanders, as demonstrated by the increased ability of plasma proteins to kill *P. aeruginosa*. Because the *P. aeruginosa* bacterium has been isolated from open sores on wild *C. alleganiensis* (Nickerson et al., 2011) and is a common cause of disease among vertebrate taxa, this response likely translates to a direct increase in fitness. Second, increased immune function was sustained over the 6 week experimental period, indicating that salamanders did not acclimate to this complex pattern of temperature change. Third, salamanders maintained normal (i.e. baseline) levels of circulating immune cells, growth and behavioral activity during exposure to rapid warming and cooling. This finding, combined with the observed increase in bacteria killing, suggests that natural patterns of temperature variation benefit amphibian health. Finally, we observed that the antimicrobial activity of *C. alleganiensis* plasma varies dramatically among species of gram-negative bacteria, highlighting the value of our comparative approach (Nickerson et al., 2011).

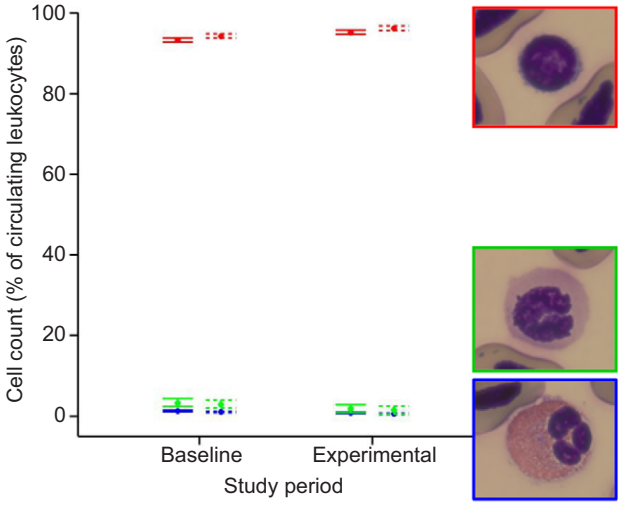

Fig. 3. Estimates and standard errors for the null model (ΔAIC_c=0, w_i>0.99) of circulating leukocyte ratios in *Cryptobranchus alleganiensis* salamanders exposed to variable temperatures (*N*=9, solid error bars) during the experimental period compared with a control group maintained at constant temperatures (*N*=9, dashed error bars). Leukocytes include lymphocytes (red markers), neutrophils (green markers) and eosinophils (blue markers), and representative images of each cell type in *C. alleganiensis* are illustrated.

Among amphibians, there is compelling evidence linking absolute temperatures to changes in immune function. For example, continued exposure to 5°C reduces T-lymphocyte proliferative ability and complement activity in *Rana pipiens* (Green and Cohen, 1977; Maniero and Carey, 1997) and inhibits antimicrobial peptide synthesis in *R. sylvatica* (Matutte et al., 2000). Conversely, warm (and constant) temperatures increase resistance of *Ambystoma tigrinum* larvae to *Ranavirus* (Rojas et al., 2005) and accelerate allograft rejection in *R. esculenta*, *Bombina bombina*, *Bufo bufo* and *Diemictylus viridescens* (Cohen, 1966; Jozkowicz and Plytycz, 1998). Yet most natural habitats do not maintain constant temperatures. Given that a modest temperature change [e.g. 3°C (Cohen, 1966)] can have a significant impact on amphibian immune function, it seems logical that continuously fluctuating temperatures would have important consequences for disease resistance. Variable temperatures might influence amphibian immune function by: (1) triggering a thermal stress response, (2) directly affecting immune cell or protein activity, or (3) allowing a pathogen to acclimate to the thermal environment more quickly than its amphibian host [i.e. the 'lag' hypothesis (Raffel et al., 2006)]. We tested the first two mechanisms, and our data supported the second possibility, as we documented an increase in protein complement activity without corresponding changes in leukocyte ratios. This response could have been driven by increased production of one or more key complement proteins (e.g. C3) and/or changes in the expression of regulatory proteins (e.g. decay-accelerating factor) (Janeway et al., 2001). Our data also revealed a latency of 1–3 weeks between the onset or

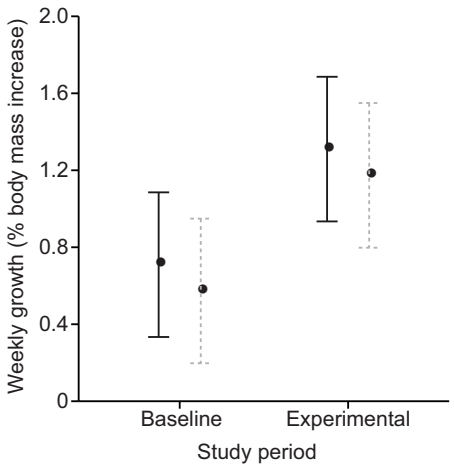

Fig. 4. Estimates and standard errors for the null model (ΔAIC_c=0, w_i>0.99) of growth rates in *Cryptobranchus alleganiensis* salamanders exposed to variable temperatures (*N*=9, solid error bars) during the experimental period compared with a control group maintained at constant temperatures (*N*=9, dashed error bars).

cessation of temperature fluctuations and a corresponding change in complement activity. This delay may reflect the time needed to cross a physiological response threshold and/or the time required for protein synthesis/degradation.

Although we observed elevated immune function, we found no evidence of an acclimation response in salamanders exposed to 6 weeks of temperature fluctuations. This contrasts with recent findings that Cuban tree frogs (*O. septentrionalis*) acclimate to daily temperature shifts (25°C↔15°C) within 4 weeks of continued exposure (Raffel et al., 2012). However, these frogs were exposed to immediate 10°C temperature changes, whereas the salamanders in our study experienced fine-scale, naturalistic patterns of warming and cooling with temperatures programmed at 30 min intervals. Taken together, these findings raise the question of whether the complexity of a temperature pattern can influence an amphibian's thermal acclimation response. This question may be particularly important for temperate-region ectotherms that experience relatively unstable temperatures compared with tropical counterparts. In the present study, we observed periods of substantial stream temperature variation over hourly, daily, weekly and monthly time scales (Table 1), particularly during the spring and summer (Table 2). In contrast to simple temperature shifts, amphibians may be unable to acclimate to more complex, naturalistic patterns of thermal variation because these changes are relatively harder to predict.

Eco-physiological studies often focus on markers of biological stress as indices of individual or population-level fitness. Yet corticosteroid hormones are ambiguous indicators of health, and even chronic stress may be adaptive (Boonstra, 2013). Changes in the proportions of circulating leukocytes (specifically, more neutrophils and fewer lymphocytes) are also widely used indicators of physiological stress. However, this response is also ambiguous and is believed to result from the redistribution of cells in blood *versus* tissue, rather than a change in cellular production (Davis et al., 2008). Furthermore, these and other conventional indicators of health (e.g. growth or behavior) are less likely to reflect a positive effect of environmental change, particularly for laboratory animals maintained under ideal conditions. That is, any departure from normal or baseline values would likely reflect a negative effect on stress, growth or behavior. Yet the value of these indicators is that they are relatively easy to quantify and can be assessed in parallel with more direct measures of fitness to generate a better understanding of physiological function. The consistency of leukocyte counts, growth rates and behavioral data in the present study, combined with an increase in innate immune function, provides compelling evidence that amphibians can tolerate, and perhaps benefit from, natural patterns of temperature variation.

To our knowledge, this study represents the first published information on circulating leukocyte ratios in juvenile *C. alleganiensis*. We were surprised by the predominance of lymphocytes (>80% of immune cells in every sample), as well as the consistency of leukocyte ratios among individuals and over time. These findings contrast with the more variable proportions of circulating lymphocytes (~35–65%), neutrophils (~20–45%) and eosinophils (~3–20%) previously reported in *C. alleganiensis* (Huang et al., 2010; Jerrett and Mays, 1973; Solís et al., 2007). Because previous studies have focused on wild-caught adults, these differences could be related to environmental variables or animal age. Regardless, our findings highlight the need to better understand both developmental immunology and eco-physiology in this species.

The final insight from our study was that bacteria-killing ability of *C. alleganiensis* plasma differed substantially against different pathogens. Although *E. coli* is a valuable model species and the pathogen most commonly used in bacterial-killing assays, it was also the easiest to defend against. Similar findings were reported in a recent study of complement activity in little brown bats (*Myotis*

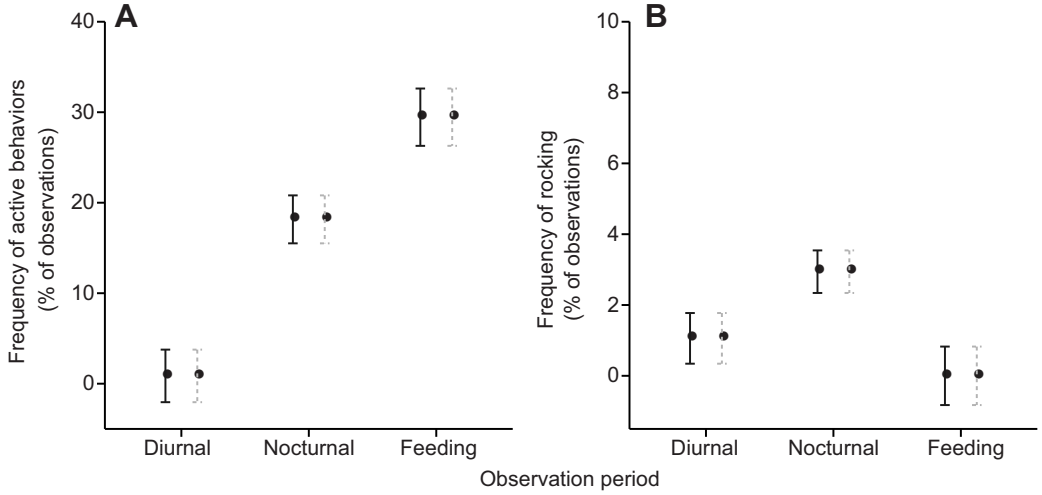

Fig. 5. Means and standard errors for frequency of active behaviors (A) and rocking (B) in *Cryptobranchus alleganiensis* salamanders exposed to variable temperatures (*N*=9, solid error bars) during the experimental period compared with a control group maintained at constant temperatures (*N*=9, dashed error bars). There were no differences between treatment groups ($P \geq 0.207$).

lucifugus) in which a greater proportion of *E. coli* was killed relative to *Staphylococcus aureus* and *Candida albicans* (Moore et al., 2011). Thus, *E. coli* may represent an 'easy test' for the innate immune system, allowing deficient individuals to achieve a high score. This idea could explain why our statistical model provided relatively weak evidence for an influence of temperature variation on *E. coli* killing, despite robust support for an effect on *P. aeruginosa*. In contrast to these two bacterial species, *C. alleganiensis* plasma was completely ineffective against *A. hydrophila*. This lack of plasma-based immune defense may contribute to the high virulence of this pathogen in wild amphibian populations, where it causes red-leg disease (Bradford, 1991; Nyman, 1986). Alternately, a different component of the amphibian immune system (e.g. neutrophils or the classical complement pathway) may play a crucial role in *A. hydrophila* resistance. Regardless, the observed differences among bacterial species illustrate the value of comparative physiology and non-traditional model species in understanding environmental drivers of animal health.

The need for a physiologically based, mechanistic understanding of the impacts of environmental change in species conservation is becoming increasingly recognized (Seebacher and Franklin, 2012; Stevenson et al., 2005; Wikelski and Cooke, 2006), and the field of vertebrate eco-physiology has grown rapidly in the past decade (e.g. Blaustein et al., 2012; Cooke et al., 2012; Klaassen et al., 2012; Raubenheimer et al., 2012). To accurately predict the physiological impacts of temperature variation on amphibians *in situ*, we need a better understanding of the distinct effects of variable weather patterns *versus* changes in absolute temperatures. Like dozens of Appalachian species, *C. alleganiensis* is adapted to relatively cool temperatures and will likely be influenced by a warmer climate (Milanovich et al., 2010). Annual temperatures are predicted to increase between ~3°C (low emissions B1 scenario) and ~5°C (high emissions A1fi scenario) throughout the Appalachian region by 2099 (Karl et al., 2009). Identifying and predicting climate change impacts on freshwater systems is difficult because of the lack of historical data in these habitats (Arismendi et al., 2012), but stream temperatures are strongly influenced by climate and typically warm by 0.6–0.8°C for every 1°C increase in air temperature (Morrill et al., 2005; Webb and Nobilis, 2007). Evidence of long-term warming and its biological impacts have been observed for streams in many of the United States (Isaak et al., 2010; Isaak et al., 2012; Kaushal et al., 2010), and models predict that this trend will continue, both within the USA (Ruesch et al., 2012; Wenger et al., 2011) and globally (Punzet et al., 2012). Warmer temperatures may increase disease susceptibility and compromise overall physiological function for species that are pushed beyond their thermal optimum (Huey et al., 2012). This threat is particularly acute for stream-dwelling species whose movements are constrained within linear networks (Fagan, 2002). Yet while the impacts of absolute temperatures are relatively well studied and can be (to some extent) inferred from a species' current range (Angilletta, 2009b), the effects of temperature variation itself are less obvious. Our present findings suggest that salamander immune function (specifically, complement activity) might be maintained, or perhaps even enhanced, if warm periods were punctuated by cooling events. Importantly, our data also reveal that temperature effects on amphibian health may be cryptic (i.e. undetected by conventional measures), underscoring the importance of non-traditional and more direct fitness indicators. Collectively, our findings illustrate the value of a comprehensive and ecologically relevant approach to investigating thermal physiology. A better understanding of amphibian thermal physiology is crucial to sustaining this remarkably diverse taxon in the face of a rapidly changing climate.

ACKNOWLEDGEMENTS

The authors thank Ken Roblee (NY Department of Environmental Conservation) and Penny Felski (Buffalo Zoo) for access to the research animals, and Keith Turner (Siemens Engineering Inc.) for designing and providing the programmable thermostat system. Kerry Grisewood provided access to her property and valuable assistance in stream temperature data collection. We also thank our colleagues from Smithsonian Conservation Biology Institute: Dr Janine Brown (interpretation of results), Veronica Acosta (salamander blood collections), Dr Jeff Hostetler (assistance with statistical analyses), Ed Bronikowski (research space and resources), Lauren Augustine (salamander husbandry) and Emma Johnson (data entry). Finally, we are grateful to two anonymous reviewers whose feedback helped us significantly improve this manuscript.

AUTHOR CONTRIBUTIONS

K.A.T. designed and performed research, analyzed and interpreted data, and wrote the paper. R.P.Q. designed and fabricated the aquatic system and performed research. S.M. designed research and performed veterinary procedures. J.D.K. designed research and interpreted data. J.B.M. designed research and provided laboratory space. M.J.E. designed and performed research. B.D.N. performed research. B.G. designed research. All authors improved the paper.

COMPETING INTERESTS

No competing interests declared.

FUNDING

This research was supported by a David H. Smith Fellowship (to K.A.T.), which is funded by the Cedar Tree Foundation and administered by the Society for Conservation Biology.

REFERENCES

Addo-Bediako, A., Chown, S. L. and Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proc. Biol. Sci.* **267**, 739-745.

Angilletta, M. J., Jr (2009a). Thermal acclimation. In *Thermal Adaptation: a Theoretical and Empirical Synthesis*, pp. 126-156. New York: Oxford University Press.

Angilletta, M. J., Jr (2009b). Thermal sensitivity. In *Thermal Adaptation: a Theoretical and Empirical Synthesis*, pp. 35-87. New York: Oxford University Press.

Arismendi, I., Johnson, S. L., Dunham, J. B., Haggerty, R. and Hockman-Wert, D. (2012). The paradox of cooling streams in a warming world: Regional climate trends do not parallel variable local trends in stream temperature in the Pacific continental United States. *Geophys. Res. Lett.* **39**, L10401.

Bates, D., Maechler, M. and Bolker, B. (2012). *lme4: Linear Mixed-Effects Models Using Eigen and S4*. R package version 0.999902344-0. Available at http://lme4.r-forge.r-project.org/.

Blaustein, A. R., Gervasi, S. S., Johnson, P. T. J., Hoverman, J. T., Belden, L. K., Bradley, P. W. and Xie, G. Y. (2012). Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. *Philos. Trans. R. Soc. B* **367**, 1688-1707.

Boonstra, R. (2013). Reality as the leading cause of stress: rethinking the impact of chronic stress in nature. *Funct. Ecol.* **27**, 11-23.

Bradford, D. F. (1991). Mass mortality and extinction in a high-elevation population of *Rana muscosa*. *J. Herpetol.* **25**, 174-177.

Caruso, N. M. and Lips, K. R. (2013). Truly enigmatic declines in terrestrial salamander populations in Great Smoky Mountains National Park. *Divers. Distrib.* **19**, 38-48.

Cheng, T. L., Rovito, S. M., Wake, D. B. and Vredenburg, V. T. (2011). Coincident mass extirpation of neotropical amphibians with the emergence of the infectious fungal pathogen *Batrachochytrium dendrobatidis*. *Proc. Natl. Acad. Sci. USA* **108**, 9502-9507.

Clusella-Trullas, S., Blackburn, T. M. and Chown, S. L. (2011). Climatic predictors of temperature performance curve parameters in ectotherms imply complex responses to climate change. *Am. Nat.* **177**, 738-751.

Cohen, N. (1966). Tissue transplantation immunity in adult newt *Diemictylus viridescens*. III. Effects of X-irradiation and temperature on allograft reaction. *J. Exp. Zool.* **163**, 231-239.

Cooke, S. J., Hinch, S. G., Donaldson, M. R., Clark, T. D., Eliason, E. J., Crossin, G. T., Raby, G. D., Jeffries, K. M., Lapointe, M., Miller, K. et al. (2012). Conservation physiology in practice: how physiological knowledge has improved our ability to sustainably manage Pacific salmon during up-river migration. *Philos. Trans. R. Soc. B* **367**, 1757-1769.

Davis, A. K., Maney, D. L. and Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct. Ecol.* **22**, 760-772.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci. USA* **105**, 6668-6672.

Duarte, H., Tejedo, M., Katzenberger, M., Marangoni, F., Baldo, D., Beltran, J. F., Marti, D. A., Richter-Boix, A. and Gonzalez-Voyer, A. (2012). Can amphibians take the heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. *Glob. Change Biol.* **18**, 412-421.

Fagan, W. F. (2002). Connectivity, fragmentation, and extinction risk in dendritic metapopulations. *Ecology* **83**, 3243-3249.

Furniss, L. (2003). *Conservation Assessment for Ozark Hellbender (Cryptobranchus alleganiensis bishopi* Grobman*)*. Rolla, MO: USDA Forest Service, Eastern Region.

Green, N. and Cohen, N. (1977). Effect of temperature on serum complement levels in the leopard frog, *Rana pipiens*. *Dev. Comp. Immunol.* **1**, 59-64.
Heatley, J. J. and Johnson, M. (2009). Clinical technique: amphibian hematology: a practitioner's guide. *J. Exotic Pet Medicine* **18**, 14-19.
Highton, R. (2005). Declines of eastern North American woodland salamanders (*Plethodon*). In *Amphibian Declines: Conservation Status of United States Species* (ed. M. Lanoo), pp. 34-46. Berkley, CA: University of California Press.
Hof, C., Araújo, M. B., Jetz, W. and Rahbek, C. (2011). Additive threats from pathogens, climate and land-use change for global amphibian diversity. *Nature* **480**, 516-519.
Huang, C. C., Xu, Y., Briggler, J. T., McKee, M., Nam, P. and Huang, Y. W. (2010). Heavy metals, hematology, plasma chemistry, and parasites in adult hellbenders (*Cryptobranchus alleganiensis*). *Environ. Toxicol. Chem.* **29**, 1132-1137.
Huey, R. B., Kearney, M. R., Krockenberger, A., Holtum, J. A. M., Jess, M. and Williams, S. E. (2012). Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philos. Trans. R. Soc. B* **367**, 1665-1679.
Isaak, D. J., Luce, C. H., Rieman, B. E., Nagel, D. E., Peterson, E. E., Horan, D. L., Parkes, S. and Chandler, G. L. (2010). Effects of climate change and wildfire on stream temperatures and salmonid thermal habitat in a mountain river network. *Ecol. Appl.* **20**, 1350-1371.
Isaak, D. J., Wollrab, S., Horan, D. and Chandler, G. (2012). Climate change effects on stream and river temperatures across the northwest US from 1980-2009 and implications for salmonid fishes. *Clim. Change* **113**, 499-524.
Janeway, C. A. J., Travers, P., Walport, M. and Shlomchik, M. J. (2001). *Immunobiology*. New York, NY: Garland Science.
Janzen, D. H. (1967). Why mountain passes are higher in the tropics. *Am. Nat.* **101**, 233-249.
Jerrett, D. and Mays, C. (1973). Comparative hematology of the hellbender, *Cryptobranchus alleganiensis* in Missouri. *Copeia* **1973**, 331-337.
Jozkowicz, A. and Plytycz, B. (1998). Temperature but not season affects the transplantation immunity of anuran amphibians. *J. Exp. Zool.* **281**, 58-64.
Karl, T. J., Melillo, J. M., Peterson, T. C. and Hassol, S. J. (2009). *Global Climate Change Impacts in the United States*. New York: Cambridge University Press.
Kaushal, S. S., Likens, G. E., Jaworski, N. A., Pace, M. L., Sides, A. M., Seekell, D., Belt, K. T., Secor, D. H. and Wingate, R. L. (2010). Rising stream and river temperatures in the United States. *Front. Ecol. Environ* **8**, 461-466.
Kellermann, V., Overgaard, J., Hoffmann, A. A., Fløjgaard, C., Svenning, J. C. and Loeschcke, V. (2012). Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. *Proc. Natl. Acad. Sci. USA* **109**, 16228-16233.
Kiesecker, J. M. (2011). Global stressors and the global decline of amphibians: tipping the stress immunocompetency axis. *Ecol. Res.* **26**, 897-908.
Klaassen, M., Hoye, B. J., Nolet, B. A. and Buttemer, W. A. (2012). Ecophysiology of avian migration in the face of current global hazards. *Philos. Trans. R. Soc. B* **367**, 1719-1732.
Liebl, A. L. and Martin, L. B. (2009). Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. *Funct. Ecol.* **23**, 1091-1096.
Lips, K. R., Brem, F., Brenes, R., Reeve, J. D., Alford, R. A., Voyles, J., Carey, C., Livo, L., Pessier, A. P. and Collins, J. P. (2006). Emerging infectious disease and the loss of biodiversity in a neotropical amphibian community. *Proc. Natl. Acad. Sci. USA* **103**, 3165-3170.
Maniero, G. D. and Carey, C. (1997). Changes in selected aspects of immune function in the leopard frog, *Rana pipiens*, associated with exposure to cold. *J. Comp. Physiol. B* **167**, 256-263.
Matutte, B., Storey, K. B., Knoop, F. C. and Conlon, J. M. (2000). Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica*, in response to environmental stimuli. *FEBS Lett.* **483**, 135-138.
Mayasich, J., Grandmaison, D. and Phillips, C. (2003). *Eastern Hellbender Status Assessment Report*. Duluth, MN: Natural Resources Research Institute.
Milanovich, J. R., Peterman, W. E., Nibbelink, N. P. and Maerz, J. C. (2010). Projected loss of a salamander diversity hotspot as a consequence of projected global climate change. *PLoS ONE* **5**, e12189.
Miller, D., Gray, M. and Storfer, A. (2011). Ecopathology of ranaviruses infecting amphibians. *Viruses* **3**, 2351-2373.
Moore, M. S., Reichard, J. D., Murtha, T. D., Zahedi, B., Fallier, R. M. and Kunz, T. H. (2011). Specific alterations in complement protein activity of little brown myotis (*Myotis lucifugus*) hibernating in white-nose syndrome affected sites. *PLoS ONE* **6**, e27430.
Morrill, J. C., Bales, R. C. and Conklin, M. H. (2005). Estimating stream temperature from air temperature: Implications for future water quality. *J. Environ. Eng.* **131**, 139-146.
National Climatic Data Center (2013). *Climatological Rankings*. Washington, DC: National Oceanic and Atmospheric Administration.
Naya, D. E. and Bozinovic, F. (2012). Metabolic scope of fish species increases with distributional range. *Evol. Ecol. Res.* **14**, 769-777.
Nickerson, M. and Mays, C. (1973). The hellbenders: North American 'giant' salamanders. *Milwaukee Pub. Mus. Publ. Biol. Geol.* **1**, 1-106.
Nickerson, C. A., Ott, C. M., Castro, S. L., Garcia, V. M., Molina, T. C., Briggler, J. T., Pitt, A. L., Tavano, J. J., Byram, J. K., Barrila, J. et al. (2011). Evaluation of microorganisms cultured from injured and repressed tissue regeneration sites in endangered giant aquatic Ozark hellbender salamanders. *PLoS ONE* **6**, e28906.
Niehaus, A. C., Wilson, R. S. and Franklin, C. E. (2006). Short- and long-term consequences of thermal variation in the larval environment of anurans. *J. Anim. Ecol.* **75**, 686-692.
Niehaus, A. C., Wilson, R. S., Seebacher, F. and Franklin, C. E. (2011). Striped marsh frog (*Limnodynastes peronii*) tadpoles do not acclimate metabolic performance to thermal variability. *J. Exp. Biol.* **214**, 1965-1970.
Niehaus, A. C., Angilletta, M. J., Jr, Sears, M. W., Franklin, C. E. and Wilson, R. S. (2012). Predicting the physiological performance of ectotherms in fluctuating thermal environments. *J. Exp. Biol.* **215**, 694-701.
Nyman, S. (1986). Mass mortality in larval *Rana sylvatica* attributable to the bacterium, *Aeromonas hydrophila*. *J. Herpetol.* **20**, 196-201.
Punzet, M., Voss, F., Voss, A., Kynast, E. and Barlund, I. (2012). A global approach to assess the potential impact of climate change on stream water temperatures and related in-stream first-order decay rates. *J. Hydrometeorol.* **13**, 1052-1065.
R Development Core Team (2008). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
Raffel, T. R., Rohr, J. R., Kiesecker, J. M. and Hudson, P. J. (2006). Negative effects of changing temperature on amphibian immunity under field conditions. *Funct. Ecol.* **20**, 819-828.
Raffel, T. R., Romansic, J. M., Halstead, N. T., McMahon, T. A., Venesky, M. D. and Rohr, J. R. (2012). Disease and thermal acclimation in a more variable and unpredictable climate. *Nature Clim. Chang.* **3**, 146-151.
Raubenheimer, D., Simpson, S. J. and Tait, A. H. (2012). Match and mismatch: conservation physiology, nutritional ecology and the timescales of biological adaptation. *Philos. Trans. R. Soc. B* **367**, 1628-1646.
Rojas, S., Richards, K., Jancovich, J. K. and Davidson, E. W. (2005). Influence of temperature on *Ranavirus* infection in larval salamanders Ambystoma tigrinum. *Dis. Aquat. Organ.* **63**, 95-100.
Ruesch, A. S., Torgersen, C. E., Lawler, J. J., Olden, J. D., Peterson, E. E., Volk, C. J. and Lawrence, D. J. (2012). Projected climate-induced habitat loss for salmonids in the John Day River network, Oregon, USA. *Conserv. Biol.* **26**, 873-882.
Seebacher, F. and Franklin, C. E. (2012). Determining environmental causes of biological effects: the need for a mechanistic physiological dimension in conservation biology. *Philos. Trans. R. Soc. B* **367**, 1607-1614.
Snyder, G. K. and Weathers, W. W. (1975). Temperature adaptations in amphibians. *Am. Nat.* **109**, 93-101.
Solís, M., Bandeff, J. and Huang, Y. (2007). Hematology and serum chemistry of ozark and eastern hellbenders (*Cryptobranchus alleganiensis*). *Herpetologica* **63**, 285-292.
Stevenson, R. D., Tuberty, S. R., Defur, P. L. and Wingfield, J. C. (2005). Ecophysiology and conservation: the contribution of endocrinology and immunology. *Integr. Comp. Biol.* **45**, 1-3.
Stillman, J. H. (2003). Acclimation capacity underlies susceptibility to climate change. *Science* **301**, 65.
Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L. and Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* **306**, 1783-1786.
Sunday, J. M., Bates, A. E. and Dulvy, N. K. (2011). Global analysis of thermal tolerance and latitude in ectotherms. *Proc. Biol. Sci.* **278**, 1823-1830.
Tewksbury, J. J., Huey, R. B. and Deutsch, C. A. (2008). Ecology. Putting the heat on tropical animals. *Science* **320**, 1296-1297.
van Berkum, F. H. (1988). Latitudinal patterns of the thermal sensitivity of sprint speed in lizards. *Am. Nat.* **132**, 327-343.
Webb, B. W. and Nobilis, F. (2007). Long-term changes in river temperature and the influence of climatic and hydrological factors. *Hydrol. Sci. J.* **52**, 74-85.
Wenger, S. J., Isaak, D. J., Luce, C. H., Neville, H. M., Fausch, K. D., Dunham, J. B., Dauwalter, D. C., Young, M. K., Elsner, M. M., Rieman, B. E. et al. (2011). Flow regime, temperature, and biotic interactions drive differential declines of trout species under climate change. *Proc. Natl. Acad. Sci. USA* **108**, 14175-14180.
Wheeler, B., Prosen, E., Wilkinson, R. and Mathis, A. (2003). Population declines of a long-lived salamander: a 20+ year study of hellbenders, *Cryptobranchus alleganiensis*. *Biol. Conserv.* **109**, 151-156.
Wikelski, M. and Cooke, S. J. (2006). Conservation physiology. *Trends Ecol. Evol.* **21**, 38-46.
Young, B., Stuart, S., Chanson, J., Cox, N. and Boucher, T. (2004). *Disappearing Jewels: The Status of New World Amphibians*. Arlington, VA: NatureServe.