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Alexander J. Novarro

Caitlin R. Gabor

Cory B. Goff

Tori D. Mezebish

Lily M. Thompson

*See next page for additional authors*

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**Authors**

Alexander J. Novarro, Caitlin R. Gabor, Cory B. Goff, Tori D. Mezebish, Lily M. Thompson, and Kristine L. Grayson

## RESEARCH ARTICLE

# Physiological responses to elevated temperature across the geographic range of a terrestrial salamander

Alexander J. Navarro<sup>1,\*</sup>, Caitlin R. Gabor<sup>2</sup>, Cory B. Goff<sup>2</sup>, Tori D. Mezebish<sup>3</sup>, Lily M. Thompson<sup>4</sup> and Kristine L. Grayson<sup>4</sup>

## ABSTRACT

Widespread species often possess physiological mechanisms for coping with thermal heterogeneity, and uncovering these mechanisms provides insight into species' responses to climate change. The emergence of non-invasive corticosterone (CORT) assays allows us to rapidly assess physiological responses to environmental change on a large scale. We lack, however, a basic understanding of how temperature affects CORT, and whether temperature and CORT interactively affect performance. Here, we examined the effects of elevated temperature on CORT and whole-organism performance in a terrestrial salamander, *Plethodon cinereus*, across a latitudinal gradient. Using water-borne hormone assays, we found that raising ambient temperature from 15 to 25°C increased CORT release at a similar rate for salamanders from all sites. However, CORT release rates were higher overall in the warmest, southernmost site. Elevated temperatures also affected physiological performance, but the effects differed among sites. Ingestion rate increased in salamanders from the warmer sites but remained the same for those from cooler sites. Mass gain was reduced for most individuals, although this reduction was more dramatic in salamanders from the cooler sites. We also found a temperature-dependent relationship between CORT and food conversion efficiency (i.e. the amount of mass gained per unit food ingested). CORT was negatively related to food conversion efficiency at 25°C but was unrelated at 15°C. Thus, the energetic gains of elevated ingestion rates may be counteracted by elevated CORT release rates experienced by salamanders in warmer environments. By integrating multiple physiological metrics, we highlight the complex relationships between temperature and individual responses to warming climates.

**KEY WORDS:** Corticosterone, Performance, Amphibian, Ectotherm, Plethodontid, Climate, Water-borne hormones

## INTRODUCTION

Ectothermic organisms may be disproportionately vulnerable to temperature extremes, which are expected to become more common under anthropogenic climate change. The ability of ectotherms to perform physiological tasks is temperature dependent and often co-varies with latitude as a result of adaptation to local climates or

developmental plasticity (Addo-Bediako et al., 2000; Deutsch et al., 2008; Terrell et al., 2013). Local adaptation and developmental plasticity generate geographic patterns in thermal physiology, and knowledge of these patterns is critical for assessing the vulnerability of species and populations to projected climate change (Huey et al., 2012). For example, populations that are active near, or above, their thermal optimum for performance and that have a limited acclimation capacity are highly sensitive to climate warming (Huey et al., 2012). Further, climate change may be happening too quickly for animals to evolutionarily track temperature shifts through climatic niche evolution (Quintero and Wiens, 2013). In this scenario, species that lack physiological plasticity or behavioral responses to fluctuating conditions will be the most susceptible to environmental change.

One method for detecting the physiological effects of environmental change is measuring changes in circulating glucocorticoids (GCs) via activation of the hypothalamic-pituitary-adrenal axis (hypothalamic-pituitary-interrenal axis in amphibians; Sapolsky et al., 2000). Typically studied as a metric in response to acute or chronic stress, the GC corticosterone (CORT) can also serve as a measure of physiological function under a particular set of environmental conditions. An acute GC response mobilizes energy stores, while suppressing growth, digestion and reproduction in vertebrates and is a mechanism for maintaining homeostasis (Greenberg and Wingfield, 1987; Romero, 2004; Sapolsky et al., 2000). Extreme temperatures can disrupt homeostasis and the GC response may be an important adaptive mechanism for responding to temperature extremes in ectotherms. Both temperature and GC production also affect metabolism (Preest and Cree, 2008; Sykes and Klukowski, 2009), so GC responses have the potential to mitigate or exasperate the energetic consequences of extreme temperatures. In some cases, animals that are chronically exposed to an external stimulus may downregulate GC responses to subsequent stimuli (Rich and Romero, 2005). For ectotherms that are chronically exposed to elevated temperatures, downregulating GC responses should reduce the energetic costs of warming (Narayan and Hero, 2014). Further, GC responses may differ among populations or closely related species that occupy habitats with different temperatures. For example, Telemeco and Addis (2014) found that GC responses to temperature (measured as changes in CORT levels) differed between northern and southern alligator lizards (*Elgaria coerulea* and *Elgaria multicarinata*, respectively) so that CORT was elevated at low temperatures in the southern species only. For the southern species, CORT release may act as an adaptive response to cold temperatures by increasing metabolic rates, which are generally depressed in cold environments.

Measuring multiple physiological metrics may aid in our understanding of organismal responses to temperature warming or cooling. For example, fish (creek chub, *Semotilus atromaculatus*) from streams within agricultural areas, which are warmer and have

<sup>1</sup>Department of Biology, University of Maryland, College Park, MD 20742, USA.

<sup>2</sup>Department of Biology, Texas State University, San Marcos, TX 78666, USA.

<sup>3</sup>Department of Animal and Avian Sciences, University of Maryland, College Park, MD 20742, USA. <sup>4</sup>Department of Biology, University of Richmond, Richmond, VA 23173, USA.

\*Author for correspondence (Anovarro1@gmail.com)

 A.J.N., 0000-0003-1806-7273; C.R.G., 0000-0001-7584-1451; K.L.G., 0000-0003-1710-0457

lower dissolved oxygen than forested streams, exhibited GC responses similar to those from forested areas when exposed to high-temperature conditions in the laboratory (Blevins et al., 2013). In this same study, Blevins and colleagues (2013) showed that fish from agricultural and forested populations differed in physiological performance. Fish from the agricultural area consumed 15% less energy (measured as resting metabolic rate) in response to elevated temperatures, relative to fish from the forested area. In other words, when populations are repeatedly exposed to higher temperatures, attenuation of the GC response can act as an adaptive mechanism for reducing metabolic expenditure, thereby reducing energetic costs in an otherwise costly environment. These results highlight the need to record multiple physiological metrics and raise questions regarding the interactions between temperature- and GC-induced changes in performance.

Thermal sensitivity of physiological responses often varies among individuals from geographically distinct populations. In addition to hormonal changes, these responses can include whole-organism physiological performance traits, which determine how well an individual performs a dynamic and ecologically relevant task (Lailvaux and Husak, 2014). According to the 'hotter is better' hypothesis, species from warmer localities should have higher maximal performance than species from colder localities (Huey and Kingsolver, 1989). A core assumption of this hypothesis is that cold-adapted populations cannot overcome the rate-depressing effects of low temperature on biochemical reactions (Hochachka and Somero, 2002). As a result, warm-adapted populations attain higher maximal performance. Whereas species-level comparisons largely confirm that hotter is better, population-level comparisons have mixed results (Knies et al., 2009; Phillips et al., 2014). A recent study found evidence that hotter is better for energy assimilation (i.e. the amount of energy acquired after accounting for metabolic losses) across salamander populations separated by >450 km along a latitudinal gradient, but not among populations separated by >900 m in elevation (Clay and Gifford, 2017). In other words, the spatial scale used to test the 'hotter is better' hypothesis matters. A limitation of past physiological studies is that they often measured thermal physiology in single populations or compared extreme ends of the geographic range (e.g. north versus south or high elevation versus low elevation; reviewed in Spicer and Gaston, 2009). Recent studies of physiological rates across landscapes have demonstrated the importance of range-wide sampling in identifying populations that are most susceptible to environmental change (Buckley et al., 2014; Crespi et al., 2015; Deutsch et al., 2008).

In this study, we examined the effects of elevated temperature on CORT (i.e. the dominant amphibian GC) and physiological performance (i.e. ingestion rate and mass gain) among eastern red-backed salamanders, *Plethodon cinereus* (Green 1818), across a 1100 km latitudinal gradient (Fig. 1). We predicted that CORT would increase when salamanders were exposed to elevated temperatures, but that salamanders from warmer sites would exhibit a smaller increase in CORT release rates compared with salamanders from cooler environments as an adaptive short-term response to higher temperatures. With regard to physiological performance, we predicted that ingestion rate would be greater in salamanders from warmer sites, in accordance with the hotter is better hypothesis, and that a greater ingestion rate would reduce mass loss after exposure to elevated temperatures. Additionally, we explored the relationship between CORT release rates and food conversion efficiency (i.e. the amount of mass gained per unit food ingested) and tested whether this relationship was temperature dependent. In salamanders, elevated CORT is associated with

decreased body mass and increased metabolic rate (reviewed in Woodley, 2017), so we predicted that an increase in individual CORT release rates would reduce food conversion efficiency and lead to long-term ecological consequences. Our study extends prior work by examining whether physiological traits provide insights into thermal sensitivity across the geographic range of a widespread species.

## MATERIALS AND METHODS

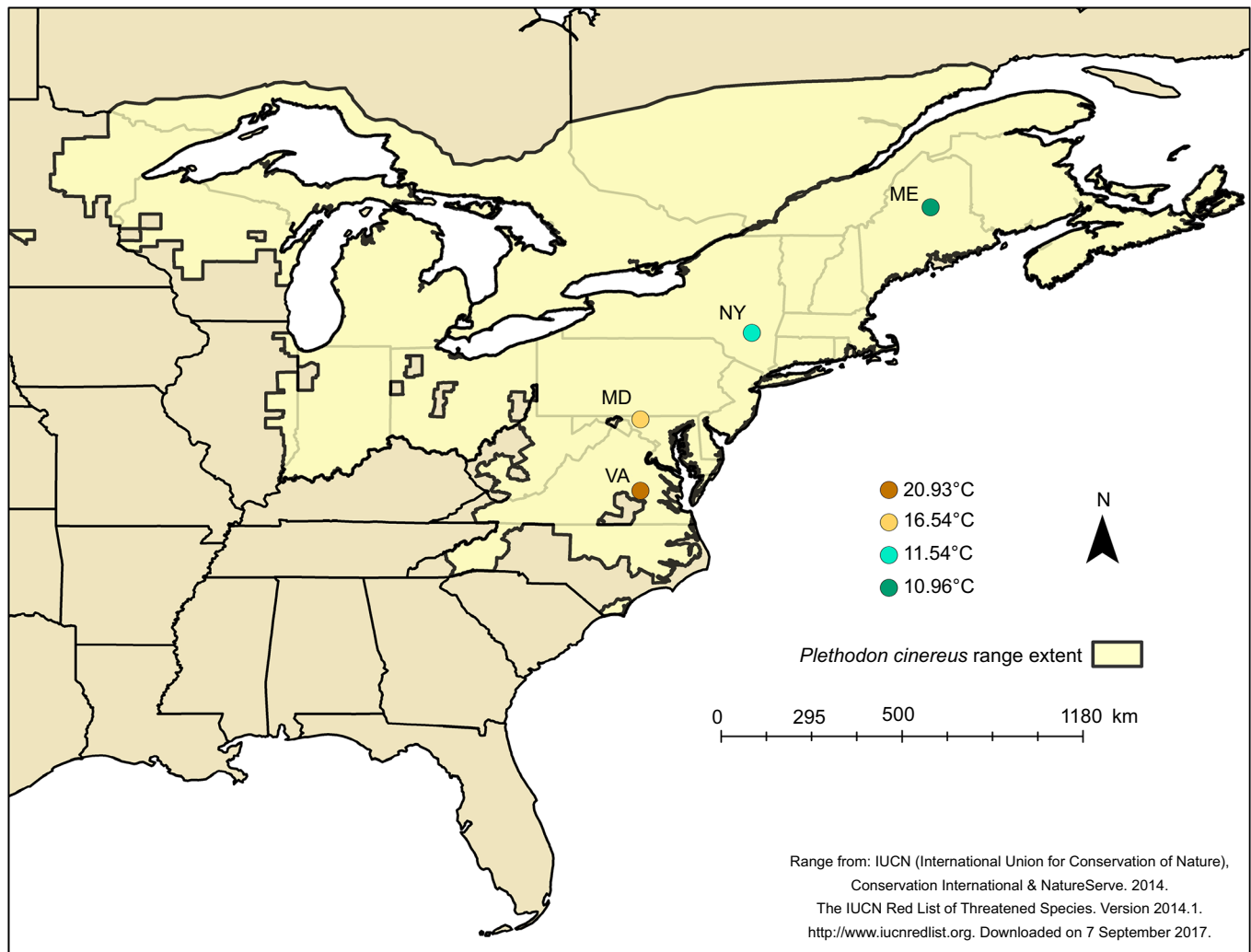
### Study species

The genus *Plethodon* consists of lungless salamanders (family Plethodontidae) that are restricted to terrestrial habitat and thus lack a larval stage (Petranka, 1998). The eastern red-backed salamander (*P. cinereus*) is the most widely distributed *Plethodon* species in the eastern USA (Fig. 1). It occupies more than half of the geographic distribution of the entire genus (1.8 million out of 3.1 million km<sup>2</sup>) and is the only *Plethodon* species found in the northernmost 1.23 million km<sup>2</sup> of the genus' range (Adams and Church, 2011). Although *P. cinereus* is a model system for plethodontid behavior (Jaeger et al., 2016), we know little about the physiological traits that allow this species to occupy such a large geographic range. Most physiological studies of *P. cinereus* are restricted to single populations (Heatwole, 1962; Homyack et al., 2010; Hutchinson, 1961; Merchant, 1970; Spotila, 1972; but see Markle, 2015, for a comparative study of critical thermal limits across the southwestern portion of the species range). Here, we compared physiological responses to elevated temperature in *P. cinereus* across a latitudinal gradient from Virginia to Maine (Fig. 1).

### Salamander collection and husbandry

In August and September 2016, we collected *P. cinereus* from 4 sites spanning 8.3° latitude and >1100 km (ME, NY, MD and VA; Fig. 1). Collection occurred prior to the breeding season, which occurs from October to December (Petranka, 1998). At each site, we hand-captured 16–17 adult salamanders (>32 mm snout–vent length, SVL; Saylor, 1966) and transported them back to the lab in individually labeled containers. We based this sample size on a previous study, which found that sample sizes of 15 or 16 salamanders provided enough statistical power to detect a significant difference in CORT release rates (Gabor et al., 2016). We did not discriminate by sex because of collecting limitations, but analyses using independent *t*-tests indicated that our variables of interest did not differ among the sexes (CORT:  $P=0.42$ ; ingestion rate:  $P=0.22$ ; mass change:  $P=0.49$ ). To avoid the potentially confounding physiological effects of color polymorphism (described in Fisher-Reid et al., 2013, and Moreno, 1989), we only collected individuals that clearly displayed the striped, rather than unstriped, phenotype. At the time of collection, we measured SVL, tail length and mass, and determined sex using the candling method (described by Gillette and Peterson, 2001). After collection, salamanders were transported to temperature-controlled chambers and held at a constant temperature of 15°C, where they underwent an acclimation period of 4 weeks. We housed each salamander individually in a plastic container (18×14×12 cm) lined with a moist unbleached paper towel and a crumpled moist paper towel to use as a retreat. Salamanders were fed 1–3 black soldier fly larvae, 3–5 crickets (6.4 mm) or 15–20 large flightless fruit flies (*Drosophila hydei*) weekly and sprayed with spring water *ad libitum*. Salamanders were held in incubators with fluorescent lighting set on a 12 h:12 h dark:light cycle throughout the entirety of this study.

Animals were collected with permission from Maine Department of Inland Fisheries and Wildlife (permit #2016-483), Maryland



**Fig. 1. Salamander collection sites in the eastern USA.** The coloration of the points indicates the site heat value, a measure of daily maximum surface temperature averaged over 35 years. We collected 16–17 salamanders from each site.

Department of Natural Resources (permit #56409), New York State Department of Environmental Conservation (permit #2007) and Virginia Department of Game and Inland Fisheries (permit #056084). Interstate transport was permitted under a Federal Fish and Wildlife injurious species permit (permit #MA90136B-0) and vertebrate research was approved by the University of Maryland (protocol FR-15-72) and University of Richmond IACUC (protocol 16-10-001).

### Thermal CORT response experiment

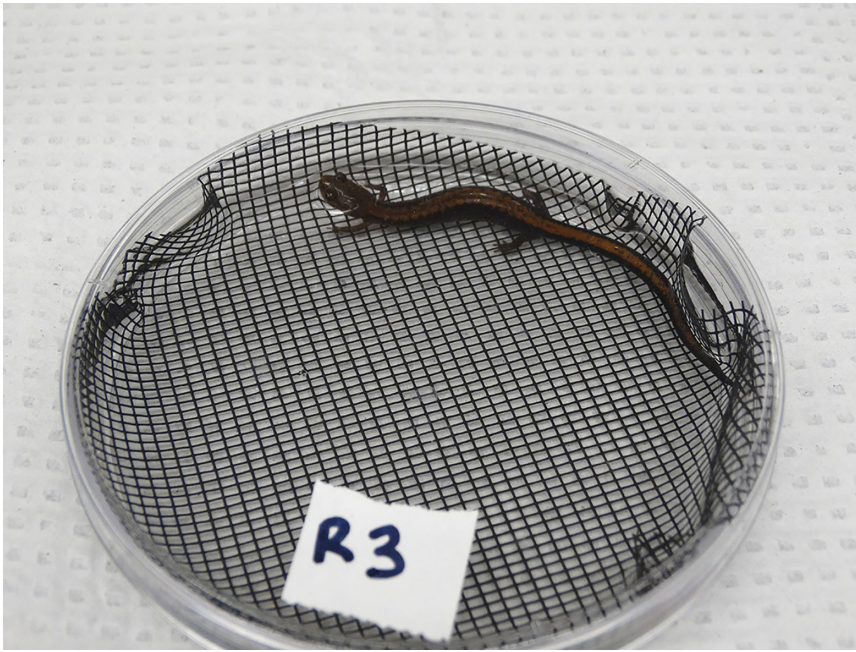
We examined the GC response of salamanders to elevated temperature using a water-borne CORT assay (Gabor et al., 2013) which provides an integrated measure reflecting an average of blood GCs that have been metabolized and excreted from urine and feces, and possibly through the skin, over a period of cumulative exposure (Santymire et al., 2018; Sheriff et al., 2011). We measured CORT release rates for each salamander at an average temperature (15°C) and an elevated temperature (25°C). The average temperature treatment was within the range of thermal preferences for *P. cinereus* in a laboratory setting (12–22°C) and is a common maintenance temperature for *Plethodon* studies (Clay and Gifford, 2017; Feder and Pough, 1975; Gabor and Jaeger, 1995). We chose to raise the temperature to 25°C for several reasons: it is above the

thermal preference of this species (Feder and Pough, 1975), it reflects a realistically high body temperature in natural conditions (A.J.N., unpublished data) and it is sublethal (Hutchinson, 1961).

After acclimating salamanders to the average temperature for 4 weeks, we collected the first CORT sample from each salamander using a water-borne assay, as described below. Salamanders remained at this temperature for an additional 24 h, and then we increased the ambient temperature by 2.5°C day<sup>-1</sup> until reaching 25°C. Salamanders remained in this elevated, likely physiologically challenging, thermal environment for 48 h, after which we repeated the water-borne assay at 25°C to collect a second CORT sample while maintaining the elevated temperature. We fed all salamanders 24 h prior to collecting each CORT sample to maintain their weekly feeding schedule and to minimize the potential effects of hunger or feeding frequency on CORT. All salamanders survived this process and did not show external signs of distress or illness.

### Water-borne hormone assays

To collect water-borne samples for hormone analysis, we placed each salamander individually in 45 ml of bottled spring water in a standard-size Petri dish (100×15 mm) for 1 h (following Gabor et al., 2016; Fig. 2). We also ran blank controls using spring water



**Fig. 2. Water-borne corticosterone (CORT)**

**collection procedure.** We collected water-borne CORT samples by placing each salamander in 45 ml of spring water in a Petri dish for 1 h. We lined the bottom of each Petri dish with mesh screen to provide a textured surface for the salamanders to stand on, and to easily remove the salamander from the Petri dish while leaving the water sample behind for collection.

samples (3 different samples as a result of different sample times) and subtracted the relevant values from the CORT release rates of each salamander (spring water CORT ranged from 2.61 to 8.79 pg ml<sup>-1</sup> of sample water). All sampling events were scheduled between 15:00 h and 17:00 h to avoid circadian fluctuations of CORT (Dunn et al., 1972). We placed a piece of mesh lining in the bottom of each Petri dish to aid in removing salamanders, while minimizing sample loss (Fig. 2). After 1 h, we lifted the mesh out of each Petri dish to transfer the salamander back to its housing container while leaving the water sample in the dish. We poured the water sample from each Petri dish into a labeled Falcon tube, and immediately stored all samples in a -20°C freezer (Ellis et al., 2004). The Petri dishes and mesh lining were cleaned with 95% ethanol and rinsed with spring water before use. We extracted CORT from water following Gabor et al. (2016) and re-suspended the CORT residue with 95% EIA buffer and 5% ethanol for a total of 220 µl. We measured CORT in duplicate for all samples using a CORT enzyme-immunoassay (EIA) kit (Cayman Chemical Company, Inc., cat. no. 501320, Ann Arbor, MI, USA) on a spectrophotometer plate reader set to 405 nm (Biotek ELX 800, Winooski, VT, USA). This assay is 100% cross-reactive with CORT, 15.8% with 11-deoxycorticosterone and 3.4% with prednisolone.

We validated the use of water-borne CORT collection methods from *P. cinereus* on Cayman Chemical EIA plates using a pooled sample of CORT from 10 non-experimental animals (following Gabor et al., 2016). We assessed parallelism of the serial dilution curve (1:1 to 1:32) using the pooled sample. The CORT dilution curve was not significantly different from the standard curve (comparison of slopes,  $t_0 = -0.894$ ,  $P = 0.39$ ). To determine quantitative recovery, we spiked the pooled sample with each of eight standards in addition to the un-spiked pooled sample. The minimum observed recovery was 89%. We found a linear relationship between observed and expected slopes ( $\beta = 1.2$ ,  $F_{1,6} = 457.38$ ,  $R^2 = 0.99$ ,  $P < 0.001$ ). Using a pooled control sample run in quadruplicate on each plate, our intra-plate variation on 5 plates ranged from 0.51% to 13.7% and the overall inter-plate variation was 15.7%. The sensitivity of the CORT EIA plates ranged between 37.5 and 1004.3 pg ml<sup>-1</sup> on average.

### Physiological performance experiment

In addition to GC response, we were interested in whether temperature differentially affected ingestion rate and mass gain among sites. After collecting the second water-borne CORT sample from each individual, we continued to expose salamanders to 25°C and fasted them for 10 days to ensure the clearing of gut contents. Following the fasting period, we began a controlled feeding trial in which we offered 50 fruit flies to each salamander, recorded the number of flies remaining after 24 h, and replenished flies that were eaten (adapted from Clay and Gifford, 2017). We repeated this procedure (counting flies and replenishing) for 5 consecutive days, recorded the number of remaining flies on day 6, and removed all leftover flies. We calculated ingestion rate as the total number of flies consumed during each trial, corrected for salamander mass (g), and divided by 5 days. To calculate mass gain, we weighed salamanders 24 h before and 48 h after each 5 day controlled feeding trial. Prior to each measurement, we placed salamanders in water for approximately 30 s and gently patted them dry with a paper towel to minimize variation in water mass (Fraser, 1980). To correct for among-individual variation in initial body mass, we calculated percentage change in mass.

After completing the feeding trial at 25°C, we brought the temperature back down to 15°C by decreasing it at 2.5°C day<sup>-1</sup> and fed salamanders a maintenance ration of fruit flies. Salamanders fasted for 10 days while adjusting to the 15°C environment prior to the next feeding trial. In total, we measured ingestion rate and mass gain for 8 salamanders from each site at each temperature ( $n = 32$ ). There was a single mortality from the NY population during the physiological performance experiment, so we removed that individual from the performance dataset. Because of differences in the availability of experimental salamanders, a different set of VA salamanders was used for CORT sampling to those used for controlled feeding trials. Thus, we excluded the VA salamanders for analyses that required paired CORT and performance data but used VA salamanders in population-level comparisons of performance. Overall, we had paired CORT and performance data for 23 salamanders representing 3 sites: MD ( $n = 8$ ), ME ( $n = 8$ ) and NY ( $n = 7$ ).

## Analyses

Following Gabor et al. (2016), we multiplied CORT ( $\text{pg ml}^{-1} \text{h}^{-1}$ ) by  $220 \mu\text{l}$  (the volume of the resuspension solution) to account for resuspension and divided by the mass of each individual (g) to obtain standardized CORT in  $\text{pg g}^{-1} \text{h}^{-1}$ . CORT data were ln-transformed for statistical analyses. For all analyses, we used the environmental temperature experienced at each collection site (described below) as an explanatory variable, rather than using site as a categorical variable. We chose to use continuous rather than categorical data for two reasons. First, we were interested in whether salamander populations exhibit physiological adaptations or acclimation based on the temperatures they experience in the wild. Second, differences in thermal conditions between the collection sites were not evenly distributed; therefore, it would be inappropriate to simply rank them based on latitudinal or elevational distribution.

To obtain climatological data for our field sites, we downloaded daily maximum ground surface temperatures for each collection site from 1980 to 2015 from NASA's Daily Surface Weather and Climatological Summaries (DAYMET) database (Thornton et al., 1997). We chose to use ground surface temperature, rather than air temperature, because *P. cinereus* is a small salamander that spends the majority of its time on the forest floor. Further, we chose maximum daily temperature because we were interested in relative heat exposure at each of the field sites. We used mean daily maximum temperature, averaged over a 35 year period, as a representative site temperature for all analyses. Hereafter, we refer to our metric for site temperature as the site heat value to avoid confusion with temperature treatments in the laboratory.

All models were run using packages *nlme* (version 3.1-131) and *stats* (version 3.4.0) in R statistical software (<http://www.R-project.org/>). To test whether CORT release rates were related to site heat value and whether they differed between the 15 and 25°C temperature treatments, we used a linear mixed-effects model (LMM) fitted by maximum likelihood. The model included site heat value, temperature treatment and the interaction between site heat value and temperature treatment as fixed effects. We nested salamander within temperature treatment as a random effect to account for the repeated sampling of each salamander at 15 and 25°C.

We were also interested in whether the relative change in CORT from 15 to 25°C varied among sites. Thus, we calculated a  $Q_{10}$  temperature coefficient (i.e. the rate at which a physiological response changes with a 10°C increase in temperature) for each salamander as the rate at which CORT changed from the average to the elevated temperature treatment (McNab, 2002).

$$Q_{10} = \left( \frac{\text{CORT}_{25^{\circ}\text{C}}}{\text{CORT}_{15^{\circ}\text{C}}} \right)^{10^{\circ}\text{C}/(25^{\circ}\text{C}-15^{\circ}\text{C})} \quad (1)$$

A  $Q_{10}$  value greater than 1 indicates an increase in CORT release rate after exposure to the elevated temperature, relative to CORT release rate at the average temperature, whereas a  $Q_{10}$  value less than 1 indicates a decrease in CORT release rate after exposure to the elevated temperature. To determine whether  $Q_{10}$  varied among sites, we fitted a linear model with  $Q_{10}$  as the response variable and site heat value as the explanatory variable. Additionally, we used a generalized LMM with a binomial error structure to determine whether the probability of having a  $Q_{10}$  value greater than or less than 1 was related to site heat value.

We compared ingestion rate and mass gain among sites using LMMs fitted by maximum likelihood. The models included site heat value, temperature treatment and the interaction between these

terms as fixed effects and either ingestion rate or mass gain as the response variable. We ran an additional analysis to determine whether food conversion efficiency varied with CORT release rate at the individual level, and whether the strength of this relationship was affected by temperature treatment. Food conversion efficiency has long been used as a performance metric in agricultural and fisheries sciences and is sensitive to temperature (Bedford and Classen, 1992; Brett et al., 1969; Handeland et al., 2008; Imsland et al., 2000; Jonassen et al., 2000). As an index of food conversion efficiency, we obtained residual values from a linear regression of mass gain on total ingestion (i.e. the total number of flies consumed during a feeding trial), with temperature treatment as an interactive effect ( $R^2=0.30$ , d.f.=42,  $P<0.001$ ). Thus, food conversion efficiency index reflects the amount of mass gained per unit ingestion for each individual relative to the mean in each temperature treatment. To determine whether CORT release rates and temperature were interactively related to food conversion efficiency, we ran a LMM with temperature treatment, CORT and the interaction between temperature and CORT as the explanatory variables and food conversion efficiency as the response variable. For all LMMs described above, we nested salamander within temperature treatment as a random effect to account for the repeated sampling of each salamander at 15 and 25°C. Statistical error is reported as s.e.m. consistently throughout the results.

## RESULTS

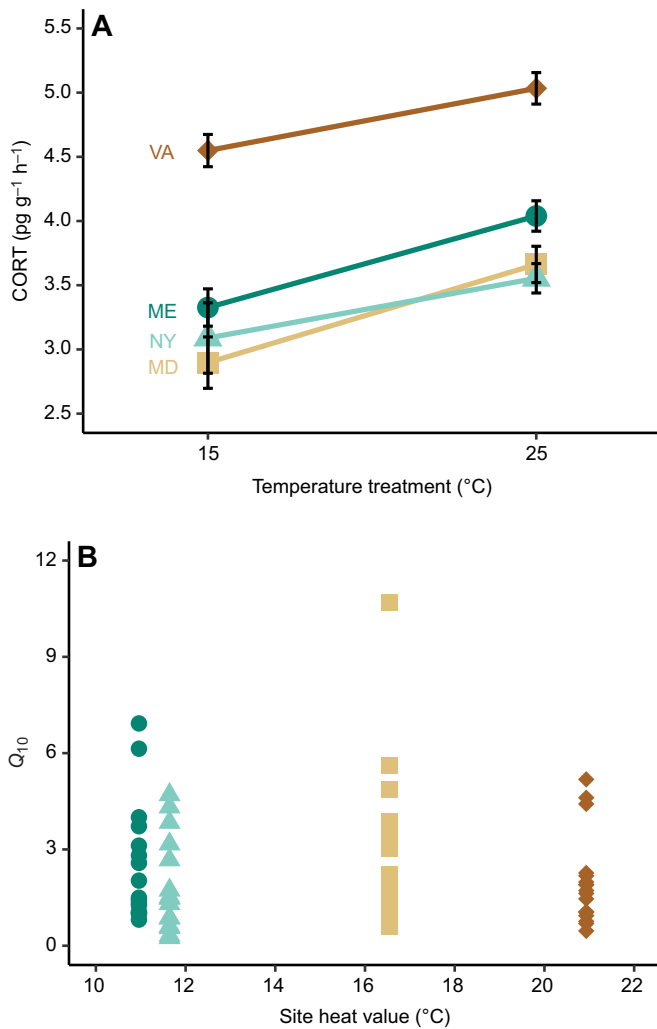
### Thermal CORT response experiment

We found that overall salamander CORT release rates were positively related to site heat value ( $\beta=0.12\pm 0.02$ ,  $\chi^2=33.07$ , d.f.=63;  $P<0.001$ ) and were significantly higher after exposure to an elevated temperature, which is outside the preferred thermal range of *P. cinereus*, than at the average temperature ( $\chi^2=33.91$ ,  $P<0.001$ ; Fig. 3A). Across all sites, exposure to an elevated temperature raised CORT release rates by an average of  $0.71\pm 0.41 \text{ pg g}^{-1} \text{ h}^{-1}$ . The interaction term (site heat value  $\times$  temperature treatment) was not significant ( $\chi^2=0.08$ ,  $P=0.78$ ), meaning that CORT release rate responded similarly to the elevated temperature across sites. Removing the random effects (individual nested within temperature treatment) significantly decreased the goodness of fit, as indicated by a likelihood ratio test ( $P<0.001$ ), suggesting that individual was an important source of variation in our data.

To better understand the effects of elevated temperature on individual CORT release rates, we calculated  $Q_{10}$  values for each salamander. A linear model determined that site heat value was not a significant predictor of  $Q_{10}$  (d.f.=63,  $P=0.41$ ). We found an average  $Q_{10}$  value of  $2.69\pm 0.42$ , indicating that CORT release rates were 2.69 times greater at the elevated temperature, compared with the average temperature treatment. However,  $Q_{10}$  values varied widely among individuals (Fig. 3B). Overall, 77% (50/65) of individuals had a  $Q_{10}$  value greater than 1 and experienced a  $3.30\pm 0.51$  rate of increase in CORT release after exposure to an elevated temperature, whereas the other 23% (15/65) had a  $Q_{10}$  value less than 1 and experienced a  $0.67\pm 0.06$  rate of decrease in CORT release after exposure to the elevated temperature. The probability of an individual having a  $Q_{10}$  value greater than or less than 1 was not significantly related to site heat value ( $z=0.04$ , d.f.=63,  $P=0.97$ ).

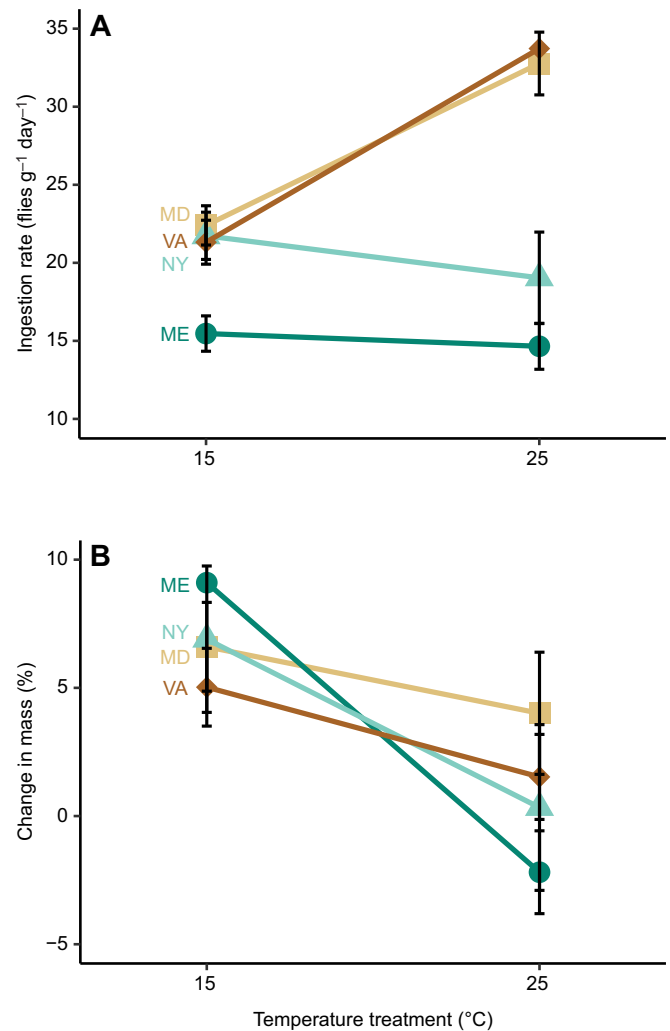
### Physiological performance experiment

We found an interactive effect of site heat value and temperature treatment on ingestion rate ( $\chi^2=33.02$ , d.f.=63,  $P<0.001$ ; Fig. 4A). The slope of the relationship between ingestion rate and site heat value was 5 times greater in the 25°C treatment than in the 15°C



**Fig. 3. CORT response to an elevated temperature.** (A) Relationship between mean CORT release rate and experimental temperature for salamanders from each collection site at 15°C ( $n=65$ ) and 25°C ( $n=65$ ). Salamander CORT release rates were positively related to site heat value ( $P<0.001$ ) and were significantly higher at 25°C than at 15°C ( $P<0.001$ ). (B) Lack of a relationship between site heat value and  $Q_{10}$  temperature coefficients, or the rate at which CORT changed with a 10°C increase in temperature ( $n=65$ ). A linear model determined that site heat value was not a significant predictor of  $Q_{10}$  ( $P=0.41$ ). A single outlier (site heat index=11.54,  $Q_{10}=24.82$ ) was excluded for visualization purposes, but was included in all analyses. Error bars represent s.e.m. and point colors indicate the site heat value (see A for locations).

treatment ( $\beta_{15^\circ\text{C}}=0.38\pm 0.25$ ,  $\beta_{25^\circ\text{C}}=1.91\pm 0.28$ ). At 25°C, salamanders from the warmest sites (MD and VA) ingested an average of  $32.77\pm 2.01$  and  $33.73\pm 2.01$  flies  $\text{g}^{-1} \text{day}^{-1}$ , respectively, whereas salamanders from the coolest sites (ME and NY) ingested an average of  $14.65\pm 1.47$  and  $19.04\pm 2.93$  flies  $\text{g}^{-1} \text{day}^{-1}$ , respectively. Similarly, we found a significant interactive effect of site heat value and temperature treatment on mass gain ( $\chi^2=3.96$ , d.f.=63,  $P=0.047$ ; Fig. 4B). The slope of the relationship between mass gain and site heat value was negative in the 15°C treatment but positive in the 25°C treatment ( $\beta_{15^\circ\text{C}}=-0.32\pm 0.25$ ,  $\beta_{25^\circ\text{C}}=0.34\pm 0.34$ ). On average, the mass of individuals increased by  $6.85\pm 0.93\%$  in the 15°C treatment and  $0.96\pm 1.13\%$  in the 25°C treatment. Across all sites, 41% (13/32) of salamanders lost mass in the 25°C treatment, whereas only 9% (3/32) of salamanders lost mass in the 15°C treatment.



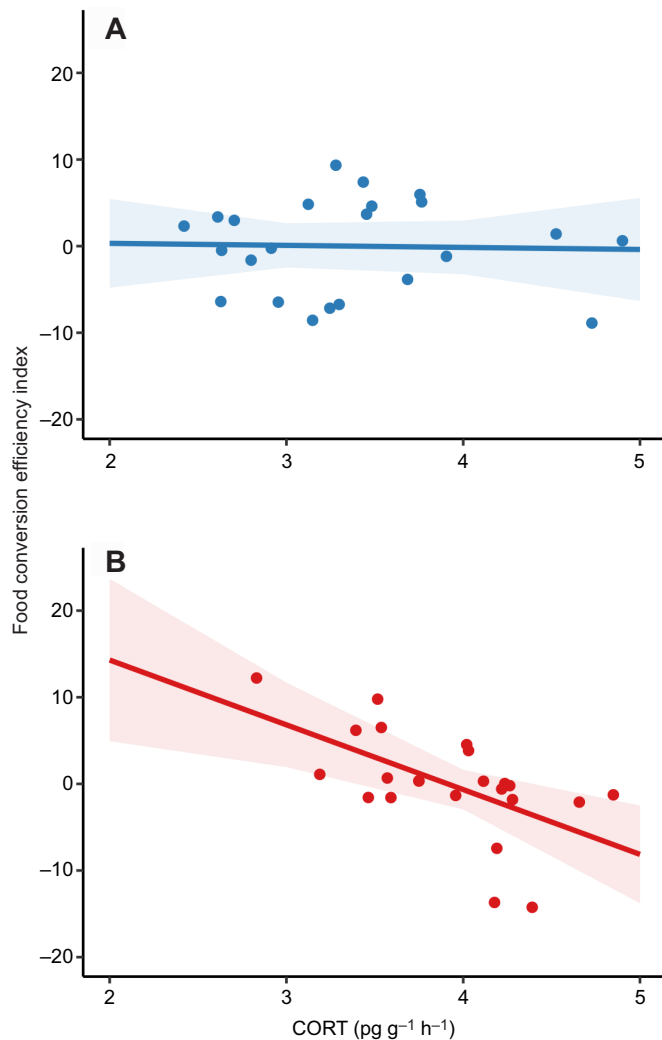
**Fig. 4. Population variation in thermal performance.** Points represent mean ingestion rate (A) and change in mass (B) for salamanders from each collection site in the 15°C ( $n=32$ ) and 25°C ( $n=32$ ) temperature treatments. Error bars represent s.e.m. and point colors indicate the site heat value. Using linear mixed-effects models, we found an interactive effect of site heat value and temperature treatment on ingestion rate ( $P<0.001$ ) and mass gain ( $P=0.047$ ).

To explore the association between CORT release rates and physiological performance, we tested the relationship between CORT and food conversion efficiency index in salamanders that underwent hormone sampling and controlled feeding trials. We found that CORT release rate and temperature treatment were interactively related to food conversion efficiency index ( $\chi^2=6.89$ , d.f.=20,  $P=0.009$ ; Fig. 5). In the 25°C treatment, an increase in CORT release rate was related to a decrease in food conversion efficiency, indicating that individuals with higher CORT release rate stored less energy per unit ingestion than individuals with a lower CORT release rate ( $\beta_{25^\circ\text{C}}=-7.48\pm 2.89$ ). In the 15°C treatment, the relationship between CORT release rate and food conversion efficiency was virtually absent ( $\beta_{15^\circ\text{C}}=-0.23\pm 1.67$ ).

## DISCUSSION

Our results suggest that thermal sensitivity differs between physiological traits and across a latitudinal gradient in *P. cinereus*. The ability of individuals to adjust CORT and performance may result in an improved ability to maintain homeostasis and allow populations to persist across a range of environmental conditions.





**Fig. 5. Relationship between individual CORT release rate and food conversion efficiency index in sites with paired hormone and performance data (ME, NY and MD).** Temperature treatments are shown separately for clarity: (A) 15°C ( $n=23$ ) and (B) 25°C ( $n=23$ ). Regression lines and 95% confidence intervals were fitted using a linear mixed-effects model with salamander nested within temperature treatment as a random effect. CORT release rates and temperature treatment were interactively related to food conversion efficiency index ( $P=0.009$ ).

Using an integrated measure of CORT (i.e. from water-borne assays), we found that CORT release rates were positively related to site heat value and were consistently higher when salamanders were exposed to an elevated temperature. Contrary to predictions, the rate at which CORT release increased when salamanders transitioned from 15 to 25°C did not vary among sites. This result suggests that salamanders from warmer sites may lack the ability to downregulate CORT, which may be maladaptive, despite living near the edge of the species' southern range. Alternatively, the higher CORT release rates observed in the southernmost population could be an adaptive response to living in overall higher temperatures than the other populations. Higher CORT may mediate a behavioral response such as retreating below ground and aid in maintaining homeostasis. Future studies could differentiate between these hypotheses by further testing whether these salamanders show a physiological response to additional stressors.

As for performance, salamanders from warmer sites responded strongly to the elevated temperature by increasing ingestion rate,

whereas salamanders from cooler sites did not. This response was predicted because both higher temperatures and higher CORT increase metabolism (Prest and Cree, 2008; Sykes and Klukowski, 2009). The elevated temperatures reduced mass gain in all four sites, although this reduction was more dramatic for salamanders from cooler sites. Finally, we found a temperature-dependent relationship between CORT release rates and food conversion efficiency where salamanders with higher CORT release had lower food conversion efficiency at 25°C but CORT release was unrelated to food conversion at 15°C. Thus, the energetic gains of elevated ingestion rates may be counteracted by higher CORT release rates experienced by salamanders exposed to an elevated temperature.

When faced with elevated temperatures, salamanders from all collection sites experienced increased CORT release rates. This finding confirms that body temperature influences CORT release rates in *P. cinereus* and that CORT release rates are greater, on average, in warmer temperatures. Very few studies have measured the effects of temperature on CORT responses in amphibians, but our results are similar to those found in cane toads (*Rhinella marina*; Narayan et al., 2012). Narayan and colleagues (2012) found that although urinary CORT metabolite concentrations (another integrated measure of CORT) rose during a 24 h acclimation period, *R. marina* consistently exhibited higher CORT at 25°C relative to 15°C. Additionally, our study also revealed geographic variation in CORT release rates, which increased on average as site heat value increased, indicating that temperatures experienced in the wild may have long-lasting effects on salamander CORT. The observed trend was largely driven by elevated CORT release rates in salamanders from the southernmost, warmest collection site (Richmond, VA). CORT is often released in response to external conditions to mobilize energy stores and initiate an escape response (Sapolsky et al., 2000). If salamanders living near the edge of the southern range have higher body temperatures and therefore higher metabolic rates, on average, then elevated CORT may have a maladaptive effect on an individual's energy budget, while also potentially mediating adaptive behavioral responses such as retreating below ground to reduce body temperatures. Additional behavioral studies would provide insights as to whether higher CORT facilitates adaptive responses to thermal fluctuations, such as retreating below ground.

Salamanders from all of our study sites experienced a similar increase in CORT release rates following the elevation in temperature. This was surprising, as amphibians have been shown to modulate or downregulate endocrine sensitivity to warming after repeated exposure to high temperatures in the laboratory (Narayan and Hero, 2014). We predicted that populations towards the edge of the southern range would downregulate CORT in response to an elevated temperature as they experience repeated exposure to heat in natural conditions. However, it is possible that behavioral avoidance of suboptimal temperatures is great enough to homogenize the frequency of extreme heat experienced among our study sites. As previously mentioned, increased CORT may mediate behavioral avoidance of high temperatures, which would aid in avoiding lethal temperatures. At our southernmost site, *P. cinereus* avoids extreme heat and desiccation by retreating below ground for a significant portion of the year, from May to September (KL.G., unpublished data). Behavioral thermoregulation may reduce thermal selection for local adaptation of physiological traits (Buckley et al., 2015), which in this case we expected to manifest as the downregulation of CORT release rates in warmer temperatures.

Although sites experienced a similar increase in mean CORT release rates when exposed to an elevated temperature, the degree to

which CORT increased from 15 to 25°C varied widely among individuals within each site. In our study, 77% of individuals experienced an increase in CORT release rate after exposure to the elevated temperature, indicated by a  $Q_{10}$  value greater than 1. For these individuals, CORT was 1.01–10.7 times higher in the elevated temperature treatment (although a single outlier from NY experienced a 24.8-fold increase in CORT release rate in the elevated temperature). When compared with another amphibian, *R. marina*, in which CORT increased 1.51-fold from 15 to 25°C and 1.43-fold from 25 to 35°C, many of the  $Q_{10}$  values observed in our study are quite high. These high  $Q_{10}$  values suggest that the CORT release rates of *P. cinereus* are relatively sensitive to elevated temperatures. The other 23% of salamanders used in the CORT experiment experienced a decrease in CORT release after exposure to the elevated temperature, indicated by a  $Q_{10}$  less than 1. Such reductions in CORT release rates with increasing temperature have also been shown in reptiles, specifically in the Children's python (*Antaresia childreni*; Dupoué et al., 2013). Researchers suggested that snakes may release more CORT as a means of coping with suboptimal temperatures. It is possible that individuals with higher thermal optima experience greater physiological consequences at lower temperatures, and potentially respond to that environmental condition with an increase in CORT. Regardless of the cause, variation in  $Q_{10}$  temperature coefficients may act as a basis for future plasticity in response to climate change (Ghalambor et al., 2007; Urban et al., 2014).

Our study revealed geographic variation in thermal performance in accordance with the 'hotter is better' hypothesis (Angilletta, 2009; Huey and Kingsolver, 1989). When exposed to an elevated temperature, *P. cinereus* from northern populations maintained low ingestion rates and consequently lost mass. In contrast, the southernmost populations effectively responded to the elevated temperature by capturing and ingesting more prey, thereby counteracting energy loss in warmer temperatures. This pattern may be explained by differences in life history strategies of *P. cinereus* across their range. Salamanders from colder localities endure longer winters and a shorter growing season than those from warmer localities. In the 15°C treatment, *P. cinereus* from the northernmost site (Millinocket, ME) gained the most mass while consuming the least amount of energy. Our findings suggest that the Maine population may have had depressed metabolic rates and/or allocated more energy towards mass gain (i.e. growth and fat storage) at 15°C relative to the other populations. There is evidence for both metabolic depression and fat storage in temperate salamanders. When acclimated to warmer temperatures, salamanders may use metabolic depression as a mechanism for coping with thermal stressors (Bernardo and Spotila, 2006; Markle, 2015). Although metabolic depression has not been explicitly tested as a means of maximizing fat storage during the limited growing season in cold-adapted salamanders, it plays an important role in torpor, hibernation and estivation in other taxa (Guppy and Withers, 1999). In addition to potential metabolic depression, *P. cinereus* from high elevations are known to allocate more energy towards tail fat storage than those from low elevations (Takahashi and Pauley, 2010). Although salamanders from Maine seem to possess successful strategies for coping with a shorter growing season, they suffered the greatest loss of mass when exposed to an elevated temperature.

An important precursor to using an integrated measure of CORT release rates as a biomarker for the effects of environmental change is linking CORT with performance and fitness consequences. We provide evidence for a temperature-dependent relationship between CORT release rates and whole-organism

physiological performance. CORT release rates were unrelated to food conversion efficiency index in the 15°C treatment but were negatively related to food conversion efficiency index in the 25°C treatment. Thus, it seems that elevated temperatures may have greater ecological consequences for individuals with higher CORT release rates. We propose that the increased metabolic demands imposed by CORT and elevated temperatures can overwhelm an individual's ability to maintain a positive energy budget. Our proposed explanation is supported by prior models of thermodynamic constraints on physiological rates described by the Boltzmann factor (Angilletta et al., 2010), as well as previous studies of metabolic rates in plethodontid salamanders. In *P. cinereus*, mass-corrected metabolic rate increases exponentially with increases in temperature (Homyack et al., 2010). Red-legged salamanders (*Plethodon shermani*) with chronically elevated plasma CORT also have higher metabolic rates (Wack et al., 2012). To date, there is mixed evidence for a relationship between CORT and performance in lungless salamanders. Salamanders with chronically elevated CORT have a weaker immune response (i.e. slower wound healing) than control individuals (Thomas and Woodley, 2015). A suppressed immune response may be a result of energetic tradeoffs, where chronically stressed individuals allocate more energy towards physiological maintenance than immune responses (Korfel et al., 2015). By contrast, increased levels of plasma CORT did not affect locomotor performance in Allegheny dusky salamanders (*Desmognathus ochrophaeus*), although other stressors (i.e. handling and low pH) reduced performance (Ricciardella et al., 2010; Woodley et al., 2014). More research on geographic patterns of physiological performance in relation to environmental change is needed to understand the mechanisms underlying the observed variation in performance and its relationship to CORT.

Environmental disturbances ultimately affect fitness in wild populations through changes in individual physiology and performance (Jeffrey et al., 2015). Thus, understanding cause-and-effect relationships between environmental disturbances and animal physiology allows us to make predictions about population-level responses to environmental change (Cooke and O'Connor, 2010; Wikelski and Cooke, 2006). Our results suggest that southern populations of *P. cinereus* may be more resilient to climate warming than northern populations, because they exhibit greater flexibility in performance (i.e. ingestion rate) when exposed to high temperatures. Although salamander CORT release rates responded similarly to an elevated temperature among our study sites, salamanders from the southern sites were able to increase ingestion rate and thereby compensate for increased metabolic demands. Taken together, our results suggest that populations may be more resilient to environmental change when they possess flexible behavioral responses for coping with elevated temperatures (Herstoff and Urban, 2013).

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.J.N., C.R.G., K.L.G.; Methodology: A.J.N., C.R.G., C.B.G., L.M.T., K.L.G.; Software: A.J.N., L.M.T.; Validation: A.J.N., C.R.G., C.B.G., L.M.T.,

K.L.G.; Formal analysis: A.J.N., C.R.G., C.B.G.; Investigation: A.J.N., C.R.G., C.B.G., T.D.M., L.M.T., K.L.G.; Resources: A.J.N., C.R.G., K.L.G.; Data curation: A.J.N., L.M.T.; Writing - original draft: A.J.N., C.R.G.; Writing - review & editing: A.J.N., C.R.G., C.B.G., T.D.M., L.M.T., K.L.G.; Visualization: A.J.N., L.M.T.; Supervision: A.J.N., C.R.G., L.M.T., K.L.G.; Project administration: A.J.N., C.R.G., L.M.T., K.L.G.; Funding acquisition: A.J.N., K.L.G.

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### Data availability

Data used in analyses have been deposited in the Dryad digital repository (Novarro et al., 2018): <https://doi.org/10.5061/dryad.g32q0h0>

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