



Oxygen concentration affects upper thermal tolerance in a terrestrial vertebrate



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ABSTRACT

We tested the oxygen limitation hypothesis, which states that animals decline in performance and reach the upper limits of their thermal tolerance when the metabolic demand for oxygen at high temperatures exceeds the circulatory system's ability to supply adequate oxygen, in air-breathing lizards exposed to air with different oxygen concentrations. Lizards exposed to hypoxic air (6% O₂) gaped, panted, and lost their righting response at significantly lower temperatures than lizards exposed to normoxic (21% O₂) or hyperoxic (35% O₂) air. A greater proportion of lizards in the hyperoxic treatment were able to withstand body temperatures above 44 °C than in the normoxic treatment. We also found that female lizards had a higher panting threshold than male lizards, while sex had no effect on gaping threshold and loss of righting response. Body size affected the temperature at which lizards lost the righting response, with larger lizards losing the response at lower temperatures than smaller lizards when exposed to hypoxic conditions. These data suggest that oxygen limitation plays a mechanistic role in the thermal tolerance of lizards.

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1. Introduction

Temperature is one of the most important environmental factors for ectotherms, influencing their biochemical and physiological processes (Cloudsley-Thompson, 1999) and affecting a range of functions from sensory input to immune response (reviewed in Angilletta et al., 2002). Global climate change imposed by anthropogenic greenhouse gas emissions poses a significant threat to terrestrial biodiversity in the twenty-first century (IPCC, 2014). The predicted global temperature increase over the next century could contribute to the extinction of 18% of all species by 2050 (Thomas et al., 2004), including up to 20% of lizard species (Sinervo et al., 2010). Ectotherms are expected to represent the bulk of this statistic because they are sensitive to micro- and macro-climatic changes due to the inherent temperature dependence of their physiological processes (Brusch et al., 2016; Sinervo et al., 2010; but see Kearney et al., 2009), especially given that their strong propensity for behavioral thermoregulation may weaken selective pressures for thermal adaptation (Buckley et al., 2015). For these reasons, the effects of temperature on the behavior and physiology of ectotherm species,

especially factors governing their thermal tolerance, is a growing area of interest.

Evaluations of thermal tolerance in terrestrial ectotherms have focused on the critical thermal maximum (CTmax¹) since Cowles and Bogert (1944) showed that reptiles demonstrate a stereotyped loss of coordinated motor function at a temperature just below the upper lethal temperature. These classic studies of CTmax also showcased the importance of reptiles as terrestrial model organisms for thermal tolerance because the highly stereotyped responses—loss of righting response followed by onset of muscular spasms at the CTmax—can be easily studied in a range of conditions and in species of varying body sizes (Lutterschmidt and Hutchison, 1997b). Despite scores of studies (reviewed in Lutterschmidt and Hutchison, 1997b) since Cowles and Bogert (1944), the physiological mechanisms responsible for the loss of coordinated motor function at high body temperatures remain unclear, largely due to the complex and differential ways in which temperature can affect proteins, cells, and organisms. These mechanisms range from effects of temperature on enzyme activity and enzyme-substrate interactions to the temperature dependence of the coordinated functioning of the nervous, endocrine, cardiovascular, and muscular systems (Schulte, 2015).

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¹ CTmax: critical thermal maximum.

An intriguing hypothesis for the physiological mechanism responsible for loss of motor function and coordination at high temperatures is the oxygen- and capacity-limited thermal tolerance hypothesis, or more simply the oxygen limitation hypothesis, which suggests that the upper thermal limits of complex animals are determined by oxygen delivery limitations that occur at high body temperatures (Frederich and Pörtner, 2000; Pörtner, 2001, 2002). That is, aerobic metabolism rises with increasing temperature, and at some point the ability of the circulatory system to deliver the necessary oxygen to the nervous system and muscles cannot keep up with oxygen demand, and the animal's ensuing anaerobic metabolism is insufficient to sustain prolonged energy demand. Support for the oxygen limitation hypothesis is mixed (reviewed in Verberk et al., 2016). Many studies on water-breathing animals have supported oxygen as a limiting factor (e.g., Pörtner, 2001; Pörtner and Knust, 2007; Verberk and Bilton, 2011; Verberk and Calosi, 2012; but see Clark et al., 2013; Ern et al., 2014; Norin et al., 2014). Fewer studies have been conducted on air-breathing organisms, where oxygen is more readily available than in water. These studies have focused mainly on invertebrates, and results have also been mixed, with some studies supporting the hypothesis (Koopman et al., 2016; Verberk and Bilton, 2013; Verberk and Bilton, 2015; Verberk et al., 2013) and others not (Boardman and Terblanche, 2015; Klok et al., 2004; McCue and De Los Santos, 2013; Stevens et al., 2010). Studies testing the oxygen limitation hypothesis in larger organisms, particularly terrestrial air-breathing vertebrates, are few, and for the most part oxygen limitation has not been supported as a mechanism behind thermal tolerance in these studies (Fobian et al., 2014; Overgaard et al., 2012; Seebacher and Franklin, 2011; Tattersall and Gerlach, 2005; but see Smith et al., 2015).

Given the paucity of experimental studies investigating the oxygen limitation hypothesis in terrestrial air-breathing organisms, we conducted an experiment in which we tested the effect of oxygen concentration of ambient air on thermal tolerance in lizards, one of the most thoroughly studied groups of terrestrial organisms in terms of thermal physiology (Angilletta et al., 2002; Brattstrom, 1965; Cowles and Bogert, 1944; Lutterschmidt and Hutchison, 1997b). We designed and built a multiplex device to deliver gases of various oxygen concentrations to lizards while heating them at a set rate. If oxygen availability limits thermal tolerance, we predicted that treatment with hypoxic air should result in lower thermal tolerance than exposure to normoxic air, and treatment with hyperoxic air should result in higher thermal tolerance than normoxic air.

2. Materials and methods

2.1. Ethical statement

All procedures were approved by the Institutional Animal Care and Use Committee of the California Polytechnic State University, which operates according to guidelines created by the National Institutes of Health.

2.2. Study site and study species

A total of 83 adult Western fence lizards (*Sceloporus occidentalis*, Baird and Girard, 1852) were collected from two sites (Logging Team facility and Poly Canyon) on the campus of California Polytechnic State University (Cal Poly), San Luis Obispo, California, USA. These sites are located on the central coast of California at an altitude of 72 m with an average annual high temperature of 21.8 °C, low of 8.5 °C, and precipitation of 48 cm (<http://www.usclimatedata.com>). This sample size was chosen to ensure sufficient power to detect potential effects of three oxygen treatments on thermal tolerance.

Sceloporus occidentalis is a heliothermic, Phrynosomatid lizard with a field active body temperature ranging from about 19 to 39 °C in its native range in the Western United States and south into Baja California

(McGinnis, 1966). It is a small to medium sized lizard, so the time needed to heat and cool the lizards to specific body temperatures in the lab is short. Because these lizards were caught from the wild and only kept in the lab for several hours before trials, they had little time to acclimate to laboratory conditions (which can happen as quickly as in the first 24 h), and results should be representative of their natural thermal tolerance (Hutchison and Dupré, 1992).

Lizards were collected by hand or by noose (fishing line on B'n'M Fishing crappie rods) and placed individually in tube socks for transport to the laboratory. Capture location and time of day were recorded for each lizard. In the lab, body mass (± 0.5 g), snout-vent length (SVL, ± 1 mm), and tail length were recorded. Sex and female reproductive state (gravid or not gravid) were recorded. After measuring thermal tolerance, blood samples were drawn from the post-orbital sinus to determine hematocrit. Blood was collected in heparinized microhematocrit tubes and centrifuged to separate the red blood cells from the plasma. These samples were taken after thermal testing was completed to avoid confounding experimental data with blood loss.

2.3. Measurements of thermal tolerance

We measured three variables: gaping threshold (lizard opens its mouth), panting threshold (lizard gapes and holds its mouth open for at least 4 s accompanied by heavy chest compressions), and loss of righting response. Gaping and panting behaviors reflect the lizards' attempt to utilize evaporative cooling to reduce body temperature (Tattersall et al., 2006), and the loss of righting response represents the failure of coordinated neural and/or muscular activity that occurs at high temperatures. The temperature at which the loss of righting response occurs, often used as a proxy for the CTmax in lizards, actually occurs just below the CTmax. The actual CTmax is better measured as the onset of muscular spasms (Lutterschmidt and Hutchison, 1997a). In a pilot study, all lizards we tested reached the upper lethal temperature without displaying any indication of spasms, so we chose to use the body temperature at which loss of righting response occurs (hereafter referred to simply as LORR²) as a measure of upper thermal tolerance. LORR is also appropriate because it represents the temperature at which performance declines, due to oxygen limitation according to our hypothesis (Verberk et al., 2016).

The three response variables were measured on the same day lizards were collected to avoid extensive acclimation to laboratory conditions (Art and Claussen, 1982; Hutchison and Dupré, 1992). To collect these data, we built a multiplex data acquisition apparatus, the Controlled Reptile Oxygen and Climate System (CROCS, Fig. 1), capable of heating animals in test chambers at a constant, designated rate with gases of varying oxygen concentration. This is accomplished via an Arduino UNO R3 microcontroller that controls flow of compressed gas from aluminum scuba tanks into an insulated metal box containing a heater (900w finned strip heater wrapped with 3/8" aluminum tubing) and digital temperature sensor (Adafruit MCP9808 High Accuracy I2C Temperature Sensor Breakout Board) and then into seven test chambers (8-inch respirometry chambers, Qubit Biology, Inc.) via silicone tubing. This arrangement, where air is heated via thermal conduction through an isolated coil of aluminum tubing contained within the heater box, was chosen to reduce the fire potential inherent when working with oxygen concentrations above 21%. Air exiting the heater passes through a diffuser manifold to disperse evenly from the heater into each of the seven chambers. When the heated air enters the chamber, additional diffusers route the heated air stream away from the lizard's snout to evenly heat the air in the chamber. Fiberglass insulation prevents excessive loss of heat to the environment from the two outer chambers. Prior to experimentation, we confirmed that the ambient temperature rises at equal rates in each of the seven chambers. Six of the test chambers

² LORR: body temperature at which loss of righting response occurs.

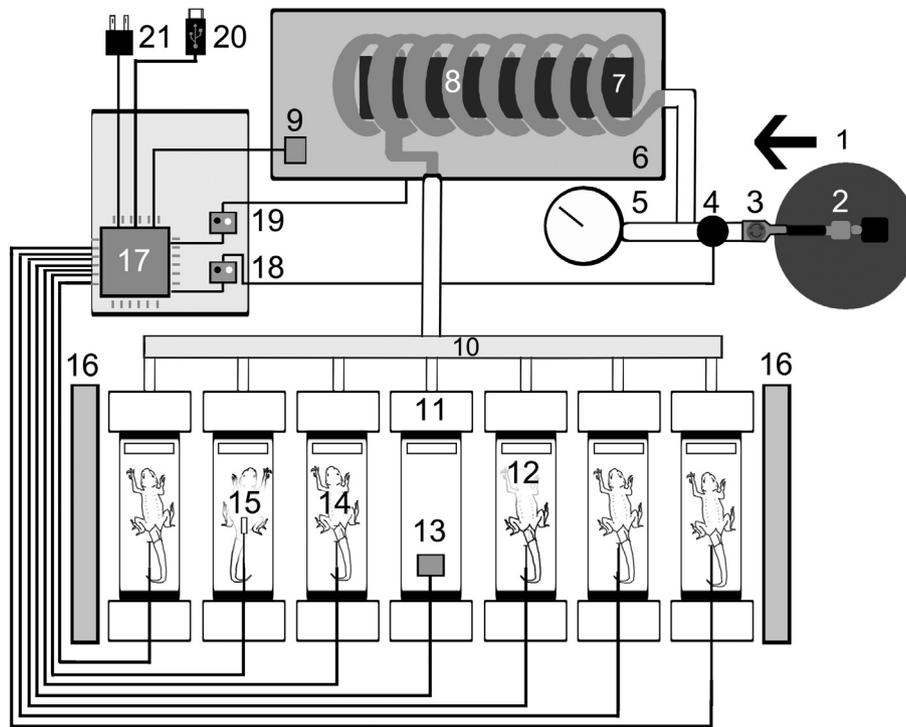


Fig. 1. Schematic of the Controlled Reptile Oxygen and Climate System (CROCS) used to evaluate the effects of gas with various concentrations of oxygen on thermal tolerance of lizards. A scuba cylinder (1) of compressed gas containing a known concentration of oxygen is routed through a first stage regulator (2) into a needle valve (3) to control airflow. The normally closed solenoid-actuated valve (4) controls air entering into the heating system. When the valve is open, airflow into the heater box is measured by a pressure gauge (5). Air flows into insulated heater box (6) containing a heater (7) wrapped in aluminum tubing (8) and a digital temperature sensor (9) that monitors the box temperature. Heated air then flows through a diffuser manifold (10) that distributes the air evenly into seven test chambers (11) via silicone tubing. Each testing chamber contains an additional plastic diffuser (12) at the entrance to help disperse the air evenly through the chamber. The central chamber contains a digital temperature sensor (13) to monitor chamber air temperature. Each of the six test chambers contains test subjects (lizards, 14) and resistance temperature detectors (15) inserted into the lizards' cloacae to measure body temperatures. Fiberglass insulation (16) prevents heat loss from the outermost two chambers. An Arduino microcontroller (17) collects data from 13 and 15 and controls a solenoid relay (18) and a heater relay (19) to maintain the desired heating rate of 1 °C. Data output (20) was saved locally on a laptop. CROCS is powered via 120 V AC power (21).

hold lizards while the central chamber contains solely a digital temperature sensor to monitor chamber air temperature (T_a). Each of the six test chambers is fitted with a resistance temperature detector (Honeywell platinum RTD) coated with waterproof and highly thermally conductive epoxy (KONA 870 FTLV-DP) to measure lizard body temperatures (T_b) when inserted into the cloacae. We wrote a logic-based Arduino program to turn the solenoid on and off to maintain the desired heating rate in the test chambers. The Arduino was programmed to stabilize to a set point temperature then to ramp 1 °C per minute and collect data from the six RTDs in the cloacae of the test lizards. By controlling the duty cycle of the solenoid, heating ramp rates within the test chambers (T_a) were maintained at an average of 0.93 ± 0.014 °C per minute. The heated air in the test chambers leaves through a small hole on the distal ends of the chambers. A laptop running the program HyperTerminal collects data from each of the temperature probes (heater box, T_a , and each of six T_b). HyperTerminal allows manual time stamping for occurrence of response variables (gaping threshold, panting threshold, LORR; see below) for each of the six study subjects individually. While each of the six subjects receives the same treatment, lizards can be tested for LORR independently and removed from test chambers without affecting the continued temperature ramping of other test chambers. All data as well as the testing parameters and response time stamps are saved as a text file on the laptop.

2.4. Oxygen treatments

Normoxic gas (compressed atmospheric air, 21% O_2) consisted of scuba breathing gas in aluminum tanks. To create hypoxic gas (6% O_2 , 94% N_2), we used the iMixer app (ARC-Insight) to calculate volumes of atmospheric air and nitrogen to mix in scuba tanks, and verified the

concentration of oxygen using a gas analyzer (ADInstruments). Hyperoxic gas (35% O_2 , 65% N_2) tanks were prepared and the concentration verified at a local scuba shop (Depth Perceptions Diving Services, San Luis Obispo, California).

Lizards were randomly assigned to either hypoxic, normoxic, or hyperoxic treatments. All lizards were tested only once. A total of 83 lizards (25 hypoxic, 29 normoxic, and 29 hyperoxic) were tested over six days. On each day, we alternated the order of gas treatments to avoid possible confounding diel effects (e.g., Chong et al., 1973; Johnson, 1972). RTDs were inserted about 1 cm into the lizards' cloacae and secured with waterproof medical tape wrapped around the tail just posterior to the cloaca to prevent cloacal discharge from removing the probes. Lizards were then placed into test chambers.

To standardize the treatments across groups, we chose a system temperature set point of 30 °C. CROCS was activated and normoxic air was circulated through the system to bring the entire apparatus to equilibrium. We then waited for the lizards to heat to within 2 °C of the set point before we began to ramp the chamber temperature. At this time, we switched to treatment gas (hypoxic, normoxic, or hyperoxic), and a heating rate of 1 °C was initiated. This heating rate is optimal because it avoids potential acclimation at lower rates and a core T_b lag at higher rates due to thermal inertia (Lutterschmidt and Hutchison, 1997b). In two additional trials, we repeated the same steps without the 1 °C heating rate to serve as a qualitative control for the effects of hypoxic gas in the absence of increased temperature.

2.5. Testing procedure

During heating, at least two observers constantly monitored the lizards for three response variables. Gaping threshold was defined and

time stamped on the laptop as the body temperature at which the first time a lizard opened its mouth. Panting threshold was defined and time stamped as the body temperature at which a lizard held its mouth open for at least 4 s accompanied by heavy chest compressions. Once lizards began gaping, we regularly performed rapid inversions of the test chambers such that lizards were flipped onto their backs, whereupon they rapidly righted themselves. LORR was defined and time stamped as the T_b at which lizards were unable to or made no attempt to right themselves despite vigorous manual shaking of the chambers. As soon as LORR occurred, lizards were removed from their test chambers to cool. Over the course of the study, no lizards died from the heating treatment, so all data were included in the analysis. During cooling, lizards were toe-clipped for permanent identification to avoid future recapture and then released that day to their original capture sites.

2.6. Data analysis

Data analyses were performed in JMP version 11.0. To meet the assumption of normality, the LORR data were transformed with a square power transformation. The gaping and panting data did not require transformation. All data met the assumptions for homogeneity of variance. We analyzed the data for the response variables of gaping threshold, panting threshold, and LORR with three separate ANOVA or ANCOVA models. The main effects tested included oxygen treatment (hypoxic, normoxic, or hyperoxic) and sex, along with the covariates SVL (mm), mass (g), holding time (the time from capture to testing, min), time of day, and hematocrit (%) and their interactions with treatment. After this initial model was built, covariates that were not significant were removed to generate a final model. In one case, we used a X^2 contingency test to compare the proportion of lizards in different treatments whose LORR surpassed 44 °C. We chose this as a biologically relevant extreme temperature because in a pilot study, most lizards exposed to normoxic air died when T_b exceeded 44 °C. To investigate whether thermal inertia could play a role in responses of lizards of varying body sizes, we ran two ANCOVAs testing the effects of oxygen treatment and either mass or SVL on the heat exposure time (the length of time lizards were heated until they lost the righting response). All results were considered significant at an alpha of 0.05.

3. Results

The final models for the analyses of gaping threshold, panting threshold, and LORR are shown in Table 1. Oxygen treatment had a significant effect on the gaping threshold temperature ($F_{2,61} = 6.3$, $p = 0.003$, Fig. 2) and the panting threshold temperature ($F_{2,61} = 14.9$, $p < 0.001$, Fig. 3). Tukey posthoc tests revealed that lizards subjected to hypoxic treatments had significantly lower gaping and panting thresholds than lizards breathing either normoxic or hyperoxic air, but that there was no difference in gaping or panting thresholds between lizards treated with normoxic and hyperoxic air. For panting, sex was also a significant factor ($F_{1,61} = 8.12$, $p = 0.006$, Fig. 4), with females having a higher panting threshold than males at all oxygen levels.

Table 1

Final models for the effects of oxygen treatment (hypoxic, normoxic, or hyperoxic gas), sex, and the covariates snout-vent length (mm) and holding time (min) on each of three response variables in *Sceloporus occidentalis*: gaping threshold, panting threshold, and loss of righting response (LORR).

Response variable	Factor/covariate	F	df	p
Gaping threshold	Treatment	6.3	2	0.003
	Sex	8.12	1	0.006
Panting threshold	Treatment	14.9	2	<0.001
	Sex	8.12	1	0.006
Loss of righting response (LORR)	Treatment	36.99	2	<0.0001
	Snout-vent length	4.61	1	0.04
	Holding time	2.89	1	0.09

Other factors and covariates did not significantly affect gaping or panting thresholds.

Oxygen treatment had a highly significant effect on LORR ($F_{2,58} = 36.99$, $p < 0.0001$, Fig. 5). Snout-vent length was a significant covariate ($F_{1,58} = 4.61$, $p = 0.04$), with larger lizards having lower LORR. Mass was also a significant covariate but was left out of the model because it was significantly related to SVL ($R = 0.48$, $p < 0.0001$) but accounted for less variation in the model than SVL. Body size did not significantly affect the heat exposure time (SVL: $F_{1,77} = 2.22$, $p = 0.14$; mass: $F_{1,77} = 1.47$, $p = 0.23$), and there were no significant interactions between body size variables and oxygen treatment (SVL * treatment: $F_{2,77} = 0.35$, $p = 0.71$; mass * treatment: $F_{2,77} = 1.86$, $p = 0.16$), indicating that differences in thermal inertia do not account for the observed effect of body size of LORR. Holding time was included as a covariate in the model because it approached significance ($F_{1,58} = 2.89$, $p = 0.09$), with lizards held longer before testing having higher LORR. Holding time was correlated with time of day ($R = 0.84$, $p < 0.0001$), so any potential effect could be due to either variable. Hematocrit and sex were not significant covariates and were left out of the model. Tukey posthoc tests showed that lizards subjected to the hypoxic treatment had significantly lower LORR than lizards breathing either normoxic or hyperoxic air, but that there was no difference in mean LORR between lizards treated with normoxic and hyperoxic air (Fig. 5). A X^2 contingency test revealed that a significantly larger proportion of lizards exposed to the hyperoxic treatment exhibited LORR above 44 °C than lizards exposed to the normoxic treatment ($N = 8$ or 27.6% of hyperoxic lizards vs. $N = 1$ or 3.4% of normoxic lizards, $X^2 = 6.44$, $p = 0.01$, Fig. 5).

We also tested ten additional lizards using unheated hypoxic gas (25 °C) to evaluate the effects of hypoxic air on LORR in the absence of heating. Only one lizard lost its righting response, indicating that inhalation of severely hypoxic air can occasionally affect this response variable in the absence of heating, but that heating dramatically increases its incidence (i.e., all lizards exposed to hypoxia and heated lost their righting response, Figs. 2, 3, 5).

4. Discussion

Our experiment provides support for the oxygen limitation hypothesis, which states that oxygen limitation is the mechanism behind ectotherms' responses to high temperatures, including gaping, panting, and LORR (often a proxy for the CTmax). Lizards exposed to hypoxic air gaped, panted, and reached the LORR at significantly lower temperatures than lizards exposed to normoxic or hyperoxic air. The mean gaping and panting thresholds and the LORR of lizards exposed to hyperoxic air were not significantly higher than those of lizards in normoxic conditions, partly due to high variability among lizards. The fact that a significantly higher proportion of lizards in the hyperoxic treatment was able to surpass 44 °C before losing the righting response is noteworthy and, together with the robust data on the effects of exposure to hypoxic conditions, provides support for the oxygen limitation hypothesis.

The oxygen limitation hypothesis has been supported in many studies of aquatic organisms, where oxygen availability is far lower than in air and is therefore expected to be a strong limiting variable (Eliason et al., 2013; Frederich and Pörtner, 2000; Pörtner, 2001, 2002; Pörtner and Knust, 2007; Verberk and Bilton, 2011; Verberk and Calosi, 2012). In marine fishes, thermal limitations on oxygen delivery in warming seas may comprise a primary factor responsible for fish extinction in coming years (Pörtner and Knust, 2007). However, Clark et al. (2013) cautions about the generality of the hypothesis and methodology, and indeed oxygen does not always limit thermal tolerance in aquatic organisms (Ern et al., 2014; Norin et al., 2014). In air-breathing organisms, studies have found various levels of support for the oxygen limitation hypothesis (Boardman and Terblanche, 2015; Bjelde et al., 2015; Klok et al., 2004; Koopman et al., 2016; Stevens et al., 2010; Verberk and

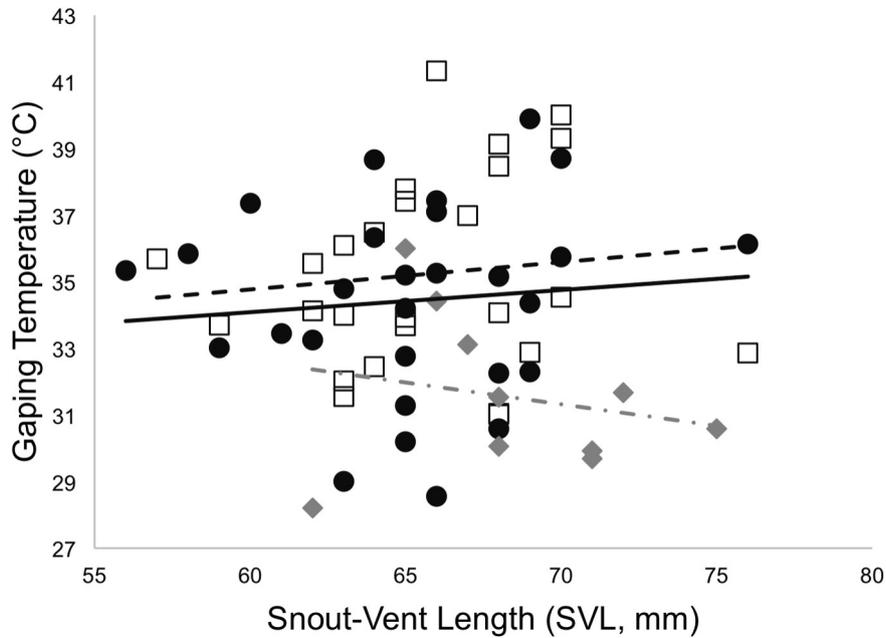


Fig. 2. Effect of oxygen concentration on the gaping threshold of *Sceloporus occidentalis*. Lizards exposed to 6% oxygen ($N = 25$) had significantly lower gaping thresholds than lizards exposed to 21% ($N = 29$) or 35% ($N = 29$) oxygen (ANOVA: $F = 6.3$, $df = 2$, $p = 0.003$). Body size (snout-vent length) did not affect gaping threshold.

Bilton, 2015; Verberk et al., 2016), including a recent study showing that lizard embryos die at lower temperatures when exposed to hypoxic conditions (Smith et al., 2015). Other studies have not supported this hypothesis (Fobian et al., 2014; McCue and De Los Santos, 2013; Overgaard et al., 2012; Seebacher and Franklin, 2011). For example, toads and pythons exposed to high temperatures did not exhibit reduced blood oxygen levels, which contradicts predictions of the oxygen limitation hypothesis (Fobian et al., 2014; Overgaard et al., 2012; Seebacher and Franklin, 2011). These studies examined how extreme temperatures affect cardiovascular function, but notably did not test animals for a drop in performance and did not manipulate oxygen levels. Other studies (Dupré et al., 1986; Tattersall and Gerlach, 2005) similarly did not test for LORR, but instead showed that hypoxic conditions (6–7% oxygen) reduced the gaping threshold of lizards. Lizards typically gape

and then pant when exposed to progressively increasing temperatures as a means of reducing body temperature via evaporative cooling (Tattersall et al., 2006), and these variables can therefore be used as measures of thermal tolerance. We similarly found that lizards gaped and panted at lower temperatures in hypoxic conditions than in normoxic conditions, showing that under hypoxia, animals experience heat stress at less extreme temperatures. The ecological relevance of this observation lies in the fact that under prolonged exposure to extreme heat (e.g., due to global climate change), animals may experience oxygen limitation even under normoxic conditions when their metabolic rates are elevated as they forage and digest, engage in mating activities, and attempt to escape from predators (Verberk et al., 2016). The mechanism by which low oxygen reduces gaping and panting thresholds as well as LORR would be further elucidated by measuring blood

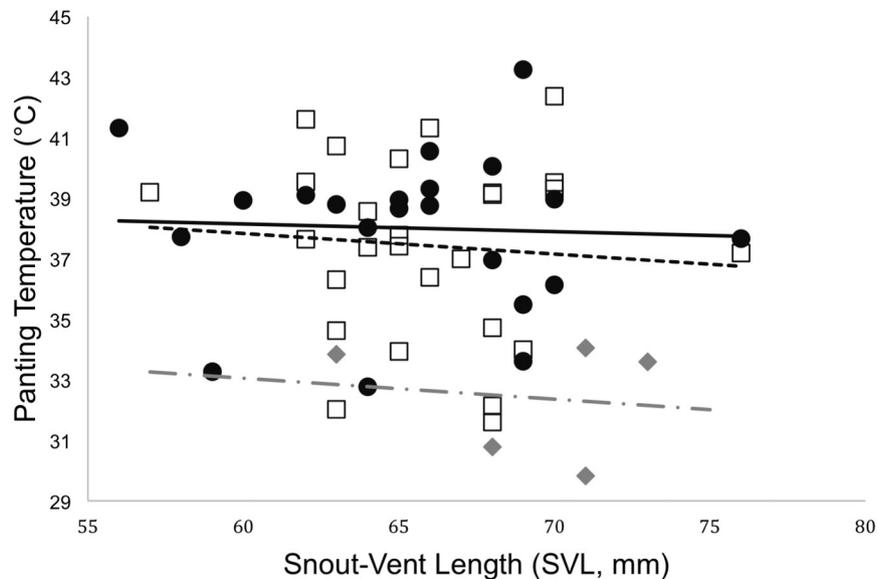


Fig. 3. Effect of oxygen concentration on the panting threshold of *Sceloporus occidentalis*. Lizards exposed to 6% oxygen ($N = 25$) had significantly lower panting thresholds than lizards exposed to 21% ($N = 29$) or 35% ($N = 29$) oxygen (ANOVA: $F = 14.9$, $df = 2$, $p < 0.001$). Body size (snout-vent length) did not affect panting threshold.

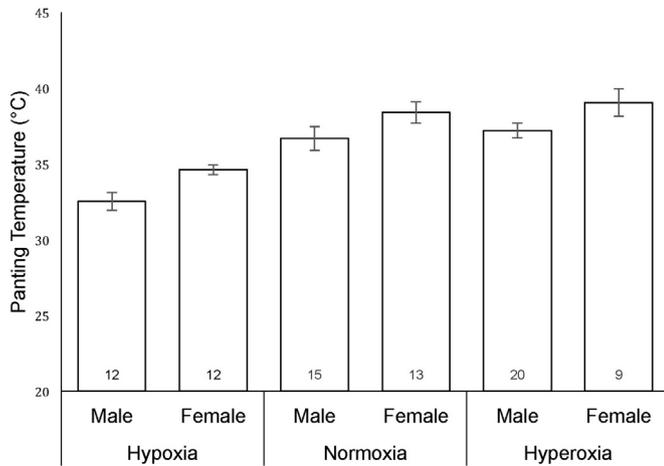


Fig. 4. Female *Sceloporus occidentalis* exhibited higher thermal tolerance in the form of the panting threshold than males, at each of three oxygen concentrations: hypoxic = 6%, normoxic = 21%, and hyperoxic = 35% (ANOVA: $F = 8.12$, $df = 1$, $p = 0.006$). Sample sizes of each sex are shown in the bars. Data are shown as means \pm s.e.m.

oxygen levels. It is possible that low blood oxygen imparted by breathing hypoxic air could be detected by peripheral or central chemoreceptors and signaled to the brain, and/or could directly limit central nervous system or muscle function, resulting in reduced thermal tolerance and the inability to right themselves.

The LORR and CTmax have long been used to examine effects of sex, acclimation, heating rate, feeding, and many other variables on thermal tolerance in lizards (reviewed in Lutterschmidt and Hutchison, 1997a, 1997b). Lutterschmidt and Hutchison (1997a) advocated using the body temperature associated with onset of spasms as the CTmax. The onset of spasms may result from altered protein structure and thus could be less variable than the LORR (Lutterschmidt and Hutchison, 1997a). However, the LORR is an ecologically relevant indicator because it represents the point at which performance declines due to oxygen limitation and at which lizards would be unable to move away from a heat source or predator. In line with our results for the gaping and panting thresholds, we found that lizards experienced LORR at far lower body temperatures in hypoxic conditions than in normoxic conditions, providing support for the oxygen limitation hypothesis.

We found that female fence lizards had higher panting thresholds than males at all three oxygen concentrations, but gaping and LORR were not different between the sexes. Although Heatwole et al. (1973)

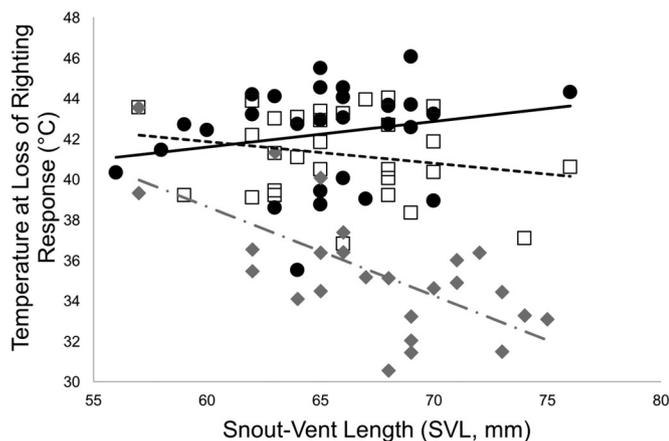


Fig. 5. Effect of oxygen concentration on the temperature at which the loss of righting response (LORR) occurred in *Sceloporus occidentalis*. Lizards exposed to 6% oxygen ($N = 25$) had significantly lower LORR than lizards exposed to 21% ($N = 29$) or 35% ($N = 29$) oxygen (ANCOVA: $F = 36.99$, $df = 2$, $p < 0.0001$). Larger lizards had lower LORR than smaller lizards ($F = 4.61$, $df = 1$, $p = 0.04$).

found no sex differences in panting thresholds in jacky dragons (under normoxic conditions), Tattersall and Gerlach (2005) found that female bearded dragons had lower gaping thresholds than males both in normoxic and hypoxic conditions. In our study, gravidity and hematocrit were not significant and were therefore not included in the models, so sex differences in reproductive investment or oxygen carrying capacity are unlikely explanations. Other factors such as potential sex differences in metabolic rate and muscle mass may explain the results. Alternatively, sex differences in thermal tolerance could reflect recent and differential thermal history of males and females in the field rather than inherent, physiological differences between the sexes (Hutchison and Dupré, 1992). For examples, if females were basking more than males prior to capture, their thermal tolerance might be temporarily higher than that of males. Such results are ecologically relevant because they represent natural conditions experienced by the lizards, but the sex effect could be dynamic and change according to multiple ecological variables. If this is true, then acclimation to laboratory conditions should abolish any sex differences observed in recently captured individuals. This would be a fruitful area for continued study.

Larger lizards had significantly lower LORR, showing that larger lizards are more susceptible to oxygen limitation than smaller lizards. This could be due to greater oxygen transport distance in the circulatory system of larger lizards, limiting the rate of oxygen delivery to tissues. Among terrestrial invertebrates, hypoxia has a much greater effect on thermal tolerance in species that rely on circulatory transport of oxygen than those that utilize a tracheal system (Klok et al., 2004; McCue and De Los Santos, 2013; Verberk and Bilton, 2013; Verberk and Bilton, 2015), suggesting that aspects of circulatory transport including transport distance, rate, and blood oxygen capacity may be important factors in how thermal tolerance is affected by hypoxia in terrestrial vertebrates. Another possibility is that the observed effect of body size on LORR is an artifact of the greater thermal inertia of larger lizards, where they would be expected to take longer to heat to than small lizards, which could reduce their thermal tolerance (Rezende et al., 2014). However, we found that neither SVL nor affected the length of time it took lizards to lose the righting response. These results suggest that the relatively dramatic effect of body size on LORR in hypoxic conditions is not likely the result of differential heat exposure times in individuals of different body sizes, but rather could be directly related to oxygen transport distance, metabolic rate, or other size-dependent variables.

Similar to results from other studies exposing air-breathing animals to hyperoxia (McCue and De Los Santos, 2013; Koopman et al., 2016), we did not detect differences in average thermal tolerance between lizards exposed to normoxic and hyperoxic air. This result was not surprising given how close the LORR and CTmax are to the upper lethal temperature in lizards (Cowles and Bogert, 1944) and given the amount of variation in LORR in lizards in the hyperoxic treatment group (Fig. 5). Additionally, normoxic treatment might have resulted in nearly or fully saturated hemoglobin such that treating subjects with hyperoxic gas would not further increase the level of oxygen in their blood. However, under hyperoxic conditions more lizards were able to exceed a temperature that had killed most lizards in a pilot study. This suggests the intriguing possibility that thermal tolerance in some lizards was elevated due to hyperoxic treatment. The mechanistic importance of this result merits additional study.

The experimental setup of CROCS was key to our ability to accurately detect the effects of oxygen on the responses of lizards to high temperatures. We were initially motivated to build CROCS so that we could provide individual lizards with specific concentrations of oxygen, something that cannot easily be done using traditional methods to evaluate thermal tolerance. Tattersall and Gerlach (2005) exposed lizards to varying concentrations of oxygen using larger chambers; however, CROCS is a multiplex, allowing rapid (~30 min) measurements of up to six lizards at once. CROCS also provides the additional benefits of improved control of two important variables: heating rate and accuracy of

lizard body temperature measurement. The rate at which test chambers and animals are heated can affect measurements of thermal tolerance (reviewed in Lutterschmidt and Hutchison, 1997b); however, the vast majority of studies simply attempt to manually control heating rate within a narrow range of values and fail to report how successful they were in doing so. In contrast, CROCS allows extremely consistent heating rates; in this study, heating rate was on average 0.93 ± 0.014 °C per minute. In addition, thermocouples, which are used to monitor animal body temperature in most studies, may only be accurate only to ± 2 °C (Tong, 2001), which could lead to tremendous error especially when responses of treatment groups are close in value, as in the normoxic and hyperoxic treatments in this study. In contrast, the RTDs used in this study have an accuracy of ± 0.5 °C, making them very useful for accurately detecting fine-scale differences in temperature. Finally, CROCS is field portable, which will allow future experiments to be conducted in situ.

In conclusion, we have experimentally demonstrated that oxygen availability is one mechanism contributing to lizards' responses to high body temperatures, including gaping, panting, and LORR. Understanding how the oxygen treatments affect blood oxygen kinetics would improve our ability to hone in on the ways in which oxygen limits the temperatures an animal is able to withstand. Another way to manipulate oxygen availability to the tissues is to experimentally induce anemia (Wang et al., 2014); concomitant reduction of the LORR would provide further support for the oxygen limitation hypothesis. In addition, it would be interesting to examine how exposure to hypoxic or hyperoxic conditions interacts with adaptation to different thermal (e.g., warm- versus cold-adapted populations) and oxic (e.g., low versus high altitude) environments to affect animals' responses to high temperatures. Given that recent models predict that increasing global temperatures could induce massive extinctions (Thomas et al., 2004), including as many as 20% of lizard species (Sinervo et al., 2010), it is essential that we continue to explore the mechanisms that reduce the performance of individuals as they are heated. Understanding these mechanisms will allow us to better predict animals' responses to global climate change, which will help inform conservation efforts and identify at-risk species and potential subjects of future studies on thermal physiology.

Conflicts of interest

None.

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