Title: Hormonal and metabolic responses to upper temperature extremes in divergent life-history ecotypes of a garter snake

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Author Contributions

AMB, EJG, KGH, and RST conducted the experiment; EJG and KGH performed lab work; EJG performed data analysis and drafted manuscript; all authors contributed to experimental design, interpretation of results, and manuscript revisions.

Competing interests

No competing interests declared.

Data availability

All data are available in the Dryad digital repository: doi:10.5061/dryad.cs5th (provisional)

Abstract

Extreme temperatures constrain organismal physiology and impose both acute and chronic effects. Additionally, temperature-induced hormone-mediated stress response pathways and energetic trade-offs are important drivers of life-history variation. This study employs an integrative approach to quantify acute physiological responses to high temperatures in divergent life-history ecotypes of the western terrestrial garter snake (*Thamnophis elegans*). Using wild-caught animals, we measured oxygen consumption rate and physiological markers of hormonal stress response, energy availability, and anaerobic respiration in blood plasma across five ecologically relevant temperatures (24, 28, 32, 35, and 38º C; 3-hour exposure). Corticosterone, insulin, and glucose concentrations all increased with temperature,

but with different thermal response curves, suggesting that high temperatures differently affect energy-regulation pathways. Additionally, oxygen consumption rate increased without plateau and lactate concentration did not increase with temperature, challenging the recent hypothesis that oxygen limitation sets upper thermal tolerance limits. Finally, animals had similar physiological thermal responses to high-temperature exposure regardless of genetic background, suggesting that local adaptation has not resulted in fixed differences between ecotypes. Together, these results identify some of the mechanisms by which higher temperatures alter hormonal-mediated energy balance in reptiles and potential limits to the flexibility of this response.

Introduction

Temperature constrains organismal performance in a wide array of traits (Angilletta, 2009; Huey, 1982; Huey et al., 2012). Although ectotherms can tolerate a broad range of body temperatures, many species might soon experience temperatures above those optimal for performance, including growth and reproduction, but below lethal limits as a consequence of ongoing climate change (Diamond et al., 2012; Visser, 2008). Temperatures outside of an organism's optimal range challenge homeostasis and result in reduced performance and the suspension of normal activity (Huey and Stevenson, 1979). These effects in turn reduce individual fitness and often impact population growth and persistence (Huey et al., 2012; Lemoine and Burkepile, 2012; Pörtner, 2002; Pörtner and Knust, 2007). Because physiology largely mediates organismal responses to stressful environments, understanding the physiological mechanisms responsible for declining fitness at environmental temperatures above thermal optima is critical to quantifying organismal responses to environmental change (Angilletta, 2009). Additionally, characterizing the processes that set thermal tolerance limits in ectotherms, both in terms of acute and long-term maintenance of energy balance, is essential for developing predictions of how organisms will respond to projected future environments (Williams et al., 2016). Our goals in the present study are to quantify the metabolic and hormonal responses to high temperatures, to test for local adaptation in the thermal stress response, and to test the hypothesis that oxygen limitation is a proximate mechanism setting thermal tolerance limits.

Central to all three of these goals are measures of organismal energetics, such as metabolic rate, which are highly temperature dependent and central to recent models of organismal response to climate change (Bozinovic and Pörtner, 2015; Dillon et al., 2010; Gillooly et al.,

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2001; Glazier, 2015; Pörtner and Farrell, 2008). Even so, vertebrate ectotherms such as squamate reptiles (i.e., snakes and lizards) exhibit intraspecific variation in metabolic rate beyond temperature-dependence, suggesting that additional physiological mechanisms can modulate metabolic rates. For example, metabolic rate can be temperature-insensitive within a range (Aleksiuk, 1971; Davies et al., 1981; Seidel and Lindeborg, 1973), be dependent on ecological role (e.g., foraging strategy; Andrews and Pough, 1985; Angilletta, 2001; Beaupre, 1993; Sears, 2005; Taylor and Davies, 1981; Zaidan, 2003; Zari, 2013), or exhibit temperature-dependent scaling with body size (Buikema and Armitage, 1969; Davies and Bennett, 1981; Dmi'el, 1972; Gangloff et al., 2015; Vinegar et al., 1970). Both the standard effect of temperature to increase metabolic rate and the capacity of organisms to adjust that relationship, for example via the effects of glucocorticoids, will determine the energetic cost of high temperature exposure.

Characterizing organismal responses to increased temperatures requires examining the endocrine system, which likely mediates metabolic plasticity in squamates. For example, glucocorticoids, such as corticosterone (CORT) in reptiles, can increase metabolic rate independent of temperature (Bradshaw, 2003; Durant et al., 2008; Preest and Cree, 2008). In general, we expect ambient temperatures that approach critical limits will induce an acute endocrine-driven stress response as an individual attempts to both survive and avoid permanent damage (McEwen and Wingfield, 2010; Romero et al., 2009). Glucocorticoids are major mediators of energy balance during the stress response in vertebrates, serving to both mobilize energy stores and to maintain longer-term energetic homeostasis (Johnstone et al., 2012; Wingfield and Kitaysky, 2002). Even so, relatively few studies have examined the temperature dependence of glucocorticoids in reptiles (but see Dupoué et al., 2013; Sykes and Klukowski, 2009; Telemeco and Addis, 2014). Moreover, CORT and insulin generally

antagonistically regulate circulating glucose concentrations (Dallman et al., 1995; Kaplan, 1996; Strack et al., 1995). In birds, typical CORT and insulin functions are maintained throughout a capture-restraint stress response (Remage-Healey and Romero, 2001). However, heat stress seems to de-couple this counter-regulatory relationship between glucocorticoids and insulin in many mammals. Despite reduced nutrient intake, the glucocorticoid response is accompanied by increased insulin levels, which may aid in the activation and upregulation of heat shock proteins (reviewed in Baumgard and Rhoads, 2013; Li et al., 2006). Few studies have measured insulin in reptiles and none have categorized the insulin response to temperature extremes. By measuring both CORT and insulin concentrations, and their subsequent effects on glucose concentration, we can quantify the impact of heat stress on a specific aspect of energy regulation.

Our third goal was to assess the role of oxygen limitation in setting thermal limits. The recent oxygen and capacity-limited thermal tolerance (OCLTT) hypothesis posits that ectotherms suffer from a mismatch between oxygen demand and supply at high temperatures, leading to reduced energetic capacity and ultimately physiological collapse (Pörtner, 2002). A number of physiological indicators identify this limit, including reduction of whole-organism metabolic rate and an increase in circulating anaerobic metabolites, such as lactate (Frederich and Pörtner, 2000; Pörtner, 2002). Thus, by simultaneously measuring oxygen consumption rate and markers of anaerobic respiration across increasing temperatures, we may elucidate the proximate cause of organismal failure (i.e., death) at critically high temperatures.

The divergent life-history ecotypes in populations of the western terrestrial garter snake (*Thamnophis elegans*), around Eagle Lake, CA, USA provide an excellent system to study physiological response to high temperatures and thermal adaptation. Here, replicate fast-

living lakeshore populations (hereafter, “L-fast”) and slow-living meadow populations (hereafter, “M-slow”) share a common ancestral source (Manier and Arnold, 2005) but have evolved distinct life-history ecotypes on the pace-of-life continuum (sensu Ricklefs and Wikelski, 2002), diverging in growth, reproduction, lifespan, immune investment, and endocrine function (Bronikowski, 2000; Bronikowski and Arnold, 1999; Palacios et al., 2011; Schwartz and Bronikowski, 2011; Sparkman et al., 2007). The selective forces driving these ecotypic differences likely include thermal environment, with air temperature at the lakeshore habitats averaging 5-10ºC warmer during the active season than the montane meadows (Bronikowski, 2000; Bronikowski and Arnold, 1999) and corresponding differences in retreat-site temperatures (Huey et al., 1989; Peterson et al., 1993). Additionally, stress physiology pathways likely contribute to the divergent life-history ecotypes (Schwartz and Bronikowski, 2013). This system of locally adapted populations provides a unique opportunity to test the potential of the thermal environment to shape aerobic performance curves, shifts in the temperature-dependence of energetic trade-offs, and divergences in endocrine pathways shaping the evolution of life histories (Andrews and Pough, 1985; Angilletta, 2001; Beaupre, 1995; Dunham et al., 1989).

We exposed snakes from both ecotypes to ecologically-relevant ambient temperatures ranging from moderate to near-lethal and measured metabolic rate (oxygen consumption rate) and physiological markers of energy regulation and processing (plasma CORT, insulin, glucose, and lactate concentrations). We tested hypotheses focusing on three aspects of the physiological response to high temperatures: First, we hypothesized that temperatures above natural activity temperatures (35°C and above) induce a hormonal stress response (Hypothesis #1). Specifically, we predicted that both circulating CORT and glucose concentrations would increase with temperature (glucose: Baumgard and Rhoads, 2013;

CORT: Schwartz and Bronikowski, 2013). Additionally, we predicted that insulin concentration would increase with increasing temperatures independent of hyperglycemia, similar to the pattern observed in mammals. That is, we predicted that insulin and glucose concentrations would respond similarly in overall pattern, but exhibit different within-individual dynamics. Second, we hypothesized that the thermal tolerances of the ecotypes are locally adapted to their divergent thermal environments, as are other aspects of their biology, and that this divergence would be maintained in adult snakes even after an extended period in common-garden conditions (Hypothesis #2). Thus, we predicted that L-fast snakes would tolerate higher temperatures before displaying physiological stress and would show an attenuated stress response at high temperatures in comparison with M-slow snakes. Finally, we tested the OCLTT hypothesis for the mechanism setting thermal limits in our *T. elegans* populations (Hypothesis #3). If thermal tolerance results from oxygen limitation, we predict that oxygen consumption rate will plateau at near-critical high temperatures, plasma glucose concentration will drop, and lactate concentration will increase (Frederich and Pörtner, 2000; Verberk et al., 2015). In quantifying both the hormonal effects on energy mobilization and the whole-organism metabolic response across a range of high temperatures, this study provides insight into the impact of acute exposure to high temperatures on the regulation of homeostasis and energy stores.

Methods

Experimental Animals

Female *T. elegans* were wild-caught from 3 fast pace-of-life lakeshore populations (L-fast; N = 13) and 4 slow pace-of-life meadow populations (M-slow; N = 15) around Eagle Lake, Lassen County, California, USA in June 2010. All snakes were caught as reproductive-sized adults (snout-vent length (SVL) in mm; M-slow range: 416–568; L-fast range: 460-692).

Snakes were transported to Iowa State University and individually housed in 40 gal glass aquaria with ground corn-cob substrate and a plastic bowl that served as both water dish and retreat site. They were placed on a thermal gradient for 24 hr/d (range: 25-34ºC), and kept on a 12:12 light:dark schedule. We offered frozen/thawed mice once per week, recording the amount eaten at each feeding and increasing the amount offered weekly according to the amount eaten and in relation to body size. For each of the 4 years in captivity, snakes were hibernated uniformly in the dark at 4ºC from January-May. Four days before the experiment began, we measured body mass (in g; M-slow range: 35.6–86.2, S.E.M. = 3.3; L-fast range: 63.2–120.5, S.E.M. = 5.3) and snout-vent length (SVL in mm; M-slow range: 454–590, S.E.M. = 9.6; L-fast range: 545–736, S.E.M. = 13.4). Body condition was calculated as the residual of the log-mass on log-SVL regression (Palacios et al., 2013; Weatherhead and Brown, 1996) and did not differ between the ecotypes at the start of the experiment (*t*-test, $P = 0.88$). Utilizing animals maintained under identical conditions allowed us to control for any immediate external effects on physiology, which is known to vary seasonally and with environmental conditions (Palacios et al., 2013; Palacios et al., 2012). Fieldwork was conducted under permit from the California Department of Fish and Game and treatment of all experimental animals was in accordance with Iowa State University Institutional Animal Care and Use Committee protocol #3-2-5125-J.

Experimental Procedure

We conducted the experiment across 15 consecutive days beginning 18 May 2014, with snakes recently emerged from hibernation (5 May 2015) and fasted throughout hibernation and the experiment. Although this time roughly corresponds to the reproductive period and vitellogenesis in wild snakes, which can be energetically costly (e.g., Van Dyke and Beaupre, 2011), females likely did not vary in reproductive status due to similarities in age, body

condition, and time in captivity. Snakes were divided into five blocks of 4-6 snakes and each block was subjected to a three-hour treatment at each of five ambient temperatures (24, 28, 32, 35, and 38ºC) with temperature order randomized, allowing for a complete, randomized, repeated-measures statistical design. Thus, there were 28 snakes × 5 temperatures for $N = 140$ measures of metabolism and physiology. *Thamnophis elegans* demonstrates a preferred activity range in the wild of 26-32ºC (Scott et al., 1982) with a mean field-active body temperature of 29.9ºC (Peterson, 1987). Snakes become inactive at 35ºC (Scott et al., 1982) with a maximum recorded field body temperature of 36.0ºC (Stevenson et al., 1985). Scott et al. (1982) report a critical thermal maximum (muscle spasms and failure of righting response) of between 43-44ºC and Stevenson et al. (1985) identified a median survival temperature of 42.8ºC. It is not known, however, how snakes from these ecotypes differ in preferred or maximal temperatures.

Originally, the intent of our experiment was to measure responses at temperatures up to 40ºC; however, two snakes exposed to this temperature during preliminary trials both died within several days of treatment. We therefore concluded that an upper extreme of 38ºC represents a stressful treatment near the lethal limit for fasted, post-hibernation subjects. Repeated measures were separated by 72 h, with two blocks subjected to temperature treatments from 0830–1130 h and three blocks from 1230–1530 h. We chose to randomize order of temperature exposure but not time of day within blocks to avoid confounding the effects of time of day and temperature on individual snakes. The evening preceding each treatment, snakes were moved from their home cages into 1.5 L plastic boxes fitted with a rubber stopcock and tubing, covered with a mesh lid, and kept at 24ºC overnight. At the time of the experiment, each container was sealed with an airtight plastic lid and in- and out-flow tubing was attached to the respirometry equipment. Containers were then placed in an incubator

(Econotemp incubator, Thermo Fisher Scientific, Waltham, MA, USA) at the specified temperature treatment. Snakes were maintained in containers in the incubator, undisturbed and in the dark, for 3 h during oxygen consumption rate measurements. Qualitatively, we observed little movement of snakes immediately before and after the experimental procedure. Incubators were equipped with customized fans to ensure air flow and uniform temperature distribution. Incubator temperature was monitored using thermistor probes (model SENT-TH, Sable Systems, Las Vegas, NV, USA), corroborated with a calibrated electric thermometer.

Immediately following each temperature treatment, we collected blood samples (about 150 µl) from the caudal vein of each snake using heparin-rinsed syringes. Blood was immediately transferred to cold centrifuge tubes and kept on ice for a short time (< 50 min) then centrifuged at 3000 *g* for 10 min at 4ºC. We then aliquoted the plasma into separate tubes for each assay (CORT, insulin, glucose, and lactate), immediately snap-froze them in liquid nitrogen, and stored them at -80ºC until analysis. We recorded the time from the end of the experimental treatment to the time each snake was bled and the total handling time for each snake. All handling times were less than 10 min, which is before circulating CORT is elevated due to handling stress in *T. elegans* (Palacios et al., 2012).

Oxygen Consumption Rate

Instantaneous oxygen consumption rate ($\dot{V}O_2$) and carbon dioxide production rate ($\dot{V}CO_2$) were measured using a push mode flow-through respirometry system (Lighton, 2008; Lighton and Halsey, 2011). $\dot{V}O_2$ is a proxy for resting metabolic rate (RMR), which is a measure of steady-state maintenance in post-absorptive individuals at rest during normal activity periods (Andrews and Pough, 1985; Bennett and Dawson, 1976). Standard operating protocols were used for this system: Air flow (400 mL/min) was maintained by an MFS-2

mass flow generator and cycled through eight chambers at 5-min intervals with an RM-8 flow multiplexer. One chamber in each block served as a blank to control baseline values during the experiment. Each chamber was sampled for four 5-min intervals, with the blank chamber sampled for eight intervals. Air outflow was scrubbed of water using Drierite desiccant (Hammond Drierite, Xenio, OH) and then measured for oxygen and carbon dioxide content with CA-10 and FC-10 analyzers, respectively (Sable Systems, Las Vegas, NV, USA). Data were recorded using a UI-2 analog-digital interface. Oxygen and carbon dioxide levels were corrected for variation in barometric pressure and then converted to $\dot{V}O_2$ and $\dot{V}CO_2$ using ExpeData 1.7.30 software (Sable Systems). Measurements from the first intervals (i.e., the first 45 min of the treatment) were excluded from analysis to allow time for snake body temperature to equilibrate with incubator temperature. For analysis of $\dot{V}O_2$, we mathematically corrected for $\dot{V}CO_2$ (following equations in Lighton, 2008) and averaged across the entire 5 min interval, which accounts for any potential periods when the snake was not breathing. Values from the three 5-min readings were then averaged, resulting in a single value of $\dot{V}O_2$ for analysis for each individual at each temperature. Analysis of $\dot{V}CO_2$ demonstrated the same qualitative patterns as the analysis of $\dot{V}O_2$, so we present only the latter here for simplicity. During analysis, we identified a malfunction whereby the multiplexer machine did not switch between channels as programmed, leaving us with $N = 82$ observations. Because the order of individuals was randomized within each trial, the resulting lost data are equally distributed between ecotypes and among temperature treatments (χ^2 test $P = 0.75$ and $P = 0.54$, respectively).

Corticosterone

The concentration of CORT in blood plasma was measured using double-antibody radioimmunoassay kits (Kit #07120102, MP Biomedical, Orangeburg, NY, USA). We followed previously described protocols (Robert et al., 2009), except samples were diluted 1:160 (instead of 1:40) to account for high CORT concentrations. All experimental measurements were made with a single kit over two days. The kit-provided controls were used to assess overall variability in measurements. Each control was run seven times on each day, yielding coefficients of variation (CV%) of 6.67% and 6.48% for the low and high controls, respectively. All samples (N = 140) were run in duplicate with a CV% < 10%. To compare measures from this experiment with baseline laboratory CORT values, we also include measures made on blood samples from these snakes (N = 26) in normal husbandry conditions from August 2013. Because these samples were measured with a different CORT kit, we transformed the calculated concentrations using the equation from a linear regression of eight samples that were measured with both kits, which were strongly correlated (R^2 = 0.93).

Insulin

The relative change in blood plasma insulin concentration was measured using an enzyme-linked immunosorbent assay (ELISA) with a monoclonal insulin antibody assay kit (MyBioSource Cat# MBS269923, San Diego, CA, USA) following manufacturer's protocols with slight modification to sample and reagent volumes. To validate this kit for measures of snake insulin, we tested the parallelism of kit-provided controls with a serially-diluted pooled sample from a congeneric garter snake species, *Thamnophis marcianus* (heterogeneity of slopes: $F_{1,14}$: 2.04, P = 0.17). All samples were run in duplicate on three plates. A pooled

sample was run in duplicate on each plate to assess inter-assay variability, resulting in a CV% of 11.62% across plates. Because of the large amount of plasma necessary for this assay, insulin concentration could only be measured in a subset of samples and duplicate samples with a CV% > 15% (N = 8) were excluded, yielding N = 85 measurements for analysis. Caution is warranted when concluding absolute insulin concentrations from a heterologous assay; however, in interpreting patterns of relative change this approach is valid.

Glucose

The concentration of glucose in blood plasma was measured using an ELISA with a glucose assay kit (Autokit Glucose C2, Wako Chemicals, Richmond, VA, USA) following manufacturer's protocols with slight modification of reagent volumes. All samples (N = 140) were run in duplicate on eight plates and re-run if CV% > 10%. A pooled sample was run on each plate to assess inter-plate variability, resulting in a CV% of 9.9% across plates.

Lactate

The concentration of lactate in blood plasma was measured using an ELISA L-lactate assay kit (Kit #A-108L, Biomedical Research Service & Clinical Application, Buffalo, NY, USA) following the manufacturer's protocols except that sample volumes and deproteinization reagent (polyethylene glycol) were reduced to 20 µL. All samples were run in duplicate on four plates and values accepted if CV% < 20% (per manufacturer's recommendation). We did not have sufficient blood plasma to re-run 11 samples that exceeded this threshold, leaving N = 129 values for analysis. Samples with values outside the standard curve (N = 9) were assigned to the extreme ends of the standard curve. Inter-plate variability was assessed with a pooled sample run on each plate, resulting in a CV% of 4.4% across plates.

Statistical Methods

Dependent physiological variables ($\dot{V}O_2$, CORT, insulin, glucose, and lactate concentrations) were analyzed using repeated-measures mixed linear models with PROC MIXED in program SAS version 9.4 (SAS Institute, Cary, NC) with $\alpha = 0.05$. Graphics are presented as back-transformed least-squares means, using ggplot2 (Wickham, 2009) in the programming language, R (v. 3.1.2, R Core Team, 2014). Variables were $\log_{10}$-transformed to meet assumptions of normality and then standardized to a mean of zero and unit variance to allow for direct comparisons of effect sizes among the different measures (Gelman and Hill, 2006). Temperature was modeled as a categorical factor because this provided a better fit to the data than treating temperature as a continuous variable due to the non-linear relationship of temperature and some of the dependent variables, as assessed by distribution of residuals. Since we were interested in both linear and possible curvilinear effects of temperature on dependent variables such as metabolic rate (e.g., Iles, 2014), we tested the linear and quadratic effects of temperature by utilizing the ESTIMATE statement in PROC MIXED, with coefficients for orthogonal contrasts obtained using our test temperatures (24, 28, 32, 35, and 38°C) in PROC IML. The ESTIMATE statement provides a custom hypothesis test and assesses significance with a *t*-test. By modeling temperature as a categorical factor and then conducting these post hoc linear contrasts, we are able to both utilize models that best fit the data and subsequently test the linear and quadratic effects of temperature. Block and population of origin nested within ecotype were included as fixed effects to account for block and heterogeneity of populations within each ecotype, respectively. Individual-nested-within-block was treated as a repeated-measures random effect modeled with an autoregressive(1) covariance structure, which provided the best model fit as assessed by minimizing AICc values. Initial models included the fixed effects of temperature, ecotype, and body condition, as well as the two- and three-way interactions thereof. With the exception of the temperature

by ecotype interaction, which is of biological interest and influences metabolic rate in this species (Gangloff et al., 2015), we removed non-significant interactions from final models. Since the ecotypes differed in mass (L-fast snakes are larger; *t*-test, $P < 0.0001$), we accounted for mass by first normalizing the $\log_{10}$-tranformed mass to a mean of zero and unit variance within each ecotype so that we could test for the effect of different sizes without confounding this with ecotype. For analysis of plasma hormone, glucose, and lactate concentrations, two covariates were initially included in each model: time (in minutes) from the end of the trial to the time the snake was removed from its chamber and the handling time (in minutes) of the snake needed to complete blood sample collection. Since these covariates represent potential effects of experimental logistics (e.g., we were not able to draw blood from all snakes immediately and simultaneously) rather than biological questions of interest, we removed these covariates from final models if non-significant. After removing non-significant covariates and interactions (all $P \geq 0.18$), the repeated-measures model for the analysis of dependent variables took the form:

$$Y = \mu + Test\ Temperature + Ecotype + Ecotype \times Temperature + Condition + Block + Population(Ecotype) + [Ecotype\text{-}specific\ Mass] + [Handling\ Time] + \varepsilon$$

where μ represents the grand mean and ε represents the error term. Only the models for $\dot{V}O_2$ and CORT concentrations included the term for ecotype-specific mass and only the model for lactate concentration included the term for handling time.

To ensure that our measures represent the effect of temperature treatments and not the effect of handling stress or placement in metabolic chambers, we compared values of CORT concentration from blood samples taken during normal husbandry conditions nine months

prior to the experiment. We compared least-squares means from a repeated-measures ANOVA of log_{10}-transformed CORT concentration with sampling time (baseline or temperature treatment) as a categorical independent variable and individual as a repeated-measures random effect with an ar(1) covariance structure. To further elucidate the relationship between insulin and glucose concentrations within each temperature, we conducted an additional mixed-model analysis of log-transformed and normalized insulin concentration with glucose concentration, temperature, and their interaction as independent factors and individual as a repeated-measures random effect with an ar(1) covariance structure. For all mixed models, we estimated denominator degrees of freedom for *F*-tests using the Kenward-Roger Degrees of Freedom Approximation (Kenward and Roger, 1997).

Results

Oxygen Consumption Rate

Oxygen consumption rate increased with temperature, with no indication of plateau (linear effect of temperature $P < 0.0001$). The main effects of condition and ecotype, and the interaction of ecotype and test temperature, did not influence $\dot{V}O_2$ (Table 1, Fig. 1A, 2). Mass did not exhibit a significant effect on $\dot{V}O_2$, but we retained this as a covariate in the model because of the expected influence of mass on metabolic rates (see Discussion).

Plasma Hormone & Metabolite Concentrations

Temperature linearly increased CORT, insulin, and glucose concentrations (all $P \leq 0.01$) and non-linearly affected CORT, glucose, and lactate concentrations ($P < 0.0001$, $P < 0.0001$, and $P = 0.046$, respectively; Table 1, Fig. 1B-1D, Fig. 2). Body condition did not affect hormone or metabolite concentrations (all $P > 0.53$; Fig. 2). Ecotype only affected glucose concentration, with M-slow snakes maintaining higher concentrations across all temperatures

(P = 0.048; Fig. 1D, 2). Neither the main effect of ecotype nor its interaction with temperature significantly influenced other physiological variables (all P > 0.11; Table 1, Fig. 1B-E, Fig. 2). Ecotype-specific mass negatively influenced CORT concentrations (P = 0.0076; Table 1) while there was significant heterogeneity among populations within each ecotype (P = 0.022), largely driven by very low CORT concentrations from one of the M-slow populations. In the analysis of lactate concentration, the handling time to bleed snakes was marginally significant in initial models of (P = 0.07), which is not surprising given how quickly activity can induce increases in lactate concentrations in squamate reptiles (e.g., Bennett and Licht, 1972), and so was retained the final model.

Baseline CORT concentrations from normal husbandry conditions differed significantly only from CORT concentrations at 38°C (back-transformed least-squares mean: 44.1 ng/mL; Tukey-Kramer adjusted difference between baseline and 38°C treatment, P < 0.0001; all other comparisons with baseline, P > 0.13; Fig. 1B). Additionally, glucose concentration significantly influenced insulin concentration (P =0.0039) and this relationship was independent of temperature (interaction of glucose and temperature: P = 0.90; Table 2, Fig. 3).

Discussion

In this experiment, we measured whole-organism response to acute exposure to ecologically-relevant temperatures and compared norms of reaction between two recently-diverged lineages adapted to differing thermal environments (Bronikowski, 2000; Bronikowski and Arnold, 1999). Our results revealed a universal temperature-dependence of the physiological systems we measured involved in energy processing and allocation. Oxygen consumption rate, as well as CORT, insulin, and glucose concentrations, all increased monotonically with

increasing temperature, without evidence of plateauing at the highest temperatures. Body condition, as a metric of present energy stores, did not influence any physiological parameter (Fig. 2). We found support for Hypothesis #1: a 3-hr exposure to temperatures ≥ 38°C constitutes a physiological stress. Insulin concentration increased in response to hyperglycemia (as indicated by the significant effect of glucose on insulin) and in response to temperature in a glucose-independent manner (as indicated by the shape difference in the reaction norms for insulin and glucose; Fig. 1). Even so, insulin and glucose maintained a positive relationship at all temperatures, suggesting that insulin maintains its role in glucose regulation but perhaps plays additional roles in other functional pathways at high temperatures. Hypothesis #2 was not supported: our results did not demonstrate evidence of local adaptation or canalized developmental plasticity in the thermal reaction norms of these two ecotypes (i.e., no ecotype × temperature interaction) in the measured indicators of physiological response to upper temperature extremes. Finally, our results refute the hypothesis that thermal limits in *T. elegans* result from oxygen limitation at high temperatures (OCLTT hypothesis; Hypothesis #3).

In this experiment, individuals maintained physiological functionality at temperatures just below the lethal limit. Such extreme temperatures, however, dramatically affected regulation of important aspects of energy metabolism, specifically glucose regulation pathways. CORT concentration was thermally dependent, with temperatures near the upper thermal limit inducing elevation above baseline. Somewhat surprisingly, CORT concentrations at all temperatures were well below maximum levels attained by wild-caught Eagle Lake *T. elegans* under handling stress (CORT concentration mean ± S.E.M.: 338.9 ± 44.7 ng/mL; Palacios et al., 2012). Though we are unable to make a direct comparison of absolute values across studies, the large difference in maximal values from the two studies as well as the

proportional increase from baseline indicate that the capture-restraint protocol of Palacios et al. (2012) induced a much greater CORT response than the temperature treatments of the present experiment. That the CORT response was elevated but not maximal indicates that, even at the highest temperatures, individuals have the capacity to mount a response to additional stressors and that exposure to high temperatures for this time period did not bring snakes across a critical threshold. Even at the highest temperatures snakes were likely in the "reactive homeostasis" range of Romero et al. (2009) or in "type I allostatic overload" of McEwen and Wingfield (2010). However, prolonged exposure to the highest temperatures that we measured (38ºC), exposure to higher temperatures, or exposure to additional stressors could potentially continue to elevate CORT concentration beyond the capacity for further response. Furthermore, we found that larger snakes exhibited lower CORT concentrations across the temperature range (Table 1), though post-hoc analysis revealed no mass-by-temperature interaction ($F_{4,91}$ = 0.72, P = 0.58). Since size can be used as a proxy for age in garter snakes (Sparkman et al., 2007), this result implies that either individual snakes reduce concentrations of circulating CORT as they age or that larger snakes have been able to obtain larger sizes by virtue of reduced hormonal reactivity. This distinction – whether hormonal profiles change with age or whether certain profiles permit greater growth – has important implications for understanding the role of hormonal regulation of life-history strategies (e.g., Reding et al., 2016).

Stressful stimuli elevate both glucocorticoids, such as CORT, and catecholamines, such as epinephrine and norepinephrine (Akbar et al., 1978; Sapolsky et al., 2000). These hormones trigger pathways to mobilize glucose stores, produce glucose via glycogenolysis and gluconeogenesis (reviewed in Sapolsky et al., 2000), and inhibit glucose uptake into non-essential tissues (Kaplan, 1996). Accordingly, we observed similar patterns in the increases

of CORT and glucose concentrations, with both compounds maintaining baseline levels at moderate temperatures and elevating dramatically in response to near-critical temperatures, as demonstrated by the significant quadratic effect of temperature (Table 1, Fig. 1B, 1D). Under homeostatic circumstances, insulin modulates the glucose-relevant effects of both catecholamines and glucocorticoids by reducing glucose production and promoting uptake into tissues (Foster and McGarry, 1996; Strack et al., 1995). With increasing heat stress, we observed both elevated insulin and glucose concentrations (Table 1, Fig. 1B-1C). We also found evidence that insulin was responding to hyperglycemia and that the general relationship between glucose and insulin concentrations did not change across temperatures (Table 2, Fig 3). Notably, insulin concentration rose linearly across the range of test temperatures, while glucose concentration increased non-linearly, as indicated by the significant quadratic effect of temperature (Table 1, Fig. 1B-1C). This result suggests that that insulin may play a role under heat stress in addition to glucose regulation, similar to that in mammals under conditions of heat stress (Baumgard and Rhoads, 2013). For example, insulin may enhance protection from heat stress via its effect on the transcription of heat-shock proteins (Li et al., 2006) and potentially decreased muscle proteolysis (Foster and McGarry, 1996). The temperature-dependence of insulin and its role in energy balance maintenance in ectotherms is an important avenue of future research.

Our data demonstrate that garter snakes from these populations of divergent life-history ecotypes adjust their physiology and deliver the required oxygen to their tissues at sufficient rates to maintain metabolic and physiological competence when acutely exposed to near-lethal temperatures. Neither a metabolic-rate plateau at high temperatures nor a temperature-insensitive range was apparent, in contrast to a temperature-insensitive range of oxygen consumption rate from 28–34C° reported in a previous study of *T. elegans* (Seidel and

Lindeborg, 1973). Notably, the steep increase in oxygen consumption rate by L-fast snakes at the highest temperature (38°C), as well as the large variance associated with the least-squares mean estimate (Fig. 1A), was driven by two snakes with an extreme response in oxygen consumption rate to high temperatures. The importance of individual variation in temperature-sensitivity of these physiological traits in shaping the evolution of the stress response to high temperatures represents another important direction for future research (Angelier and Wingfield, 2013; Cockrem, 2013). Surprisingly, the dependence of oxygen consumption rate on ecotype-specific body mass was not statistically significant. In an analysis of data across all temperatures, oxygen consumption rate scales positively with body mass (in grams) to an exponent of 0.37 ± S.E. 0.28. Though low, this estimate does not differ significantly from estimates for scaling exponents in males of this species (0.59, Bronikowski and Vleck, 2010) or from squamates generally (0.7-0.8, Andrews and Pough, 1985; McNab, 2002). We attribute the large variance around this estimate, rendering it insignificant in our models, to a combination of small sample sizes, a limited range of body masses sampled from each ecotype, and the incomplete $\dot{V}O_2$ data.

In contrast to the temperature-dependence of oxygen consumption rate, we found no evidence that the concentration of lactate, a by-product of anaerobic metabolism, increased with increasing temperature, as might be expected if high temperatures disrupt oxygen supply and induce anaerobic metabolism (Frederich and Pörtner, 2000). We did find a significant quadratic effect of temperature on lactate, however, due to high lactate concentrations at the low and high experimental temperatures (24 and 38°C). The increases we observe in lactate concentrations at these temperatures are likely not driven by energetic demand outpacing aerobic capacity, given that the relative increases are far below lactate increases resulting from exercise in squamates (e.g., Bennett and Licht, 1972). Rather, this pattern might result

from the temperature-dependence of lactate dehydrogenase (e.g., Johansson, 1969; Mendiola and Costa, 1990), though the activity of this enzyme this has not been quantified in *T. elegans*. Given that neither $\dot{V}O_2$ nor glucose concentration plateaued at high temperatures and that lactate concentration did not escalate with temperature, these snakes are not limited by oxygen acquisition or transport at temperatures near their thermal maximum. This result is in accordance with recent studies that refute the OCLTT hypothesis in free-living stages of terrestrial ectotherms (Fobian et al., 2014; He et al., 2013; McCue and Santos, 2013; Overgaard et al., 2012; but see Shea et al., 2016). Our data do not address other possible mechanisms which may contribute to thermal tolerance limits, including protein denaturation, membrane fluidity, enzyme-substrate interactions, mitochondrial failure, and failure of neural processes (reviewed in Angilletta, 2009; Schulte, 2015). Regardless of the mechanism setting thermal tolerance limits, our results demonstrate that high temperatures increase energetic expenditure, increase resting oxidative metabolism, and affect energy-regulating pathways, which in turn could influence individual activity patterns, response to additional stressors, and ultimately fitness.

The current study enhances our understanding of the complex interplay of genetic background and developmental environments in shaping physiology in *T. elegans* from Eagle Lake. First, a previous study found ecotypic differences in metabolic rate in wild-caught adult males after 4 months in captivity (contrasting with the four years in captivity in this study). Male snakes from the L-fast ecotype had higher mass-independent oxygen consumption rates across a range of non-stressful temperatures (15-32ºC; Bronikowski and Vleck, 2010). Second, studies in lab-born offspring from these two ecotypes indicate no difference in oxygen consumption rates between neonates at 28ºC (Robert and Bronikowski, 2010), but identified ecotype-specific thermal response curves in older snakes (Gangloff et al., 2015),

suggesting that genetic background exerts developmental effects on resting metabolic rates. Captive-born snakes from both ecotypes have similar CORT thermal response curves, but differ in their responses to high ambient temperatures for both liver mitochondrial H_2O_2 production and erythrocyte superoxide concentration, two measures related to mitochondrial respiration (Schwartz and Bronikowski, 2013). The present study detected no ecotypic differences in the responses of metabolic rate and hormone concentration to increasing temperature in wild-caught, lab-acclimated adult female snakes under heat stress, though we did identify an ecotypic difference in glucose concentrations across the range of measured temperatures. Given that the glucose concentration differences were parallel across temperature treatments (i.e., there is no ecotype by temperature interaction), the data suggest that there is variation in the maintenance of available energy between the ecotypes and that this difference is maintained in both normative and stressful conditions. After being caught as adults, these snakes were housed under identical laboratory conditions for several years, which can affect endocrine profiles (Sparkman et al., 2014). Thus, either adult females from these ecotypes do not differ in resting metabolic rates or environmental effects on thermal reaction norms remain plastic throughout adulthood (Angilletta, 2009). Although previous studies in garter snakes have showed that stress response and endocrine function are affected by current energetic stores (Dayger et al., 2013; Palacios et al., 2013), we found that body condition, which was largely homogenous among individuals in our study, did not impact physiological variables. These animals, maintained in favorable conditions with ample food stores for four years, may not represent enough variation in body condition to reveal trade-offs as expected under resource-limited conditions (Glazier, 1999; van Noordwijk and de Jong, 1986).

Snakes from these locally-adapted ecotypes did not differ in response to stressfully high ambient temperatures, despite being adapted to differing thermal environments in life-history traits (Bronikowski, 2000). This fits observations that upper thermal tolerance limits in terrestrial ectotherms display limited variance, and thus may have little capacity to adapt to changing local conditions (Huey et al., 2012; Sunday et al., 2011). That upper limits are constrained, however, does not mean that all thermal tolerances are fixed: in studies of other snakes in this genus, the critical thermal minima vary among and within species and is tied to temperatures experienced by populations in the field (Burghardt and Schwartz, 1999; Doughty, 1994). Garter snakes behaviorally avoid thermal extremes (Scott et al., 1982), which reduces selection on thermal physiology at high temperatures (Buckley et al., 2015; Huey et al., 2003). While behavioral thermoregulation may provide relief from extreme high temperatures, increasing environmental temperatures could still reduce the number of suitable retreat sites (Huey et al., 1989), increase the distance between retreat sites, and increase the resting metabolic rates of inactive snakes. Nonetheless, it is possible that snakes might benefit from the increased foraging capacity and energy input of warmer temperatures (Huang et al., 2013; Weatherhead et al., 2012), especially in habitats that are less restricted by high temperatures (i.e., meadow populations). In general, studies that quantify long-term energy budgets at increased ambient temperatures and test for temperature-induced mismatches between metabolic rate and consumption (e.g., Iles, 2014; Lemoine and Burkepile, 2012) are needed to assess the possible effects on individual fitness and population persistence. By utilizing an integrative, mechanistic approach in a broader energetic and ecological context, we can further develop our understanding of the processes that set thermal limits and the consequences of exposure to extreme but sub-lethal temperatures.

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Table 1. Parameter estimates (partial regression coefficients) and repeated-measures mixed linear model analysis across five temperatures of: $\log_{10}$-transformed and standardized oxygen consumption rate ($\dot{V}O_2$ in ml O_2 h^{-1}), ; and $\log_{10}$-transformed and standardized plasma CORT concentration (ng/mL), insulin concentration (μIU/L), glucose concentration (mg/dL), and lactate concentration (mg/mL) measured after a 3-hr temperature exposure in adult female *T. elegans*. Temperature was treated as a categorical effect in the model, so parameter estimates and *t*-test results for temperature are from orthogonal linear contrasts (see text for details). Denominator degrees of freedom for *F*-tests were estimated using the Kenward-Roger Degrees of Freedom Approximation. Significant effects designated in bold with a single ($P < 0.05$) or double ($P < 0.001$) asterisk.

Source of Variation	$\dot{V}O_2$	CORT	Insulin	Glucose	Lactate
Temperature: Linear					
Partial regression coefficient	0.97	1.04	0.63	1.02	0.0047
t (*d.f.*)	4.28 (48.3)	5.97 (73.7)	2.65 (48.6)	5.80 (77.6)	0.02 (63.5)
Pr > t	**< 0.0001****	**< 0.0001****	**0.011***	**< 0.0001****	0.98
Temperature: Quadratic					
Partial regression coefficient	0.37	0.67	0.034	0.58	0.38
t (*d.f.*)	1.64 (57)	4.65 (116)	0.15 (64.9)	4.03 (117)	2.02 (108)
Pr > t	0.11	**< 0.0001****	0.88	**< 0.0001****	**0.046***
Ecotype					
Partial regression coefficient	1.26	-0.89	0.12	0.37	0.066
F (df_n, df_d)	2.55 (1, 28.2)	0.52 (1, 24.2)	1.21 (1, 26.6)	4.32 (1, 24.7)	0.09 (1, 26.7)
Pr > F	0.12	0.48	0.28	**0.048***	0.77
Temperature × Ecotype					
Partial regression coefficient	--	--	--	--	--
F (df_n, df_d)	2.02 (4, 47.5)	0.57 (4, 92.8)	0.30 (4, 51.7)	0.33 (4, 96.1)	0.93 (4, 80.4)
Pr > F	0.11	0.69	0.88	0.85	0.45
Condition					
Partial regression coefficient	-0.053	-0.037	0.072	0.039	-0.070
F (df_n, df_d)	0.20 (1, 21.2)	0.10 (1, 25.5)	0.34 (1, 23)	0.11 (1, 25.9)	0.41 (1, 30.6)
Pr > F	0.66	0.76	0.56	0.74	0.53
Ecotype-specific mass					

Partial regression coefficient	0.011	-0.42	--	--	--
F (df_n, df_d)	0.01 (1, 19)	8.40 (1, 25.4)	--	--	--
Pr > F	0.94	**0.0076***	--	--	--
Block					
Partial regression coefficient	--	--	--	--	--
F (df_n, df_d)	1.62 (4, 23.4)	1.35 (4, 25.8)	1.82 (4, 24.4)	0.38 (4, 25.9)	1.78 (4, 29.4)
Pr > F	0.20	0.28	0.16	0.82	0.16
Population(Ecotype)					
Partial regression coefficient	--	--	--	--	--
F (df_n, df_d)	0.29 (5, 21.5)	3.17 (5, 26.7)	2.35 (5, 25.6)	1.88 (5, 25.9)	0.76 (5, 28.9)
Pr > F	0.91	**0.022***	0.070	0.13	0.59
Handling time					
Partial regression coefficient	--	--	--	--	0.065
F (df_n, df_d)	--	--	--	--	3.54 (1, 106)
Pr > F	--	--	--	--	0.063

Table 2. Results of repeated-measures mixed linear model of $\log_{10}$-transformed and normalized insulin concentration in adult female *T. elegans*, after a 3-hour treatment at each of five temperatures. Temperature was treated as a categorical effect in the model and denominator degrees of freedom for *F*-tests were estimated using the Kenward-Roger Degrees of Freedom Approximation. Significant effects ($P < 0.05$) designated in bold with a single asterisk.

Source of variation	F (df_n, df_d)	Pr > F
Temperature	0.26 (4, 64.3)	0.90
Glucose	8.88 (1, 71.6)	**0.0039***
Temperature* × *Glucose	0.27 (4, 61.8)	0.90

Figures

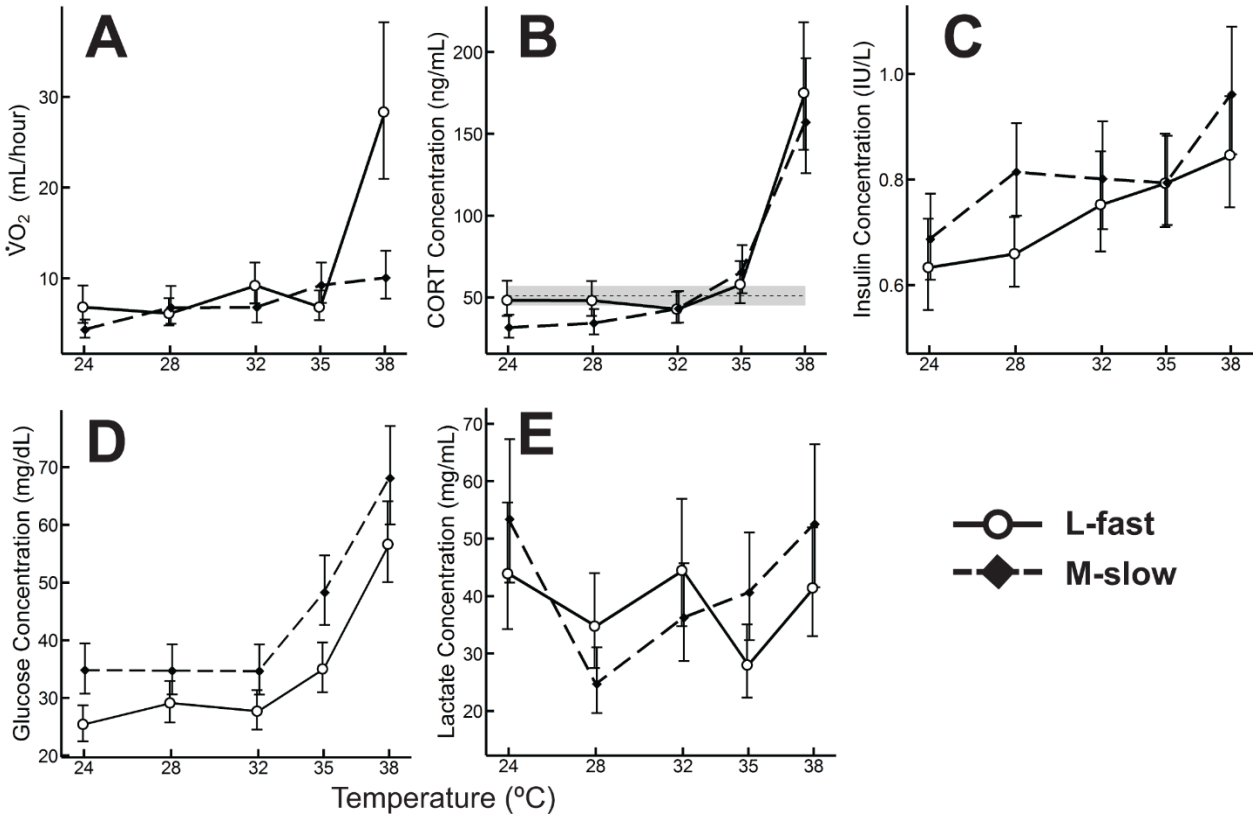

Fig. 1. Temperature had a linear effect on $\dot{V}O_2$, CORT, insulin, and glucose concentrations (all $P \leq 0.01$) and a quadratic effect on CORT, glucose, and lactate concentrations ($P < 0.0001$, $P < 0.0001$, and $P = 0.046$, respectively) in adult female *T. elegans*, but ecotypes only differed in glucose concentration ($P = 0.048$). Thermal reaction norms are for (A) oxygen consumption rate ($N = 82$ data points), (B) plasma CORT concentration ($N = 140$ data points), (C) plasma insulin concentration ($N = 85$ data points), (D) plasma glucose concentration ($N = 140$ data points), and (E) plasma lactate concentration ($N = 129$ data points) measured at five temperatures. Plasma concentrations were measured after a 3-hr exposure to each temperature. In panel (B), the dotted line and corresponding grey shading represent mean ± S.E.M. of baseline

CORT concentration values of these same individuals from common garden conditions nine months prior to the experiment. Data are back-transformed least-squares means from repeated-measures mixed linear models (see text for details). Error bars represent ± S.E.

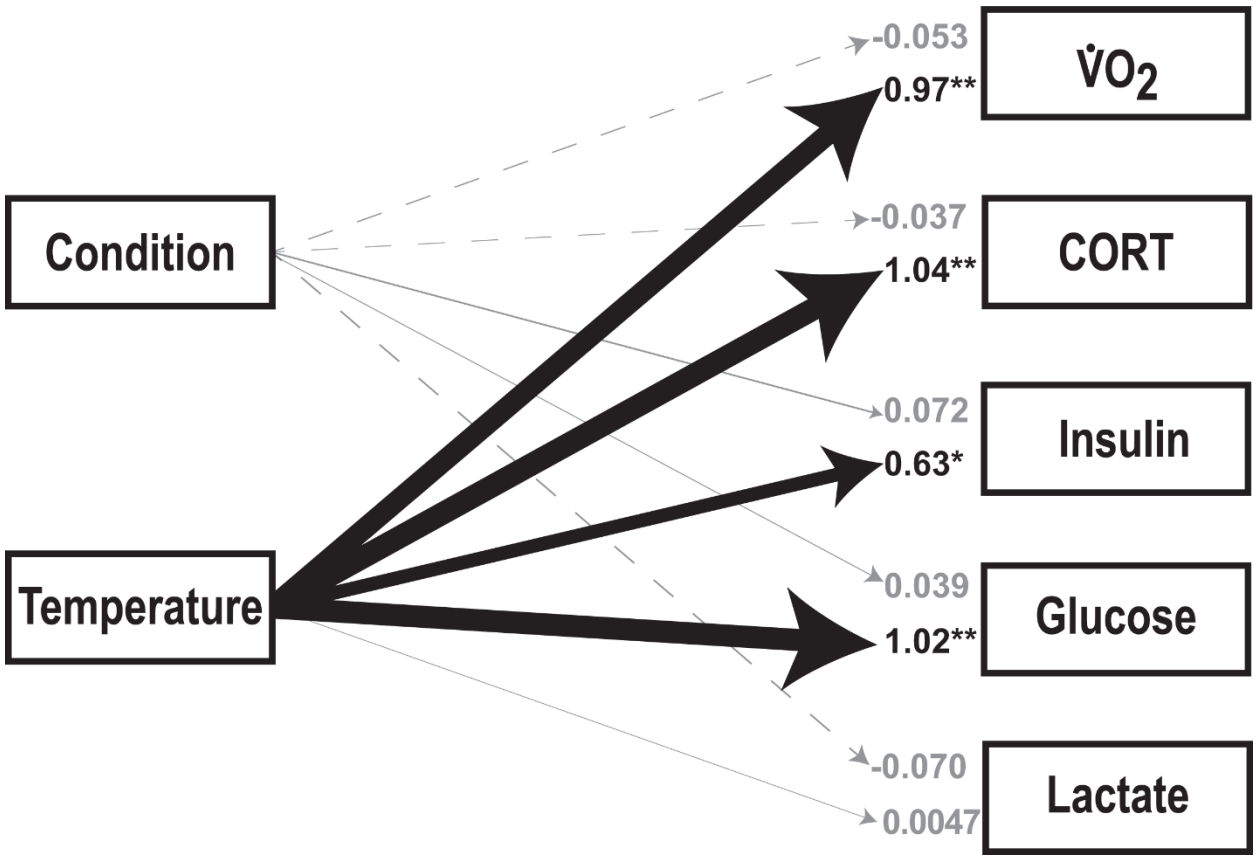

Fig. 2. Temperature exhibits strong linear effects on physiological variables ($\dot{V}O_2$, CORT, insulin, and glucose concentrations) while body condition does not. Path diagram shows partial regression coefficients for linear influences of temperature and body condition on measured physiological variables in wild-caught adult female *T. elegans* after a 3-hr exposure to five temperatures. Significant paths (all $P \leq 0.01$) shown in black, non-significant paths (all $P > 0.41$) shown in gray; positive paths shown with solid line, negative with dotted line. Line size is proportionate to effect size on standardized variables. Significant estimates designated with one ($P < 0.05$) or two ($P < 0.001$) asterisks. See text and Table 1 for model details and results.

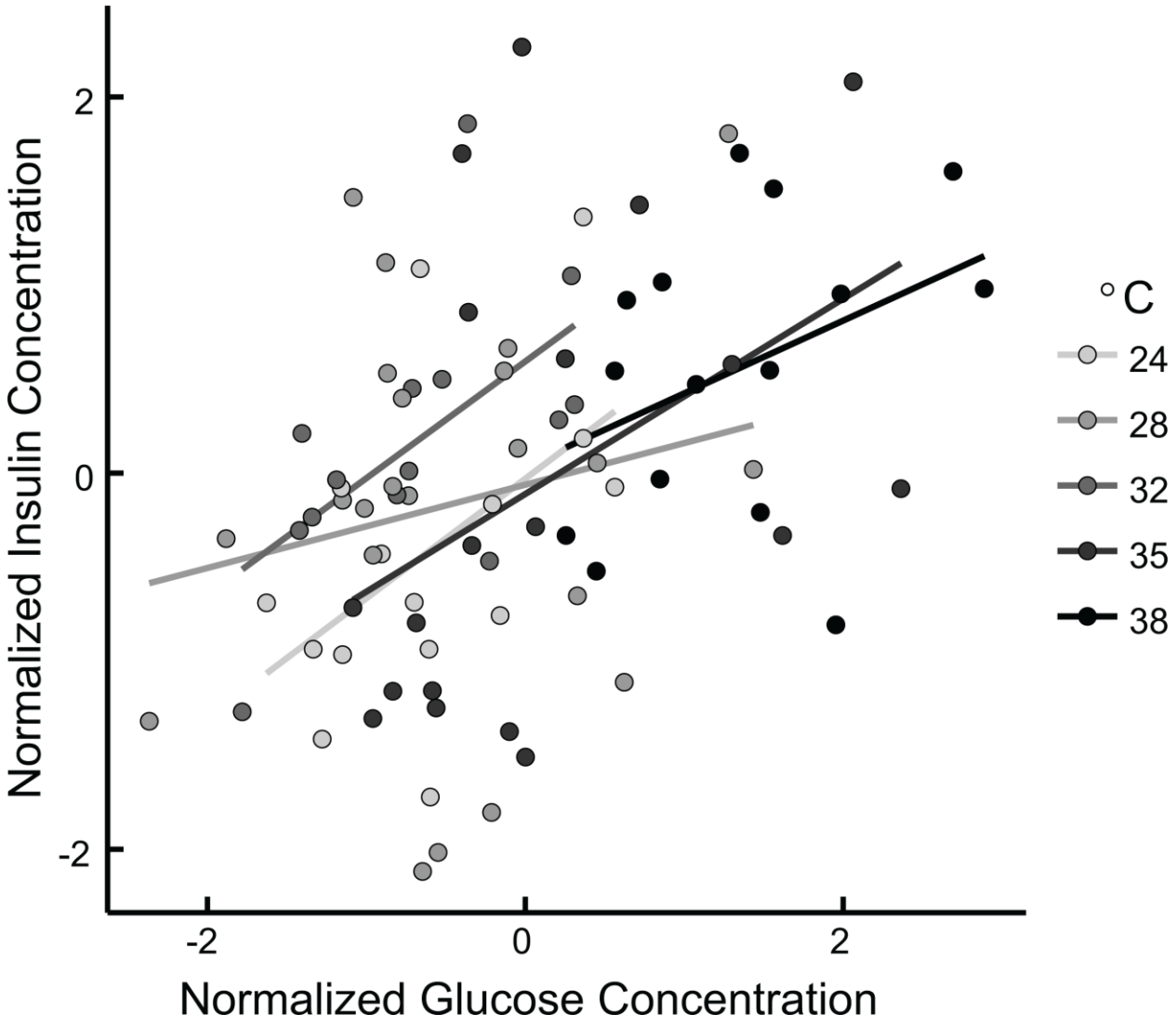

Fig. 3. Relationship between insulin and glucose concentrations within adult female *T. elegans* does not change with temperature. Scatterplot shows relationship between log-transformed and normalized glucose and insulin concentrations after a 3-hr exposure at five test temperatures. Overall effect of glucose concentration on insulin concentration is significant from repeated-measures linear model ($P = 0.0040$; see text for details). Regression lines are shown for

each temperature. Data are from individuals for which we measured both plasma glucose and insulin concentrations at a given temperature (N = 85).