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Article type : Research Article
Editor : Dehua Wang

Section heading: Physiological Ecology

Physiology at near-critical temperatures, but not critical limits, varies between two lizard species that partition the thermal environment

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Manuscript for consideration as a Standard Paper in *Journal of Animal Ecology*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1365-2656.12738

Summary

1. The mechanisms that mediate the interaction between the thermal environment and species' ranges are generally uncertain. Thermal environments may directly restrict species when environments exceed tolerance limits (i.e. the fundamental niche). However, thermal environments might also differentially affect relative performance among species prior to fundamental tolerances being met (i.e. the realized niche).
2. We examined stress physiology (plasma glucose and corticosterone), mitochondrial performance, and the muscle metabolome of congeneric lizards that naturally partition the thermal niche, *Elgaria multicarinata* (southern alligator lizards; SAL) and *E. coerulea* (northern alligator lizards; NAL), in response to a thermal challenge to quantify variation in physiological performance and tolerance.
3. Both NAL and SAL displayed physiological stress in response to high temperature, but neither showed signs of irreversible damage. NAL displayed a higher baseline mitochondrial respiration rate than SAL. Moreover, NAL substantially adjusted their physiology in response to thermal challenge whereas SAL did not. For example, the metabolite profile of NAL shifted with changes in key energetic molecules, whereas these were unaffected in SAL.
4. Our results indicate that near-critical high temperatures should incur greater energetic cost in NAL than SAL via an elevated metabolic rate and changes to the metabolome. Thus, SAL displace NAL in warm environments that are within NAL's fundamental thermal niche, but relatively costly.

5. Our results suggest that sub-critical thermal events can contribute to biogeographic patterns via physiological differences that alter the relative costs of living in warm or cool environments.

Key-words Corticosterone, OCLTT, Pejus temperatures, Reactive oxygen species, State III respiration

Introduction

While temperature has long been recognized as an important component of the fundamental niche that affects macro-ecological patterns (Addo-Bediako, Chown & Gaston 2000; Pörtner 2002; Cruz *et al.* 2005; Angilletta 2009), the need to predict the impact of climate change on species' distributions has reinvigorated interest in understanding the mechanisms underlying temperature's influence on organisms (e.g. Deutsch *et al.* 2008; Kearney & Porter 2009; Sunday, Bates & Dulvy 2012; Levy *et al.* 2015). Only with an understanding of these mechanisms can we extrapolate beyond simple correlations to predict the impacts of novel thermal environments on species (Deutsch *et al.* 2008; Sunday, Bates & Dulvy 2011; Levy *et al.* 2015; Telemeco *et al.* 2016). Even so, the mechanisms that translate the thermal environment into fitness are poorly understood (reviewed in Angilletta 2009; Dowd, King & Denny 2015).

Perhaps the best example of our limited understanding of the mechanisms underlying thermal tolerance is the fact that the proximate mechanism responsible for setting high-temperature limits is not known for most animals. Classically, animals were thought to die at high temperatures because their proteins or membranes lost structural integrity ("denaturation hypothesis" hereafter, Tansey & Brock 1972; Fields 2001; Pörtner 2002; Schulte 2015).

However, most animals die at temperatures below those that melt proteins or membranes (Hochachka & Somero 2002; Pörtner 2002). More conservatively, loss of organismal function might result from reversible loss of subcellular function that could be restored if the animal survived to be cooled (Somero 1995; Hochachka & Somero 2002; Schulte 2015). Alternatively, organisms might lose function at high-temperatures because of organ-system failures (Pörtner 2002; Schulte 2015). For example, the oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis proposes that circulatory and respiratory systems are unable to deliver sufficient oxygen to meet the elevated demand of tissues at high temperature, resulting in system failure and death (Pörtner 2001; Pörtner 2002; Pörtner *et al.* 2006).

In addition to our limited understanding of the mechanisms setting thermal limits, the relative importance of such limits for determining geographic distributions are uncertain (Addo-Bediako, Chown & Gaston 2000; Sunday, Bates & Dulvy 2012). Importantly, critical limits describe but one component of how temperature affects performance and responses to sub-critical temperatures might have greater effects on species' distributions. The effect of temperature on performance is classically depicted as a left-skewed, hump-shaped curve (i.e. thermal performance curve, Huey & Stevenson 1979), with the range between optimal (T_{OPT}) and critical thermal maximum (CT_{MAX}) temperatures referred to as the upper pejus ("getting worse") range (Frederich & Pörtner 2000; Pörtner 2002). The relative fitness consequences of pejus temperatures should be especially important if the outcomes of species interactions are altered (Dunson & Travis 1991; Gilbert *et al.* 2014). For example, asymmetric responses among competing species to temperature can cause species to transition from locally superior to inferior competitors even when temperatures are within critical limits (Gilman *et al.* 2010; Finstad *et al.* 2011; Carmona-Catot, Magellan & Garcia-Berthou 2013; Olsen *et al.* 2016; Liles *et al.* 2017).

Thus, variable responses to pejus temperatures could lead to local extinctions via increased competitor colonization and competitive exclusion (Gilman *et al.* 2010). Knowing both the thermal limits and relative consequences of sub-critical temperature exposure are necessary for understanding how the thermal environment affects species' biogeography.

We examined mechanisms underlying thermal tolerance and the physiological consequences of sub-critical temperature exposure in congeneric, sympatric lizard species that occupy different thermal habitats to illuminate the potential importance of these physiological parameters for biogeography. We examined northern alligator lizards (*Elgaria coerulea*; Wiegmann 1828; NAL) and southern alligator lizards (*E. multicarinata*; Blainville 1835; SAL). Although these species diverged ~6.6 mya (Macey *et al.* 1999), they display similar morphology, generalist diet, and habitat choice (Cunningham 1956; Beck 2009a; Beck 2009b). Both species occur along the Pacific coast of North America, but SAL has a more southern range than NAL (Stebbins 2003, see Fig. S1 in Supporting Information). In regions where the coarse geographic range overlaps, these lizards are rarely syntopic with NAL occurring at higher elevations (Beck 2009a; Beck 2009b, and personal observation). Given their ecological similarity but divergent distributions, thermal niche partitioning is hypothesized to underlie their biogeography. However, these lizards have similar thermal activity ranges (mean active temperature of ~24-25°C with an observed range of 10-35°C for both species; Brattstrom 1965; Cunningham 1966; Stewart 1984; Kingsbury 1994; Sheen 2001; Table 1), and both thermoregulate to ~28°C in the laboratory (Dawson & Templeton 1966; Telemeco & Addis 2014). Moreover, NAL and SAL display remarkably similar CT_{MAX} (estimates range from 38.2-41.4°C with no discernable difference between species, Licht 1964a; Brattstrom 1965; Cunningham 1966; Dawson & Templeton 1966, Table 1). Thus, despite living in different thermal habitats, classic descriptors

of thermal preference, performance, and tolerance fail to distinguish NAL and SAL (summarized in Table 1). Notably, these animals differ in reproductive mode (NAL is viviparous and SAL is oviparous), which may explain NAL occurring in cold habitats unsuitable for SAL (Blackburn 1982; Stewart 1984; Shine 2002; Telemeco 2014). Nonetheless, both SAL and NAL can successfully reproduce in warm environments. Thus adult thermal performance within fundamental limits could underlie SAL occurring in warmer environments than NAL.

We hypothesize that variation in the physiological response to high temperatures, particularly those in the pejus range, contribute to biogeographic differences of NAL and SAL. We first tested the hypothesis that SAL naturally occupies warmer environments than NAL using occurrence data. Next, we exposed lizards to control and near-critical hot treatments and quantified physiological stress (plasma glucose and corticosteroid concentrations), mitochondrial function, and the skeletal muscle metabolome. We examined subcellular performance because whole-organism performance rapidly fails near critical limits making precise quantification difficult (Huey 1982; Angilletta 2009) and coarse whole-organism data are already available for comparison (Table 1). Using these physiological data, we first attempted to elucidate the mechanism responsible for setting fundamental thermal limits in these species and assessed variation in tolerance. If fundamental limits result from protein or membrane denaturation, we predicted that near-critical temperature exposure would damage mitochondria, resulting in lasting functional reduction and increased reactive oxygen species (ROS) production. By contrast, if fundamental limits result from oxygen limitation, we predicted near-critical temperature exposure would increase anaerobic metabolites and decrease aerobic metabolites in the muscle metabolome (Verberk *et al.* 2013; Verberk *et al.* 2016). Additionally, if variation in the fundamental thermal niche underlies NAL and SAL biogeography (i.e. SAL tolerate higher

temperatures than NAL), we predicted increased mortality under prolonged exposure to acutely near-critical temperatures, a loss of mitochondrial function, or a shift to anaerobic metabolism in NAL but not SAL. Finally, we tested the hypothesis that SAL and NAL differ in their physiological response to pejus temperatures. If so, we predicted no mitochondrial damage from exposure to hot temperatures in either species, but 1) greater energy demand in NAL than SAL resulting from elevated mitochondrial metabolic rate as a consequence of metabolic compensation to cold environments, and 2) greater physiological stress or defense in NAL than SAL, as demonstrated by shifts in the metabolome. When considered with prior whole-organism data (Table 1), our subcellular performance measures illuminate the mechanisms responsible for limiting function at high temperatures and suggest subtle differences in physiological performance, rather than fundamental tolerances, contribute to the biogeography of alligator lizards.

Materials and Methods

THERMAL ENVIRONMENTS OF NAL AND SAL

We leveraged the large number of occurrence records available for NAL and SAL to confirm that NAL generally occupy cooler environments than SAL. We downloaded all occurrence records for each species from the VertNet data portal (version 2016-09-29, www.vertnet.org), and cleaned these records by removing obvious inaccuracies and eggs. This left 3996 NAL and 4306 SAL observations (Fig. S1). Next, we downloaded BIOCLIM data at 30 arc-second (~ 1km) resolution from the WorldClim database (www.worldclim.org, Hijmans *et al.* 2005). The BIOCLIM data are composed of 19 biologically-relevant climate variables derived from monthly temperature and precipitation data, although we focused on the seven that describe the thermal

environment (Table 2). For each NAL and SAL occurrence, we extracted the BIOCLIM variables for the corresponding location. All GIS analyses were performed using the *sp* and *raster* packages in R (Bivand, Pebesma & Gomez-Rubio 2013; Hijmans 2015).

ANIMAL COLLECTION AND LABORATORY MAINTENANCE

We collected adult NAL (*N*=19) and SAL (*N*=20) during April–July of 2010–2012 from California and transported them to a captive colony at Iowa State University (Table S1, Fig. S1). Our sampling allowed us to examine responses to high temperature where the species overlap as well as where animals might naturally experience pejus temperatures. NAL were collected near the southern-most portion of their range where they overlap with SAL, and SAL were collected where they overlap NAL and farther south (Fig. S1A). All SAL derive from the "southern" SAL clade, as described by Feldman and Spicer (2006, mitochondrial DNA evidence suggest SAL are divided into "southern" and "northern" clades), and represent a single broadly-defined population. Given how conserved CT_{MAX}, preferred body temperature, and pejus temperatures are for these animals (Table 1), and how little high-temperature tolerance varies among ectotherms in general (Addo-Bediako, Chown & Gaston 2000; Sunday, Bates & Dulvy 2011), we expect minimal local adaptation. Animals were housed in the laboratory following standard husbandry protocols (see Telemeco and Addis 2014 for details). At the onset of our thermal experiments, lizards were in captivity between 7.5 and 29 months (Table S1). Including year of capture in our statistical models did not alter any of our conclusions and thus was not included in final models.

HEAT STRESS EXPERIMENTS

We exposed each lizard to either a control or heat stress treatment (24 or 38°C, respectively) for 2.5 h prior to euthanasia and tissue collection. These temperatures were chosen because 24°C approximates the average activity temperature for both species, and 38°C is well above the pejus threshold but just below CT_{MAX} (Table 1). We assigned lizards of each species uniformly to temperature treatments. To minimize potential external stressors, we kept contact between lizards and researchers to a minimum. We last weighed lizards on 25 September 2012 and provided their last meal on 10 December 2012 before the experiments began on 17 December 2012. Because of the logistical constraints associated with assaying live tissue, we arrayed the experiments over 5 d with lizards randomly assigned to an experiment day (6–10 lizards daily). During experiments, lizards were housed individually in plastic test chambers (15.6 × 15.6 × 5.7 cm) with air holes. Within their test chambers, we moved lizards to a dark incubation chamber set at 24°C the day prior to their experiment to allow 18 h acclimation. Lizards and test chambers were then moved to large incubators pre-set to the treatment temperature and illuminated with LED lights. Lizards were not disturbed during treatment and could not be observed. However, we staggered animals in and out of the thermal treatments at 10-min intervals to allow time for post-treatment processing which resulted in brief periods of disturbance at the beginning or end of treatment. We confirmed all chamber temperatures using iButton thermal data loggers (Model DS1921-F5, factory accuracy ± 1°C, Maxim Integrated, San Jose, CA, USA). After 2.5 h in their treatment, lizards were immediately euthanized by decapitation, pithed, and exsanguinated (death within seconds of removal). We first collected whole blood and placed it on ice, then collected liver tissue and placed it in STE buffer solution (250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.4) on ice, and finally collected muscle tissue

from the right-rear leg and snap-froze it in liquid nitrogen for storage at -80°C. For all animals, <10 min passed between the end of treatment and collection of all tissues.

PLASMA PHYSIOLOGY

To assess physiological stress at the time of euthanasia, we quantified plasma circulating glucose and corticosterone (CORT; the primary glucocorticoid in reptiles). Immediately following euthanasia and collection of whole blood, we centrifuged the samples (7000 rpm for 10 min), and aliquoted plasma for storage at -80°C. We measured glucose from 1.5 µL of the thawed plasma using a FreeStyle Lite Glucometer (Abbott Diabetes Care, Alameda, CA, USA). CORT was assayed as in Telemeco and Addis (2014) using the Immun-Chem Double Antibody Corticosterone I-125 RIA kit (Cat #07-120103, MP Biomedical, Orangeburg, NY, USA). We also examined CORT from 11 additional SAL, housed under identical conditions and exposed to 24°C. These animals were excluded from other experiments (mitochondria, metabolomics) because of time constraints. Their inclusion did not qualitatively alter our CORT results.

MITOCHONDRIAL PHYSIOLOGY

We examined respiration rates and reactive oxygen species (ROS) production of fresh mitochondria isolated from liver tissue. We chose liver mitochondria because of the high metabolic activity and large size of livers, which allowed isolation of sufficient mitochondria from individual lizards. All mitochondrial assays were completed within 8 h of death. To isolate fresh mitochondria, we homogenized the liver tissue (Robert, Brunet-Rossinni & Bronikowski 2007) and used differential centrifugation to separate mitochondria from the cellular debris (Palloti & Lenaz 2001; Pon & Schon 2007). We estimated the concentration of mitochondria

within our isolates via Bradford protein determination and used this estimate to standardize our assays (Robert, Brunet-Rossinni & Bronikowski 2007).

We estimated mitochondrial respiration rates by measuring oxygen consumption within an airtight chamber using a Clark-type oxygen electrode (Hansatech, Norfolk, UK) according to established protocols (Brand, Harper & Taylor 1993; Trounce *et al.* 1996; Herrero & Barja 1997) optimized for reptiles (Robert, Brunet-Rossinni & Bronikowski 2007). Using these data, we calculated State III respiration (oxidative phosphorylation), State IV respiration (proton leak), and respiratory control ratio (RCR) for each individual using the equations of Robert and Bronikowski (2010). We included analyses of RCR to ensure functional mitochondria in all groups. All mitochondrial respiration rates were assayed at 24°C, regardless of whole-animal thermal treatment. Thus, any effects of thermal treatment on mitochondrial respiration indicate plasticity (e.g. acclimation) or damage rather than the direct effect of temperature on metabolism. As an indicator of ROS production by the mitochondria, we measured mitochondrial H_2O_2 production using the Amplex® red hydrogen peroxide/peroxidase assay kit (Invitrogen Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions for a 96-well microplate as optimized for reptiles (Schwartz & Bronikowski 2013). For analyses, we calculated the rate of mitochondrial H_2O_2 production (μmol H_2O_2 min^{-1} mg^{-1}) as the difference between the average 30- and 5-min measurements for each individual, divided by 25 minutes. For all mitochondrial assays, we assayed samples from each individual in duplicate, and averaged measurements for analyses. See Appendix S1 for further details on the mitochondrial assays.

MUSCLE METABOLOMICS

We examined the muscle metabolome to test for a global shift in metabolic physiology of NAL versus SAL when exposed to near-critical temperatures and to test the OCLTT hypothesis which predicts an increase in anaerobic metabolism at high temperatures. Metabolite extraction and quantification took place at the W. M. Keck Metabolomics Research Laboratory at Iowa State University (http://www.metabolomics.biotech.iastate.edu). We prepared skeletal muscle from the right-rear limb using two-step methane/water/chloroform extraction (A *et al.* 2005; Wu *et al.* 2008). Skeletal muscle has high energetic needs and thus may be more rapidly affected by temperature-induced oxygen limitation than other tissues. Polar and non-polar extracts from each sample were analyzed using an Agilent 6890 Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent Technologies, Santa Clara, CA, USA). Peak areas were transformed to concentration values using internal standards and tissue dry mass using ChemStation software (v. 2.0; Agilent Technologies). Additional details on metabolite extraction and quantification are provided in Appendix S1.

We first filtered the raw GC/MS data to exclude putative metabolites that were not represented in ≥ 90% of the samples from at least one species by treatment grouping. We identified remaining metabolites using AMDIS software (version 2.71; NIST) to compare peaks to the National Institutes of Standards and Technology database and a proprietary reference library maintained by the W.M. Keck Metabolomics Research Laboratory. We then consolidated the data for metabolites present in both the polar and non-polar fractions, which yielded 190 metabolites for analysis. Finally, we assigned metabolites to one or more functional categories for analyses (amino acids, anaerobic metabolites, antioxidants, endocrine metabolites, fatty acids, hydrocarbons, organic acids, steroids, sugars, sugar alcohols, TCA metabolites, and

vitamins) using online databases: Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/KEGG) and PubChem (https://pubchem.ncbi.nlm.nih.gov).

STATISTICAL ANALYSES

We performed statistical analyses and data visualization using R statistical software (version 3.1.0, R Core Team 2016). Prior to analyses, we graphically assessed each univariate dataset for normality and outliers using boxplots, histograms, and q-q plots (Zuur *et al.* 2009). We natural-log transformed the CORT, State III, and RCR data, and square-root transformed the H_2O_2 data. We identified no outliers in the plasma glucose or CORT datasets, but identified 2–5 outliers from each mitochondrial dataset, and these points were removed. For all ANOVAs, we began with a full model and used backwards selection to reduce the model using AICc and p-values (Zuur *et al.* 2009). We calculated AICc using the "aictab" function in the *AICcmodavg* package (Mazerolle 2015). We assessed significance using type-III sum of squares with the "Anova" function from the *car* package (Fox & Weisberg 2011). To explore statistically significant interactions, we employed Tukey-corrected pairwise tests using the "lsmeans" function in the *lsmeans* package (Lenth 2013).

We analyzed metabolome data using Euclidean distance-based, nonparametric multivariate analyses of variance (NP-MANOVA; also known as permutational MANOVA; see Anderson 2001 for details) via the "adonis" function in the *vegan* package (statistical signficance assessed from 1000 permutations, Oksanen *et al.* 2013). We used NP-MANOVA because, unlike traditional MANOVA, this method allows analysis when variables outnumber samples (i.e. dependent-variable matrix has more columns than rows, Anderson 2001). Prior to metabolite analyses, we used scatterplot matrices to graphically examine the multivariate

distributions and natural-log-transformed functional groupings as necessary to bring them qualitatively close to multivariate normal. Significant effects revealed by NP-MANOVA might result from either variation in the quantity or relative composition of metabolites. To distinguish between these two possibilities, any time NP-MANOVA suggested statistically significant effects, we also summed the concentrations of all metabolites tested and reanalyzed the summed data using ANOVA.

We report actual (i.e. uncorrected) p-values because each analysis is effectively the result of an independent experiment (Perneger 1998; Cabin & Mitchell 2000; Moran 2003). Multiple comparison tests, such as sequential Bonferroni, control study-wide type I error at the expense of type II error; however, both forms of error are equally undesirable for our study. Therefore, rather than employing study-wide, multiple-comparison corrections, we conceptually combat statistical error by basing conclusions on the bulk of our analyses rather than on individual tests. General agreement across independent statistical tests is extremely unlikely to occur by chance alone (Moran 2003).

Results

THERMAL ENVIRONMENTS OF NAL AND SAL

As predicted, NAL occupy cooler environments than SAL: annual mean temperatures for NAL are 4.4°C cooler than for SAL, on average (Welch two-sample t-test, Table 2). Each thermal BIOCLIM variable suggests a similar ~4°C difference (Table 2). Additionally, NAL occupy slightly more seasonal environments than SAL (annual thermal s.d. 0.3 °C greater for NAL, Table 2).

PLASMA PHYSIOLOGY

All animals survived thermal treatment. Behavioral responses varied, but lizards exposed to 38°C generally appeared lethargic compared to animals exposed to 24°C at the time of euthanasia. Temperature treatment and species interacted to affect plasma glucose levels (ANOVA, $F_{1,34} = 13.83$, $P = 0.0007$, Fig. 1A, Table S2). Plasma glucose did not differ between the species at 24°C (Tukey-corrected comparison, $t_{34} = 1.11$, $P = 0.2755$), but SAL had higher plasma glucose than NAL at 38°C (Tukey-corrected comparison, $t_{34} = -4.15$, $P = 0.0002$, Fig. 1A). Plasma glucose was higher in lizards exposed to 38°C than 24°C in both species (Tukey-corrected comparison, NAL: $t_{34} = -4.614$, $P = 0.0001$; SAL: $t_{34} = -10.267$, $P < 0.0001$, Fig. 1A). CORT tended to be higher in NAL than in SAL (ANOVA, $F_{1,49} = 3.70$, $P = 0.0601$), while temperature treatment (ANOVA, $F_{1,49} = 0.08$, $P = 0.7805$) and its interaction with species (ANOVA, $F_{1,49} = 1.32$, $P = 0.2559$, Table S2, Fig. 1B) were unimportant.

MITOCHONDRIAL PHYSIOLOGY

Neither treatment (ANOVA, $F_{1,34} = 0.10$, $P = 0.7509$) nor species (ANOVA, $F_{1,34} = 0.56$, $P = 0.4583$) affected the RCR, and there was no interaction (ANOVA, $F_{1,33} = 0.18$, $P = 0.6744$, Table S2). The mean (± s.e.m.) RCR across all samples was 2.6 ± 0.1, similar to estimates previously reported for reptiles, indicating functional mitochondria (Cassuto 1971; Robert, Brunet-Rossinni & Bronikowski 2007; Robert & Bronikowski 2010).

State III respiration (oxidative phosphorylation, ADP present) was higher in NAL than in SAL (ANOVA, $F_{1,34} = 16.04$, $P = 0.0003$, Fig. 2A) and was unaffected by temperature treatment (ANOVA, $F_{1,34} = 1.47$, $P = 0.2329$) or its interaction with species (ANOVA, $F_{1,33} = 0.34$, $P = 0.5612$, Table S2). Conversely, mitochondrial H_2O_2 production rate was higher in SAL than in

NAL (ANOVA, $F_{1,35} = 5.65$, $P = 0.0231$), while neither temperature treatment (ANOVA, $F_{1,35} = 1.46$, $P = 0.2347$) nor the interaction between temperature treatment and species (ANOVA, $F_{1,35} = 1.86$, $P = 0.1815$) was important (Fig. 2B, Table S2).

MUSCLE METABOLOMICS

We calculated mean (± s.e.m.) concentration within each treatment by species level for the 190 metabolites that met our criteria for analysis, assigned those metabolites to functional groups, and analyzed each functional group (Tables S3–S4). Temperature treatment and species interacted (NP-MANOVA, $F_{1,33} = 2.49$, $P = 0.013$) to affect metabolite profiles when all metabolites were analyzed together (Fig. 3, Table S3). This interaction was the result of temperature treatment affecting the metabolite profile of NAL (NP-MANOVA, $F_{1,16} = 2.92$, $P = 0.022$), but not SAL (NP-MANOVA, $F_{1,19} = 0.854$, $P = 0.548$). This general pattern of temperature affecting NAL metabolites but not SAL metabolites was evident for numerous functional groups. Of the 12 functional metabolite groupings that we examined, four were affected by temperature treatment in NAL (amino acids, sugars, endocrine metabolites, and organic acids), and an additional five (antioxidants, fatty acids, vitamins, sugar alcohols, and steroids) trended similarly ($0.05 < P < 0.10$, Table S3). By contrast, temperature treatment never affected metabolite profiles in SAL (all $P > 0.15$; Table S3). In all but two cases (endocrine metabolites and sugar alcohols), statistically significant effects resulted from metabolite composition rather than metabolite quantity (Table S4).

In both species, heat stress did not affect the overall profile of anaerobic-indicator metabolites (lactic acid, succinate, and alanine (Verberk *et al.* 2013), NP-MANOVA: Treatment: $F_{1,33} = 0.28$, $P = 0.794$; Species: $F_{1,33} = 1.50$, $P = 0.222$; Interaction: $F_{1,33} = 2.02$, $P = 0.132$, Fig. 4A) or Krebs cycle metabolites (phosphoric acid, fumeric acid, succinic acid, and oxalacetic acid

(Verberk *et al.* 2013), NP-MANOVA: Treatment: $F_{1,33} = 0.12$, $P = 0.791$; Species: $F_{1,33} = 0.81$, $P = 0.405$; Interaction: $F_{1,33} = 1.19$, $P = 0.300$, Fig. 4B). To confirm that high temperature failed to elicit a shift to anaerobic metabolism, we examined lactic acid and succinic acid in isolation, similar to more classic approaches (Bennett & Licht 1972). Neither treatment (ANOVA; lactic acid: $F_{1,31} = 1.16$, $P = 0.303$; succinic acid: $F_{1,33} = 0.23$, $P = 0.634$), species (ANOVA; lactic acid: $F_{1,34} = 0.11$, $P = 0.732$; succinic acid: $F_{1,33} = 0.33$, $P = 0.570$), nor their interaction (ANOVA; lactic acid: $F_{1,31} = 0.42$, $P = 0.521$; succinic acid: $F_{1,33} = 0.04$, $P = 0.849$) affected either of these primary metabolites in skeletal muscle (Figs. 4C and 4D).

Discussion

We used an integrative approach to assess relative physiological performance at high temperatures of congeneric lizards that naturally partition the thermal environment. We predicted that, because SAL occupy warmer habitats, SAL would display increased temperature tolerance (i.e. higher-temperature fundamental niche), or greater physiological performance at near-critical temperatures relative to NAL (i.e. potential for temperature to alter the outcome of biotic interactions and thus the realized niche). Our results confirm prior observations that the absolute heat tolerances of SAL and NAL are similar despite a ~4°C difference in thermal environment. Even so, SAL appears more robust to high temperatures: SAL maintained function through prolonged exposure to near-critical temperatures without making the physiological changes apparently required for NAL to maintain function. The mitochondrial metabolic rate of NAL was also higher than SAL suggesting that high temperatures incur a greater energetic cost in NAL. Such differences in robustness and energetics could affect the outcome of competitive interactions (either direct or indirect) between these ecologically similar species.

Our results demonstrate that focusing on thermal limits may obscure physiological adaptations underlying biogeographic patterns. Performance will be compromised by high-temperature exposure below critical thermal limits. Although such reductions in performance will not cause dramatic and rapid die-offs, they could lower the intrinsic rate of increase below one or cause a species to become an inferior competitor: either of which would cause local extinction (Roff 1992; Pörtner & Knust 2007; Finstad *et al.* 2011; Carmona-Catot, Magellan & Garcia-Berthou 2013). Thus, any traits affecting the rate of performance reduction at upper pejus temperatures should be under strong selection as animals are exposed to thermal stress near their low-latitude and low-elevation range boundaries, or as a consequence of global climate change. Identifying traits that underlie responses to pejus temperatures, such as mitochondrial respiration rate and metabolome homeostasis in alligator lizards, is necessary to predict high-temperature effects on ecological interactions, evolution by natural selection, and population persistence in response to warming environments.

Our observation that lizards of both species in the 38°C treatment displayed elevated plasma glucose concentrations indicate that they were in the midst of a stress response at the time of euthanasia (Broom & Johnson 1993; Bradshaw 2003). Additionally, we qualitatively observed behavioral variation among the lizards consistent with thermal stress. Immediately following perception of a stressor, vertebrate animals elevate production of catecholamine and glucocorticoid hormones, which act to elevate plasma glucose for use in emergency action (Stevenson, Coulson & Hernandez 1957; Norris 2007). Catecholamines are fast acting: elevating within 1–2 s in birds and mammals and within minutes in reptiles (Akbar, Afroz & Ali 1978; McCarty 1983; Matt *et al.* 1997; Palme *et al.* 2005). Somewhat surprisingly, we did not observe elevated plasma CORT in lizards exposed to 38°C, despite observing elevated glucose

concentrations. This result may indicate that CORT had not yet elevated and plasma glucose was only responding to catecholamine production, or that CORT had already elevated and returned to baseline despite continued heat exposure. Glucocorticoid hormones require longer to elevate than do catecholamines (Akbar, Afroz & Ali 1978; Palme *et al.* 2005; Norris 2007), potentially requiring hours to reach peak levels in reptiles (Palacios, Sparkman & Bronikowski 2012; Anderson *et al.* 2014; Gangloff *et al.* 2017). In alligator lizards, elevated CORT is detectable after 5-hr exposure to 28°C, but not 35°C (Telemeco and Addis 2014, same individuals as the present study in some cases). These data confirm that CORT responds to temperature in alligator lizards, but suggest temperature may affect the rate of the CORT response. Even so, our results indicate that the lizards exposed to 38°C were acutely stressed at the time of euthanasia, with catecholamines and possibly CORT having acted to elevate glucose.

We were unable to detect signs of either subcellular damage or reduced aerobic respiration in animals of either species exposed to the 38°C treatment, suggesting that critical limits are not set by denaturation or by oxygen and capacity-limited thermal tolerance. Additionally, surviving exposure to 38°C for 2.5 h without discernable damage corroborates prior work suggesting that the high-temperature critical limits of NAL and SAL are similar (Licht 1964a; Brattstrom 1965; Dawson & Templeton 1966). If protein or membrane denaturation were responsible for critical limits in alligator lizards, we expect the mitochondria to be compromised. However, our measurements of State III respiration (oxidative phosphorylation), RCR, and H_2O_2 production suggest no lasting damage to proteins or mitochondrial membranes. On the other hand, if critical thermal limits resulted from a limited capacity to deliver oxygen to tissues at high temperatures (OCLTT hypothesis), we would expect animals to increase anaerobic respiration to accommodate reduced aerobic capacity (Pörtner &

Knust 2007; Verberk *et al.* 2013; Fobian, Overgaard & Wang 2014; Verberk *et al.* 2016). For example, in response to limited oxygen during exercise, lizards rapidly increase anaerobic respiration, which is detectable by examining metabolites such as lactic acid (Bennett & Licht 1972). We did not detect an effect of temperature treatment on the anaerobic metabolites of either species, indicating that lizards were not oxygen limited at near-critical temperatures. This observation adds to growing evidence that the OCLTT mechanism is not important for setting critical thermal limits in adult reptiles (Fobian, Overgaard & Wang 2014; Gangloff *et al.* 2016; DuBois *et al.* 2017). Given our observations, the most parsimonious explanation for the mechanism setting critical limits is that protein or membrane functions underlying key cellular processes are reversibly disrupted at high temperatures (Hochachka & Somero 2002). Further work is needed to directly test this hypothesis.

Even though exposure to near-critical temperatures did not cause mitochondrial damage or reduce aerobic respiration, the NAL metabolome shifted in response to heat challenge whereas the SAL metabolome did not. Such physiological changes likely represent compensatory mechanisms that allowed NAL to avoid damage and survive high-temperature exposure. This effect was apparent in the entire skeletal muscle metabolome, as well as for energetically important sub-groupings, such as sugars and amino acids. Similarly, the metabolome of *Drosophila melanogaster* flies artificially selected for rapid recovery from cold-induced coma are relatively unaffected by cold exposure whereas control lines display marked metabolomic responses to cold (Williams *et al.* 2014). A robust metabolome might generally underlie improved thermal performance within the pejus ranges of animals, possibly indicating greater capacity to maintain homeostasis. However, further work is needed to assess the generality of

such metabolomic robustness for allowing animals to maintain high performance at extreme temperatures.

SAL also had higher rates of ROS production by the mitochondria than did NAL. Thus, SAL should incur greater cellular damage as a consequence of respiration at a given temperature than NAL (Finkel & Holbrook 2000; Schwartz & Bronikowski 2013), even though NAL have a higher State III respiration rate. Given that NAL and SAL are active across similar body temperatures in nature (Brattstrom 1965; Cunningham 1966; Stewart 1984; Kingsbury 1994), we predict that reduced ROS production by NAL will allow them to senesce more slowly and have longer lifespans than SAL, as observed in other taxa (Finkel & Holbrook 2000; Dowling & Simmons 2009; Schwartz & Bronikowski 2013). Unfortunately, data on the longevity of these lizards are not available.

NAL and SAL differentially responded to stressful high temperatures consistent with their biogeography, even though their critical limits and optima are indistinguishable (Brattstrom 1965; Cunningham 1966; Stewart 1984; Kingsbury 1994; Sheen 2001). These physiological differences may contribute to NAL and SAL biogeography by influencing the outcome of competitive interactions. Even so, other aspects of the biology of these species will also affect realized distributions. For example, oviparous reproduction allows SAL to produce 2–4 times more offspring than NAL are able to produce via viviparity (Goldberg 1972; Vitt 1973; Stewart 1979). This life-history difference could allow SAL to rapidly outnumber and potentially outcompete NAL in warm environments well within the NAL fundamental thermal niche (Pincheira-Donoso *et al.* 2013). The differences in thermal physiology at upper pejus temperatures that we observed between NAL and SAL should exacerbate this difference in

reproductive rate. Thus, differences in the thermal physiology of adults may facilitate biogeographic differences more proximally driven by life history and competition.

Our results highlight the importance of considering all aspects of an organism's biology when attempting to understand the mechanisms underlying biogeographic differences, even when distributions appear straightforwardly governed by an abiotic factor such as temperature. Although the specific physiological patterns and mechanisms that we observed in NAL and SAL will not be universal to other systems, they illustrate how a simple exploration of thermal tolerance can fail to capture physiological adaptation. Moreover, NAL and SAL illustrate how effects of the abiotic environment on organismal function that appear minor in isolation (e.g. high mitochondrial metabolic demand and shifts in the metabolite profile of NAL at high temperature) might have broad consequences when ecological interactions are considered, potentially altering competitive landscapes. As abiotic conditions such as temperature shift as a result of climate change, the impact of novel conditions on performance relative to interacting species and within critical limits might frequently drive natural selection and determine the ultimate viability of populations.

Data Accessibility

All data, including plasma and mitochondrial physiology, and muscle metabolome data, are available in the Dryad digital repository, http://dx.doi.org/10.5061/dryad.b5n38 (Telemeco et al. 2017).

Author Contributions

R.S.T., A.M.B., and F.J.J. conceived and designed the experiment. The thermal challenge treatment and mitochondrial physiology assays were performed by R.S.T., A.M.B., R.L.P., and G.A.C. Metabolomics data collection and processing was performed by R.S.T. and E.J.G. R.S.T. performed statistical analyses and wrote the initial draft. All authors contributed to interpreting the results and to subsequent drafts.

Acknowledgements

We thank M. Westphal and the U.S. Bureau of Land Management for access to Ft. Ord National Monument and the San Lorenzo Valley Water District for access to Zayante Quarry. For assistance collecting lizards, we thank numerous volunteers including T. Breitman, S. Deering, L. Erickson, C. Feldman, J. Homen, J. Lucas, T. Marino, K. Mondragon, P. Moravcsik, J. Richmond, R. Seymore, M. Telemeco, M. Westphal, K. Wiseman, and S. Young. For assistance with the metabolomics analyses, we thank A. Perera and Z. Song of the W.M. Keck Metabolomics Research Laboratory. We also thank B. Nikolau for allowing us access to his laboratory for extracting metabolites. For assistance in the laboratory, we thank M. Barazowski, B. Bodensteiner, A. Brouillette, E. Hernandez, K. Holden, J. Reneker, M. Telemeco, and D. Warner. The research was conducted under approved animal care protocols (IACUC #4106893J and #4106894J) and a California Department of Fish and Game permit (SC-11085). The research was supported by grants from the Chicago Herpetological Society, the Ecology, Evolution, and Organismal Biology Department at Iowa State University (ISU), and Sigma Xi to R.S.T, and National Science Foundation grants DEB-0640932 and DEB-1242510 to F.J.J. Further support was received from an U.S. Environmental Protection Agency Science to Achieve

Results Fellowship (FP-91723101) and a National Science Foundation GK-12 Fellowship to R.S.T. Additionally, R.S.T. was partially supported by a National Science Foundation grant to L.B. Buckley (EF-1065638) and E.J.G. was partially supported by a fellowship from the Office of Biotechnology at ISU.

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Figure legends

Fig. 1. Exposure to near-critical high temperature for 2.5 h elevated plasma glucose (A, $P < 0.01$) but not plasma corticosterone (B, $P = 0.78$) in both northern (NAL; *Elgaria coerulea*) and southern (SAL; *E. multicarinata*) alligator lizards. Data are back-transformed least-squares means ± s.e.m.

Fig. 2. Although species differed, temperature treatment (2.5 h exposure to control or near-critical maximum) had no lasting effect on mitochondrial physiology in alligator lizards. Panel (A) State III respiration (oxidative phosphorylation, ADP present) was higher in northern (NAL; *Elgaria coerulea*) than in southern (SAL; *E. multicarinata*) alligator lizards ($P < 0.01$), but unaffected by temperature treatment ($P = 0.23$); (B) H_2O_2 production was higher in SAL than NAL ($P < 0.01$), but unaffected by temperature treatment ($P = 0.74$). Data presented are back-transformed least-squares means ± s.e.m. Measurements were made within 8 h of treatment.

Fig. 3. Exposure to near-critical high temperature induced a change in the metabolome of northern (NAL; *Elgaria coerulea*, $P = 0.02$) but not southern (SAL; *E. multicarinata*, $P = 0.55$) alligator lizards. Plots are principal components plots of metabolites ($N = 190$) from the muscle of lizards exposed to 24°C (control) and 38°C. Points represent the mean for each group ± 95% c.i. In panel B, the mean for 24°C NAL is hidden behind the SAL points.

Fig. 4. Exposure to near-critical maximum temperature (38 °C) for 2.5 h did not alter the profile of anaerobic ($N = 3$) or Krebs cycle (TCA) metabolites ($N = 4$) in the muscle of either northern (NAL; *Elgaria coerulea*) or southern (SAL; *E. multicarinata*) alligator lizards when compared to control lizards (24 °C, $P > 0.1$ for all). Panels A and B are principal components plots for the anaerobic and TCA metabolites, respectively. Points represent mean values ± 95% c.i. for each group. Panels C and D display the effects of temperature treatment on two key primary metabolites: lactic acid and succinic acid. Data are back-transformed least-squares means ± 95% c.i.

Table 1. Literature summary of alligator lizard (*Elgaria multicarinata* [SAL] and *E. coerulea* [NAL]) thermal tolerance. Data are presented as "mean [range]", with superscripts referencing methodology, and sample size (N) below. When available, subspecies and collection location are also provided. Although historically-recognized subspecies are no longer accepted for SAL and questionable for NAL, they provide general location information. Hyphens denote unavailable data.

Species/Subspecies	Location	Active T_b (°C)	T_{Pejus} (°C)	CT_{MAX} (°C)	T_{Lethal} (°C)	Citation
***Elgaria multicarinata* [SAL]**						
E.m.ignavus	—	22.4 [16.4-22.4][A] N = 5	—	—	—	Zweifel 1958
E.m.multicarinatus	Riverside Co, CA	—	33[C]	39.5 [—][G]	—	Licht 1964b
E.m.multicarinatus	—	—	32[D]	39 [—][H] N = 6	—	Licht 1964a
E.m.webbi	—	21.2 [11.0-33.2][A] N > 15	—	40.3 [40.0-40.5][I] N > 15	—	Brattstrom 1965
E.m.webbi	Los Angeles Co, CA San Bernadino Co, CA Ventura Co, CA	21.1 [4.9-35.7][A] N = 102	—	41.4 [—][I] N = 5	43.6-43.8, N = 2	Cunningham 1966
E.m.webbi	Los Angeles Co, CA	—	33[E]	39.5 [39-40][J] N = 12	—	Dawson and Templeton 1966
—	San Diego Co, CA	23.8 [9.5-33.8][B] N = 16	33[F]	—	—	Kingsbury 1994

***Elgaria coerulea* [NAL]**						
E.c.principis	—	15.8 [11.0-19.0][A] N = 5	—	38.2 [—] N = 1	—	Brattstrom 1965
E.c.principis	Whatcom Co, WA	24.9 [20-30][A] N = 27	—	—	—	Vitt 1973
E.c.shastensis	—	19.8[—] [A] N = 1	—	40.1 [38.5-41.2][I] N = 3	—	Brattstrom 1965
—	Monterrey Co, CA	25.2 [13.6-34][A] N = 73	—	—	—	Stewart 1984
—	Manchester Co, CA Mendocino Co, CA	24.4 [11.8-31.2][A] N = 91	—	—	—	Stewart 1984
—	San Mateo, Co, CA	25.9 [16-31] N = 86	—	—	—	Levin 1967
—	King Co, CA Klickitat Co, CA Whatcom Co, CA	25.5 [20-31] N = 35	—	—	—	Vitt 1974

[A] body temperature at capture
[B] radio transmitting data loggers
[C] maximal ATPase activity
[D] reduction in muscle contraction tension
[E] breathing rate and evaporative water loss
[F] behavioral avoidance
[G] ATPase 20% denatured
[H] muscle irreversibly damaged
[I] loss of righting ability
[J] oxygen consumption and heart rate

Table 2. Summary of the thermal environment occupied by *Elgaria coerulea* (NAL) and *E. multicarinata* (SAL). For each BIOCLIM variable examined, mean ± s.d. for each species, and *t*, d.f., and *P* are presented.

BIOCLIM variable (Description)	NAL	SAL	*t*	d.f.	*P*
BIO1 (Annual mean temp, °C)	10.6 ± 2.5	15.0 ± 2.5	75.7	7501.8	< 0.0001
BIO4 (Temp seasonality [s.d.], °C)	4.5 ± 1.7	4.2 ± 1.5	-8.7	7287.4	< 0.0001
BIO5 (Maximum temp of warmest month, °C)	24.7 ± 3.7	28.4 ± 4.2	41.4	7682.7	< 0.0001
BIO6 (Minimum temp of coldest month, °C)	0.1 ± 4.2	3.8 ± 3.5	41.8	6950.9	< 0.0001
BIO7 (Annual temp range, °C)	24.6 ± 6.7	24.6 ± 6.2	-0.3	7386.4	0.8912
BIO10 (Mean temp of warmest quarter, °C)	16.6 ± 2.2	20.6 ± 2.8	69.7	7568.1	< 0.0001
BIO11 (Mean temp of coldest quarter, °C)	5.3 ± 3.8	9.9 ± 3.4	56.2	7176.0	< 0.0001

Supporting Information

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

Appendix S1. Supplemental methods

Table S1. Descriptive data for *Elgaria coerulea* and *E. multicarinata* specimens used in experiments.

Table S2. Results from ANOVAs examining effects of species and thermal treatment on plasma and mitochondrial measures of physiology.

Table S3. Results from NP-MANOVAs examining effects of species and thermal treatment on each metabolite grouping.

Table S4. Results from ANOVAs examining the total concentration of each metabolite grouping found to be significantly affected by treatment or species by NP-MANOVA.

Fig S1. Occurrence points for *Elgaria coerulea* and *E. multicarinata* and capture locations of lizards used for experiments.