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# Rapid Physiological Plasticity in Response to Cold Acclimation for Nonnative Italian Wall Lizards (*Podarcis siculus*) from New York

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## ABSTRACT

Thermal physiology helps us understand how ectotherms respond to novel environments and how they persist when introduced to new locations. Researchers generally measure thermal physiology traits immediately after animal collection or after a short acclimation period. Because many of these traits are plastic, the conclusions drawn from such research can vary depending on the duration of the acclimation period. In this study, we measured the rate of change and extent to which cold tolerance (critical thermal minimum [ $CT_{min}$ ]) of nonnative Italian wall lizards (*Podarcis siculus*) from Hempstead, New York, changed during a cold acclimation treatment. We also examined how cold acclimation affected heat tolerance (critical thermal maximum [ $CT_{max}$ ]), thermal preference ( $T_{pref}$ ), evaporative water loss (EWL), resting metabolic rate (RMR), and respiratory exchange ratio (RER). We predicted that  $CT_{min}$ ,  $CT_{max}$ , and  $T_{pref}$  would decrease with cold acclimation but that EWL and RMR would increase with cold acclimation. We found that  $CT_{min}$  decreased within 2 wk and that it remained low during the cold acclimation treatment; we suspect that this cold tolerance plasticity reduces risk of exposure to lethal temperatures during winter for lizards that have not yet found suitable refugia.  $CT_{max}$  and  $T_{pref}$  also decreased after cold acclimation, while EWL, RMR, and RER increased after cold acclimation, suggesting trade-offs with cold acclimation in the form of decreased heat tolerance and increased energy demands. Taken together, our findings suggest that cold tolerance plasticity aids the persistence of an established population of invasive lizards.

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More generally, our findings highlight the importance of accounting for the plasticity of physiological traits when investigating how invasive species respond to novel environments.

**Keywords:** acclimation, cold tolerance, critical thermal maximum, critical thermal minimum, evaporative water loss, invasive species, lacertid, metabolic rate, thermal preference.

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## Introduction

Temperature has a profound impact on terrestrial ectotherms at multiple biological scales. Consequently, behavioral thermoregulation and tolerance to extreme temperatures are commonly studied when investigating an ectotherm's adaptation to its thermal environment (Kearney and Porter 2004; Huey et al. 2009; Clusella-Trullas et al. 2011). These physiological traits can be used to estimate how ectotherms will respond to novel stressful conditions (Kearney and Porter 2004; Deutsch et al. 2008; Sinervo et al. 2010). The capacity for change in physiological traits within an individual's lifetime (i.e., reversible plasticity) can also alter an organism's fitness and be informative as to how ectotherms will respond to novel environments (Fusco and Minelli 2010; Gunderson and Stillman 2015).

One way that organisms are exposed to novel environments is through introduction to a new region. The ability of an introduced species to rapidly acclimatize to novel stressors can quickly facilitate its successful establishment in a novel environment (Gabriel et al. 2005). One meta-analysis found that nonnative species generally exhibit greater plasticity than co-occurring native species (Davidson et al. 2011). Plasticity of a trait is therefore important to consider when using a trait to investigate how some species survive introductions to stressful environments. For example, McCann et al. (2018) found that in nonnative populations of cane toads (*Rhinella marina*), populations from cool climates showed a decrease in cold tolerance (critical thermal minimum [ $CT_{min}$ ]) after an overnight acclimation treatment to cooler temperatures, whereas populations from warmer climates did not show this plasticity. Had cold tolerance been measured in only the toads from the warm climate or without any acclimation treatment at all, this seemingly adaptive plastic response would not have been discovered. When researchers do incorporate the plasticity of a physiological trait, however, they tend to focus on whether a value changes in response to a temperature change in a lab setting (e.g.,

studies reviewed by Clusella-Trullas and Chown 2014; Gunderson and Stillman 2015) or in response to a seasonal temperature change in a wild setting (Litmer and Murray 2020). The rate of the change and/or the extent to which a trait has changed is rarely determined or considered.

For a trait such as cold tolerance, which correlates with ambient temperature (Araújo et al. 2013) and tends to show high variability and plasticity (Clusella-Trullas and Chown 2014; Gunderson and Stillman 2015; Litmer and Murray 2019), taking the duration of acclimation into account when measuring and reporting values is particularly important. Pintor et al. (2016) found that because of short experimental acclimation times of 0–2 wk, most studies measuring cold tolerance of reptiles may overestimate the  $CT_{min}$  that could be reached given an extended acclimation treatment. Overestimating  $CT_{min}$  would lead one to misinterpret the negative effect of cold exposure and to possibly overlook cold tolerance plasticity as a key factor in an ectotherm's winter survival. One could account for plasticity of  $CT_{min}$  by holding animals for a relatively long period before testing and/or by comparing only animals that have experienced similar thermal regimes before testing. For example, Leal and Gunderson (2012) found that a nonnative population of Puerto Rican anoles (*Anolis cristatellus*) that experienced a colder climate than the native source population had a lower  $CT_{min}$  than the source population, even after extensive exposure (~49 d) to nearly identical conditions. Although one can make comparisons following extensive exposure to similar conditions under the assumption that  $CT_{min}$  has reached a stable value, it is necessary to measure  $CT_{min}$  throughout acclimation to determine whether there was a plastic response in the first place. Determining this can also inform how incipient populations of lizards will respond to colder environments.

Nonnative species introduced to cold climates provide test cases for how an ectotherm will respond to extreme cold as a stressor. One such ectotherm, the Italian wall lizard (*Podarcis siculus*, Rafinesque 1810), has been remarkably successful at establishing populations in various locations across the northern United States (Kolbe et al. 2013; Donihue et al. 2015; Donihue 2017). A population introduced to Hempstead, Long Island, New York, in 1966 is thought to be the source of more recently established populations in and outside of New York (Burke and Deichsel 2008; Donihue et al. 2015). Given the extreme winter weather of the northeastern United States, we were interested in whether plasticity of physiological traits may help these lizards survive extreme cold. We focused on lizards from Hempstead, New York, because of their relatively long-term success in a region that experiences prolonged freezing winter conditions. We investigated how cold acclimation would affect cold tolerance ( $CT_{min}$ ), heat tolerance (critical thermal maximum [ $CT_{max}$ ]), selection of body temperatures ( $T_b$ 's; thermal preference [ $T_{pref}$ ]), evaporative water loss (EWL), and resting metabolic rate (RMR). From metabolic rate measurements, we also calculated respiratory exchange ratios (RERs; ratio of  $CO_2$  volume produced to  $O_2$  volume consumed). We hypothesized that cold tolerance plasticity is one of the mechanisms allowing *P. siculus* to persist in Hempstead, New York. We therefore expected lizards to change cold tolerance

in response to a cold acclimation treatment. Because thermal physiology often involves trade-offs, such that acclimation to one extreme results in loss of performance at the other extreme, we expected heat tolerance and  $T_{pref}$  to decrease as well. Similarly, because fluidity of biological systems tends to increase with cold acclimation to compensate for the rigidity caused by cold (Hochachka and Somero 2002), we expected EWL rate and RMR to increase following cold acclimation.

## Material and Methods

### *Study Organism, Husbandry, and Cold Acclimation*

In July 2016, we collected 24 *Podarcis siculus campestris* (12 males, 12 females) from Hempstead, New York (for a summary of mass and snout-vent length, see appendix; table A1). We used only adults above the minimum size for reproductive maturity (55 mm for females, 57 mm for males; G.B. Pauly and S.R. Goldberg, unpublished data) because thermal physiology differs between adults and juveniles of a *Podarcis siculus siculus* population from San Pedro, California (Liwanag et al. 2018). Lizards were housed individually in 10-L plastic terraria (25 cm × 16 cm × 16 cm, Lee's Aquarium and Pet Products, San Marcos, CA) with approximately 1.5 cm of shredded bark used as a substrate (Bark Blend, Zilla, Franklin, WI). Each terrarium was placed halfway atop a heating pad (Sunbeam, Boca Raton, FL), creating a 27°C–32°C gradient across the terrarium. A water bowl and a hide were placed on the cooler side of each terrarium to reduce evaporation. All terraria were situated under full-spectrum lighting set to a 12L:12D cycle. Lizards were fed four to five crickets twice weekly and given water ad lib. Crickets were dusted with vitamins (calcium and vitamin D) every third feeding. Lizards were housed under identical laboratory conditions for at least 8 mo before any testing to ensure that measurements did not reflect recent thermal history before capture. Timings of laboratory conditions and assays are presented in the appendix (see fig. A1). All animal use protocols were evaluated and approved by the Institutional Animal Care and Use Committee at California Polytechnic State University, San Luis Obispo (protocol 1514).

Before cold acclimation, lizards were tested for cold tolerance, heat tolerance,  $T_{pref}$ , EWL, and RMR. After initial testing, all lizards were moved with their original cages into large environmental chambers (plant growth chamber, Precision model 818, Thermo Scientific, Waltham, MA) with no heating pads. Our acclimation treatment was designed to mimic average surface temperatures reported by the National Oceanic and Atmospheric Administration's 1981–2010 climate normals for Hempstead, New York, from September to October, a time when *P. siculus* activity is severely decreased (Burke and Ner 2005; Arguez et al. 2012). This temperature acclimation treatment began with 1 wk of 20°C:18°C (12L:12D) to avoid shocking the lizards with a ≥10°C drop in temperature, followed by 4 wk of 17°C:16.5°C (12L:12D). We initially decreased temperatures to 17°C:15°C after the 20°C:18°C weeklong treatment, but we increased the nighttime temperature after the first night because of observed lethargy in lizards the following day; this lethargy resolved with the increase in nighttime temperature to 16.5°C. Temperatures during the dark

photoperiod were only slightly lower than daytime temperatures so as to mimic field conditions in which lizards behaviorally avoid exposure to surface air temperatures at night. The photoperiod remained the same as before acclimation. During cold acclimation, lizards were offered one to two crickets weekly and given water ad lib. Cold tolerance was tested on all lizards weekly for 5 wk during the acclimation period, with the exception of four lizards that were tested only at week 5 to determine whether repeated testing had an effect on cold tolerance. After 5 wk of cold acclimation, we remeasured heat tolerance,  $T_{\text{pref}}$ , EWL, and RMR in addition to cold tolerance. Lizards were maintained at 17°C:16.5°C for an additional 7 wk, and cold tolerance was tested once more at the end of the extended cold acclimation period to determine final cold tolerance values. The full temperature acclimation treatment lasted 12 wk (1 wk at 20°C:18°C, 11 wk at 17°C:16.5°C). After the treatment, lizards were returned to room temperature (~27°C) for 2 wk to allow for gradual reacclimation, and then they were provided heat mats to achieve the full preacclimation range of temperatures.

#### Data Collection

We measured cold tolerance ( $CT_{\text{min}}$ ) as the  $T_b$  at which a lizard lost mobility during a low-temperature challenge (Cowles and Bogert 1944; Huey and Stevenson 1979; Angilletta et al. 2002). We recorded  $CT_{\text{min}}$  using a custom-designed device similar to that described by Shea et al. (2016). In brief, lizards were placed inside individual acrylic chambers, and a 28-G resistance temperature detector probe (HEL-705 RTD, Honeywell, Charlotte, NC) connected to our device was inserted 3 mm into each lizard's cloaca and secured with medical tape. We then cooled each lizard's surrounding air temperature by 1°C min<sup>-1</sup>. Although this could lead to different cooling durations across treatments (i.e., for lizards from the 27°C–32°C, 20°C:18°C, and 17°C:16.5°C conditions), previous work on the tsetse fly suggests that cooling rate is a more important determinant of  $CT_{\text{min}}$  than starting temperature (Terblanche et al. 2007). When a lizard's  $T_b$  reached 15°C, that lizard's chamber was quickly turned to flip the lizard onto its back, and the lizard was given 5 s to right itself. If the lizard did not right itself within 5 s, the chamber was gently shaken. If the lizard did not right itself after gentle shaking, the lizard was righted. If the lizard immediately began walking forward within the temperature-controlled tube after it was righted, we presumed that it was still able to escape potentially lethal conditions, and we continued cooling at 1°C min<sup>-1</sup>; otherwise, we recorded the current  $T_b$  as the lizard's  $CT_{\text{min}}$ . Lizards were retested every 1.5 min two more times. After these initial 3 min, loss of righting response was tested every minute for the remainder of the trial until  $CT_{\text{min}}$  was determined.

We assessed heat tolerance ( $CT_{\text{max}}$ ) using the same equipment and in a similar manner to that described for  $CT_{\text{min}}$ , with a heating rate of 1°C min<sup>-1</sup>. For  $CT_{\text{max}}$  measurements, lizards were initially brought to a  $T_b$  of 25°C at a constant chamber temperature and subsequently warmed at 1°C min<sup>-1</sup>. Once  $T_b$  reached 36°C, lizards were tested for righting response every 1.5 min until their  $T_b$ 's reached 40°C. Lizards were then tested every 30 s because

lethal temperatures are relatively close to the warmer temperatures at which most lizard species lose righting response (Lutterschmidt and Hutchison 1997). Because we did not observe the onset of muscle spasms for any individuals in this study,  $T_b$  at loss of righting response was the only variable measured during heating and used to define  $CT_{\text{max}}$  (Lutterschmidt and Hutchison 1997).

We measured preferred  $T_b$ 's ( $T_{\text{pref}}$ ) using a linear temperature gradient (163 cm L × 46 cm W) divided into four lanes running the length of the gradient, with a temperature range from 10°C to 40°C along its length. White dividers were placed between lanes so that lizards could not see each other so as to reduce the potential effects of visual cues on spacing. A 1-cm-thick plastic panel was placed above the gradient to restrict airflow between lanes and to reduce potential effects of neighboring scents on spacing. The cold end of the gradient was maintained with cold water circulated through copper piping beneath the metal floor of the gradient, using a circulating water bath set to 10°C (SD07R-20, PolyScience, Niles, IL). The warm end of the gradient was maintained by electric heat strips beneath the metal floor of the gradient. The floor of the gradient was covered with ReptiSand (Zoo Med, San Luis Obispo, CA) so that lizards were not in direct contact with the metal floor. The gradient was encased in a Plexiglas chamber lined with Drierite (W.A. Hammond Drierite, Xenia, OH) to reduce condensation. Each lizard was fitted with a flexible 40-G type-K thermocouple (Omega Engineering, Stamford, CT) inserted into the lizard's cloaca and secured with medical tape. Four lizards were tested simultaneously, with a single lizard in each lane of the gradient, and  $T_b$  was monitored continuously. Because time of day can influence  $T_{\text{pref}}$  (Cowgell and Underwood 1979; Carretero et al. 2006), we began trials at 0900 hours for consistency and recorded  $T_b$  every 10 min for 4 h. We focused our analyses on the 50% interquartile range (values between the 75th and 25th percentiles) of body temperatures during the last 2 h to account for outliers (Huey 1982; Hertz et al. 1993). We limited data to the last 2 h because of observed exploratory behavior during the first 2 h of trials. We calculated  $T_{\text{pref}}$  as the average  $T_b$  within the resulting interquartile range for each lizard.

We estimated rates of EWL (mg H<sub>2</sub>O h<sup>-1</sup>) by observing mass lost over time at four experimental temperatures (10°C, 20°C, 30°C, and 40°C). Individual lizards were tested across multiple temperatures, and lizards were never measured at more than one temperature per day. Before each trial, lizards were fasted for 5–7 d, placed inside individual fiberglass mesh pouches (18 × 6 mesh count), and weighed to the nearest 0.001 g using a digital balance (Practicum 213, Sartorius, Bohemia, NY). Lizards in pouches were placed atop individual petri dishes on three separate shelves inside an environmental chamber (plant growth chamber, Precision model 818, Thermo Scientific) set to 10°C, 20°C, 30°C, or 40°C for 7.5 h. Every 1.5 h, we reweighed lizards and rotated their positions among the shelves to control for any potential inconsistency of temperature, humidity, and/or airflow within the chamber (four total rotations). Absolute humidity estimated from measured relative humidity was similar across temperatures (3, 4, 5, and 5 g m<sup>-3</sup> at 10°C, 20°C, 30°C, and 40°C, respectively). Although lizards may have urinated during the trials,

urate was never observed on petri dishes; on the rare occasion when feces were observed in a pouch, the feces mass was negligible (i.e., weighed less than 0.001 g), so trials were continued with the feces removed. Experimental temperatures were tested in random order. Following 5 wk of the cold acclimation treatment, the first EWL trial was randomly selected to be 40°C. This trial resulted in the death of four lizards at the first weighing (within 1.5 h). We consequently terminated the 40°C postacclimation trial to prevent further animal loss and excluded the 40°C level within the temperature factor from statistical comparisons of pre- and postacclimation values. An additional lizard died of unknown causes unrelated to the EWL trial before the cold acclimation treatment.

We measured resting metabolic rates of oxygen consumption ( $\text{RMR}_{\text{O}_2}$ ;  $\text{mL O}_2 \text{ h}^{-1}$ ) and carbon dioxide production ( $\text{RMR}_{\text{CO}_2}$ ;  $\text{mL CO}_2 \text{ h}^{-1}$ ) by using stop-flow respirometry. We made measurements on fasted lizards during the day in chambers with fluorescent lighting. Lizards were fasted 5–7 d before any RMR testing. Before each trial, fasted lizards were weighed to the nearest 0.001 g with a digital balance and then individually placed into 150-mL plastic syringes with a hole drilled into the 150-mL mark near the top of the syringe for ventilation. Syringes with lizards were then placed into an environmental chamber set to either 15°C or 35°C, and lizards were allowed to rest for 45 min with access to ambient air through the drilled hole. After this rest period, each syringe was flushed with ambient air for 5 min using an aquarium pump, sealed at the 140-mL mark, and returned to the environmental chamber for an additional 45 min. We then injected a 20-mL subsample of air from the syringe into a flow of baseline air that was being analyzed for  $\text{O}_2$  and  $\text{CO}_2$  concentrations.  $\text{O}_2$  and  $\text{CO}_2$  concentrations were measured using a Sable Systems FC-10 oxygen analyzer and CA-10 carbon dioxide analyzer (Sable Systems International, Las Vegas, NV), respectively. Using an air pump and flowmeter located at the end of our system, we pulled baseline air through the analyzers at a rate of  $100 \text{ mL min}^{-1}$  from outside the building through tubing connected to a carboy. This air was scrubbed of water using a magnesium perchlorate ( $\text{Mg}(\text{ClO}_4)_2$ ) column before entering the  $\text{CO}_2$  analyzer, and then it was scrubbed of  $\text{CO}_2$  and water again using a Drierite/Ascarite/Drierite column before entering the  $\text{O}_2$  analyzer. Following the equations of Lighton (2008), we calculated the rate of  $\text{O}_2$  consumption and  $\text{CO}_2$  production for each 20-mL sample of injected air using ExpeData analysis software (Sable Systems International). These values were used to estimate  $\text{RMR}_{\text{O}_2}$  and  $\text{RMR}_{\text{CO}_2}$  for each lizard. We divided the volume of  $\text{CO}_2$  produced by the volume of  $\text{O}_2$  used within an hour to determine RER for each lizard. We noticed a trend toward an increasing RER at 15°C after cold acclimation, so we measured  $\text{RMR}_{\text{O}_2}$  and  $\text{RMR}_{\text{CO}_2}$  at week 11 of cold acclimation to determine whether the trend was consistent.

#### Data Analysis

We performed studies on subsets of lizards for each variable because of material and time constraints and attrition of four lizards at week 5 of cold acclimation. We report the timing and

sample sizes for all measured variables in figure legends and in the appendix (see fig. A1; tables A1–A5). We compared  $\text{CT}_{\text{min}}$  values at seven different time points (preacclimation, week 1, week 2, week 3, week 4, week 5, week 12) using a mixed effects model with time as an ordered factor and lizard ID as a random effect. We made pairwise comparisons between each time point and the subsequent time point to determine whether there were changes in  $\text{CT}_{\text{min}}$  values from one time point to the next. We also used pairwise comparisons to determine whether  $\text{CT}_{\text{min}}$  values at week 5 for lizards that had been tested weekly during cold acclimation differed from  $\text{CT}_{\text{min}}$  values at week 5 for lizards that had not yet been tested during cold acclimation. To describe  $\text{CT}_{\text{min}}$  acclimation over time with a mathematical function, we fitted an exponential model to our data that included all  $\text{CT}_{\text{min}}$  data, regardless of statistical difference from a previous week; the purpose of this was to describe  $\text{CT}_{\text{min}}$  at the asymptote.

To investigate potential benefits of cold tolerance plasticity, we extracted average monthly low surface temperatures for Hempstead, New York, using the minimum temperature ( $T_{\text{min}}$ ) variable of the WorldClim dataset (ver. 1.4; Hijmans et al. 2005). We then created a polynomial equation describing  $T_{\text{min}}$  in Hempstead, New York, throughout a 365-d period, beginning with the first day of January. We used the equation describing  $T_{\text{min}}$  throughout the year to estimate the number of days when  $T_{\text{min}}$  dropped below  $\text{CT}_{\text{min}}$  before and after cold acclimation, and we used the difference in days to estimate how beneficial  $\text{CT}_{\text{min}}$  plasticity may have been within the year (i.e., how many more days of activity are conferred by the cold acclimation response).

We used linear mixed effects models to investigate the effect of acclimation period (preacclimation, week 5 of acclimation) on  $\text{CT}_{\text{max}}$ ,  $T_{\text{pref}}$ , EWL, RMR, and RER. In all models, we accounted for repeated measurements by including lizard ID as a random effect and accounted for sex by including sex as a fixed effect. We included mass as a covariate in  $\text{CT}_{\text{min}}$ ,  $\text{CT}_{\text{max}}$ ,  $T_{\text{pref}}$ , RMR, and RER models. We included surface area (estimated as  $\text{mass}^{0.67}$ ) as a covariate in the EWL model. We included temperature as a fixed effect and included an interaction between temperature and acclimation period in EWL, RMR, and RER models. Model residuals were visually inspected to ensure that data met the assumptions of parametric analysis. We log transformed EWL data to normalize residuals. We also removed a datum outlier to ensure model residuals were normally distributed in our  $T_{\text{pref}}$  model; we present the  $T_{\text{pref}}$  model diagnostics and analysis with and without the datum removed in the appendix (see tables A6, A7; figs. A2–A5). Summary statistics are presented as mean  $\pm$  SE in the text and in tables A1–A5. We used  $z$ -tests (pairwise contrasts) to compare  $\text{CT}_{\text{min}}$  values at each time point with  $\text{CT}_{\text{min}}$  values at the subsequent time point and to investigate interactions between temperature and acclimation period in EWL, RMR, and RER models. We corrected  $P$  values for multiple tests using the Holm method. Models were created and analyzed using the nlme, lme4, and lmerTest packages; post hoc tests were conducted using the multcomp package; and climate data were extracted using the raster package in R version 3.6.1 (R Development Core Team 2021). We considered  $P < 0.05$  to be statistically significant.

## Results

We found that  $CT_{\min}$  significantly decreased during the cold acclimation treatment ( $F_{1,88.0} = 9.81, P < 0.001$ ; fig. 1).  $CT_{\min}$  was  $10.2^{\circ}\text{C} \pm 0.7^{\circ}\text{C}$  after 8 mo of acclimation to laboratory housing conditions before cold acclimation. After 1 wk of cold acclimation ( $20^{\circ}\text{C}:18^{\circ}\text{C}$ ),  $CT_{\min}$  did not decrease significantly, but  $CT_{\min}$  did decrease significantly to  $8.0^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$  after 1 wk at  $17^{\circ}\text{C}:16.5^{\circ}\text{C}$  (corrected  $P = 0.007$ ; fig. 1). At week 5, the  $CT_{\min}$  of four cold-acclimating lizards that had not been previously tested during cold acclimation did not differ from the  $CT_{\min}$  of lizards that had been previously tested (corrected  $P = 0.995$ ), and the four lizards were therefore included in the week 5 analyses. Overall,  $CT_{\min}$  at week 5 of cold acclimation ( $6.5^{\circ}\text{C} \pm 0.4^{\circ}\text{C}, n = 21$ ) was significantly lower than  $CT_{\min}$  at preacclimation ( $10.2^{\circ}\text{C} \pm 0.7^{\circ}\text{C}, n = 8$ , corrected  $P < 0.001$ ; fig. 2). Neither sex nor mass was a significant predictor of  $CT_{\min}$  in our model (sex:  $F_{1,20.6} = 1.53, P = 0.230$ ; mass:  $F_{1,38.4} = 0.03, P = 0.867$ ). The exponential model of best fit took the following form, where  $w$  is week:

$$CT_{\min} = 6.61 + 4.33e^{-0.54w}.$$

According to the exponential equation, once lizards begin acclimating,  $CT_{\min}$  would decline from the preacclimation value of  $10.94^{\circ}\text{C}$  and reach asymptote at  $6.61^{\circ}\text{C}$ . Using climate data extracted for Hempstead, New York, we found that if no acclimation occurs and if  $CT_{\min}$  stays at  $10.94^{\circ}\text{C}$ , there would be 218 full days in which low surface temperatures decrease below  $CT_{\min}$  (fig. 3). If acclimation occurs and if  $CT_{\min}$  reaches  $6.61^{\circ}\text{C}$ , there would be 174 full days with low surface temperatures below  $CT_{\min}$  (fig. 3). Across the year, plasticity would result in an additional 44 full days in which average low surface temperatures are above  $CT_{\min}$ .

$CT_{\max}$  significantly decreased from  $43.0^{\circ}\text{C} \pm 0.4^{\circ}\text{C}$  at preacclimation ( $n = 8$ ) to  $41.1^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  at week 5 ( $n = 9, F_{1,7.3} = 18.17, P = 0.003$ ; fig. 2). Neither sex nor mass was a significant predictor of  $CT_{\max}$  (sex:  $F_{1,10.8} = 0.02, P = 0.900$ ; mass:  $F_{1,13.0} = 0.94, P = 0.349$ ).

Initial  $T_{\text{pref}}$  model residuals suggested that an outlier occurring approximately 2.26 SDs from the preacclimation mean was skewing

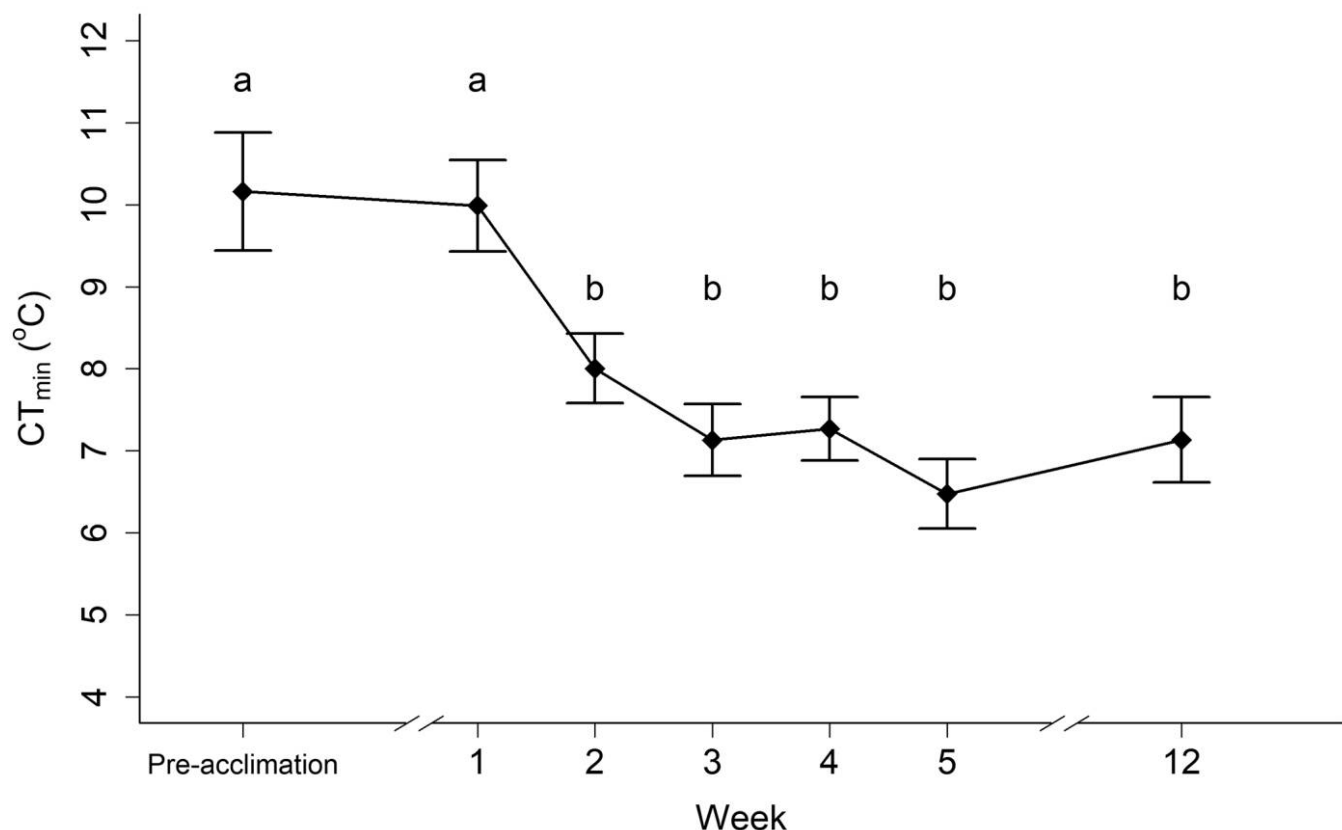


Figure 1. Change in cold tolerance (critical thermal minimum [ $CT_{\min}$ ],  $^{\circ}\text{C}$ ) for *Podarcis siculus* over time during a cold acclimation treatment. Diamonds indicate the mean, and whiskers represent standard error. Preacclimation conditions consisted of living in a gradient of  $27^{\circ}\text{C}$ – $32^{\circ}\text{C}$ . Lizards were held at preacclimation conditions for 8 mo before being tested for  $CT_{\min}$  ( $n = 8$ ). Lizards were moved to conditions at  $20^{\circ}\text{C}:18^{\circ}\text{C}$  (12L:12D) for 1 wk, and  $CT_{\min}$  was measured again ( $n = 17$ ). Lizards were then moved to conditions at  $17^{\circ}\text{C}:16.5^{\circ}\text{C}$  (12L:12D) and tested for  $CT_{\min}$  weekly from week 2 to week 5 (weeks 1–4:  $n = 17$ ; week 5:  $n = 21$ ) and again at week 12 ( $n = 15$ ). Lowercase letters above whiskers represent statistically different groups, as determined by Tukey's all-pair comparisons; groups not sharing letters are significantly different from each other at the  $P < 0.05$  level.

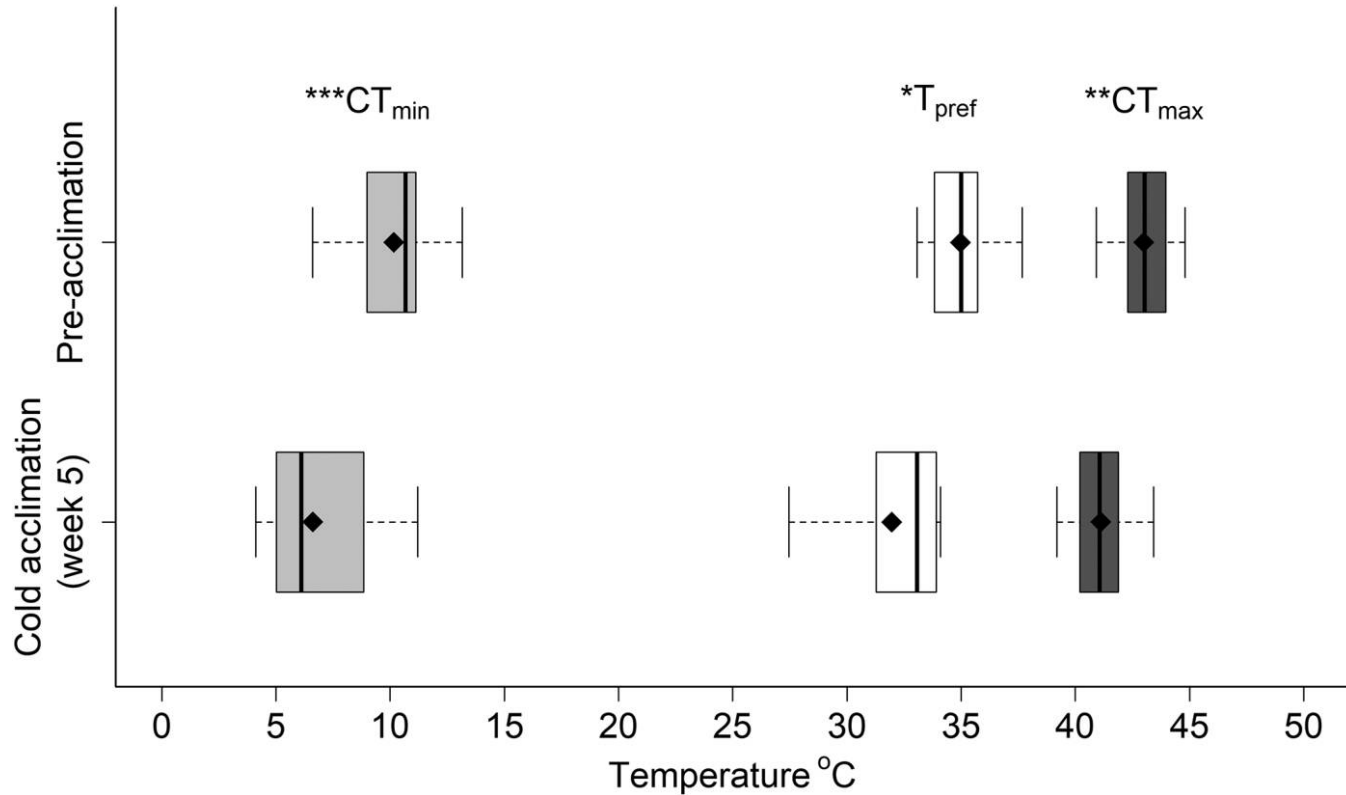


Figure 2. Cold tolerance (critical thermal minimum [CT<sub>min</sub>], °C, light gray), thermal preference ( $T_{\text{pref}}$ , °C, white), and heat tolerance (critical thermal maximum [CT<sub>max</sub>], °C, dark gray) for *Podarcis siculus* before cold acclimation treatment (upper boxplots; CT<sub>min</sub>:  $n = 8$ ;  $T_{\text{pref}}$ :  $n = 7$ ; CT<sub>max</sub>:  $n = 8$ ) and at week 5 of cold acclimation treatment (lower boxplots; CT<sub>min</sub>:  $n = 21$ ;  $T_{\text{pref}}$ :  $n = 5$ ; CT<sub>max</sub>:  $n = 9$ ). The vertical line within each box indicates the median value, and the box boundaries indicate the lower quartile (left boundary) and the upper quartile (right boundary). Whiskers indicate minimum and maximum values within a quartile  $\pm 1.5$  times the interquartile range. Diamonds within boxplots indicate the mean values. Levels of significance for thermal tolerance and thermal preference differences between pre- and postacclimation values are marked as follows: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

results and violating model assumptions (see appendix). We removed the preacclimation datum and its associated post-acclimation datum from our  $T_{\text{pref}}$  model to ensure that model residuals were normally distributed (see appendix). The resulting  $T_{\text{pref}}$  significantly decreased from  $35.0^{\circ}\text{C} \pm 0.6^{\circ}\text{C}$  at pre-acclimation ( $n = 7$ ) to  $32.0^{\circ}\text{C} \pm 1.2^{\circ}\text{C}$  at week 5 ( $n = 5$ ,  $F_{1,8} = 10.02$ ,  $P = 0.013$ ; fig. 2). Neither sex nor mass was a significant predictor of  $T_{\text{pref}}$  (sex:  $F_{1,8} = 0.03$ ,  $P = 0.872$ ; mass:  $F_{1,8} = 3.96$ ,  $P = 0.082$ ).

The effect of temperature was not significantly different between acclimation periods in our log-transformed EWL (logEWL) model at the 0.05 level, although the interaction between temperature and acclimation period approached significance ( $F_{2,52.4} = 3.12$ ,  $P = 0.053$ ; fig. 4). LogEWL significantly increased with temperature, as expected ( $F_{3,52.0} = 63.5$ ,  $P < 0.001$ ). Acclimation was also a significant predictor of logEWL ( $F_{1,58.8} = 4.50$ ,  $P = 0.038$ ). Post hoc tests revealed that logEWL at  $10^{\circ}\text{C}$  significantly increased at week 5 of cold acclimation (corrected  $P = 0.013$ ; fig. 4). Neither surface area nor sex was a significant predictor of logEWL (surface area:  $F_{1,10.3} = 0.22$ ,  $P = 0.649$ ; sex:  $F_{1,9.7} = 0.12$ ,  $P = 0.736$ ).

The effect of temperature on RMR<sub>O<sub>2</sub></sub> was not significantly different between acclimation periods, with probability of a type 1 error occurring exactly at our a priori 0.05 threshold ( $F_{1,31.0} = 4.15$ ,  $P = 0.050$ ). RMR<sub>O<sub>2</sub></sub> was significantly higher at  $35^{\circ}\text{C}$  than at  $15^{\circ}\text{C}$  ( $F_{1,31.0} = 282.69$ ,  $P < 0.001$ ; fig. 5). Acclimation was not a significant predictor of RMR<sub>O<sub>2</sub></sub> ( $F_{1,35.2} = 2.22$ ,  $P = 0.145$ ; fig. 5). Sex was not a significant predictor of RMR<sub>O<sub>2</sub></sub> ( $F_{1,9.2} = 2.79$ ,  $P = 0.128$ ), but mass was a significant predictor of RMR<sub>O<sub>2</sub></sub> ( $F_{1,10.1} = 8.16$ ,  $P = 0.017$ ). For RMR<sub>CO<sub>2</sub></sub>, the effect of temperature was significantly different between acclimation periods ( $F_{1,30.7} = 5.64$ ,  $P = 0.024$ ). RMR<sub>CO<sub>2</sub></sub> at  $15^{\circ}\text{C}$  did not differ between preacclimation and week 5 of cold acclimation (corrected  $P = 0.932$ ), but RMR<sub>CO<sub>2</sub></sub> at  $35^{\circ}\text{C}$  was significantly higher at week 5 than at preacclimation (corrected  $P = 0.007$ ; fig. 5).

The effect of temperature was not significantly different between acclimation periods in the RER model ( $F_{1,52} = 1.5$ ,  $P = 0.222$ ). Sex, mass, and temperature were not significant predictors of RER (sex:  $F_{1,52} = 0.60$ ,  $P = 0.442$ ; mass:  $F_{1,52} = 2.33$ ,  $P = 0.133$ ; temperature:  $F_{1,52} = 0.52$ ,  $P = 0.474$ ). However, acclimation period was a significant predictor of RER ( $F_{2,52} = 3.31$ ,  $P = 0.044$ ). Post hoc comparisons revealed that

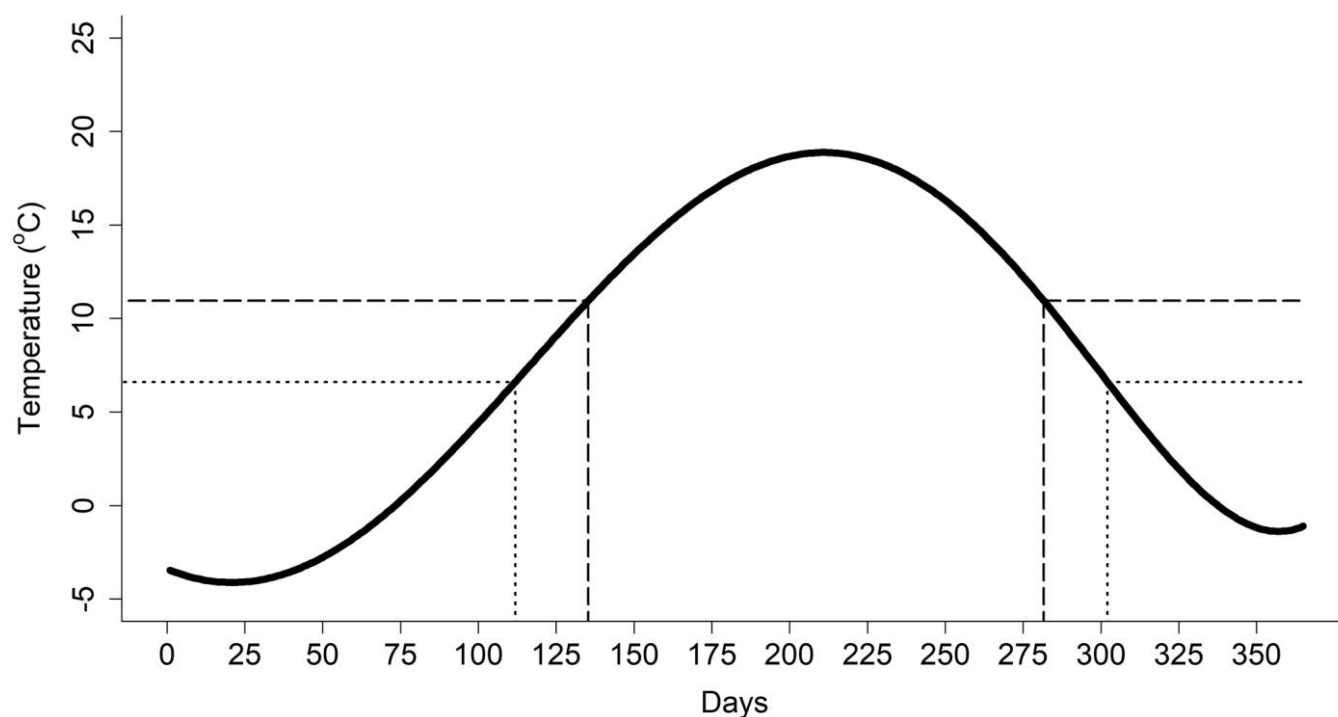


Figure 3. Predicted low surface temperatures ( $^{\circ}\text{C}$ ) throughout a year for Hempstead, New York (solid line), beginning with the first day of January. Expected temperature and corresponding day at which surface temperatures cross critical thermal minimum ( $\text{CT}_{\text{min}}$ ) are shown for unacclimated *Podarcis siculus* (dashed lines) and for fully acclimated *P. siculus* (dotted lines). Temperatures were predicted with a polynomial regression on the minimum temperature variable of the WorldClim dataset (ver. 1.4; Hijmans et al. 2005).

the only significant difference was a higher RER at  $15^{\circ}\text{C}$  at week 11 of cold acclimation than at  $15^{\circ}\text{C}$  before cold acclimation (corrected  $P = 0.018$ ; fig. 6). This effect was mainly driven by a change in  $\text{RMR}_{\text{O}_2}$ , as average  $\text{RMR}_{\text{O}_2}$  at  $15^{\circ}\text{C}$  decreased by 21.9% from preacclimation to week 11 of cold acclimation, while average  $\text{RMR}_{\text{O}_2}$  at  $15^{\circ}\text{C}$  decreased by 8.8% from preacclimation to week 11 of cold acclimation.

## Discussion

We found that *Podarcis siculus* from Hempstead, New York, showed a relatively fast cold acclimation response of  $\text{CT}_{\text{min}}$  compared with what has been found for other reptile species. Pintor et al. (2016) estimated that the  $\text{CT}_{\text{min}}$  of 22 reptile species completed 95% of the acclimation response in 7.3 wk. An exponential model of *P. siculus* acclimation in our study suggests that 95% of the acclimation response was reached in 6 wk (fig. 1). The temperatures at which other reptiles were acclimated are not reported in Pintor et al.'s (2016) meta-analysis, however, making direct comparisons difficult. Curiously,  $\text{CT}_{\text{min}}$  in our study did not decrease after lizards were initially moved from a gradient of  $27^{\circ}\text{C}$ – $32^{\circ}\text{C}$  to a cooler treatment of  $20^{\circ}\text{C}$ : $18^{\circ}\text{C}$  (12L:12D), but it did decrease after 1 wk at  $17^{\circ}\text{C}$ : $16.5^{\circ}\text{C}$  (12L:12D; i.e., after 2 wk below  $20^{\circ}\text{C}$ ; fig. 1). Given that the change from  $20^{\circ}\text{C}$ : $18^{\circ}\text{C}$  to  $17^{\circ}\text{C}$ : $16.5^{\circ}\text{C}$  is small relative to the initial conditions, it is possible that the decrease in  $\text{CT}_{\text{min}}$  was due to the overall temperature decrease and not to the specific conditions at

week 2. Closed-litter rainbow skinks (*Carlia longipes*) caught during the warm season and acclimated to  $18^{\circ}\text{C}$ – $22^{\circ}\text{C}$  also did not show a decrease in  $\text{CT}_{\text{min}}$  1 wk into an acclimation treatment, but they did show a sharp decrease in  $\text{CT}_{\text{min}}$  when measured at the second week (Pintor et al. 2016). More studies would need to be completed to determine whether this is a common trend among lizards, but the current results suggest that the mechanisms that lead to changes in  $\text{CT}_{\text{min}}$  may occur around 2 wk into prolonged cold exposure. Therefore, unless studying how acclimatization (i.e., change as a result of seasonal temperature differences) affects  $\text{CT}_{\text{min}}$ , future studies aiming to measure comparable  $\text{CT}_{\text{min}}$  values between populations should acclimate animals to lab temperatures for 2–3 wk, with repeated measurements if possible. Additionally, the effect of the magnitude of temperature change between treatments and the effect of temperature variability within treatments are ecologically relevant effects to consider when measuring  $\text{CT}_{\text{min}}$ .

The acclimation response of  $\text{CT}_{\text{min}}$  observed in our study appears to be beneficial to *P. siculus* in Hempstead, New York. Given the lack of freeze tolerance in this species (Burke et al. 2002), once behavioral thermoregulation can no longer overcome winter temperatures, the main defense against winter temperatures is to seek suitable winter refugia, which seems to occur between October and November in Hempstead (Burke and Ner 2005). Based on climate data for Hempstead, by October 1, average temperatures at the surface have decreased to  $12.3^{\circ}\text{C}$ , suggesting that lizards already need to avoid stressful conditions at this time.  $\text{CT}_{\text{min}}$  plasticity may therefore reduce risk of exposure

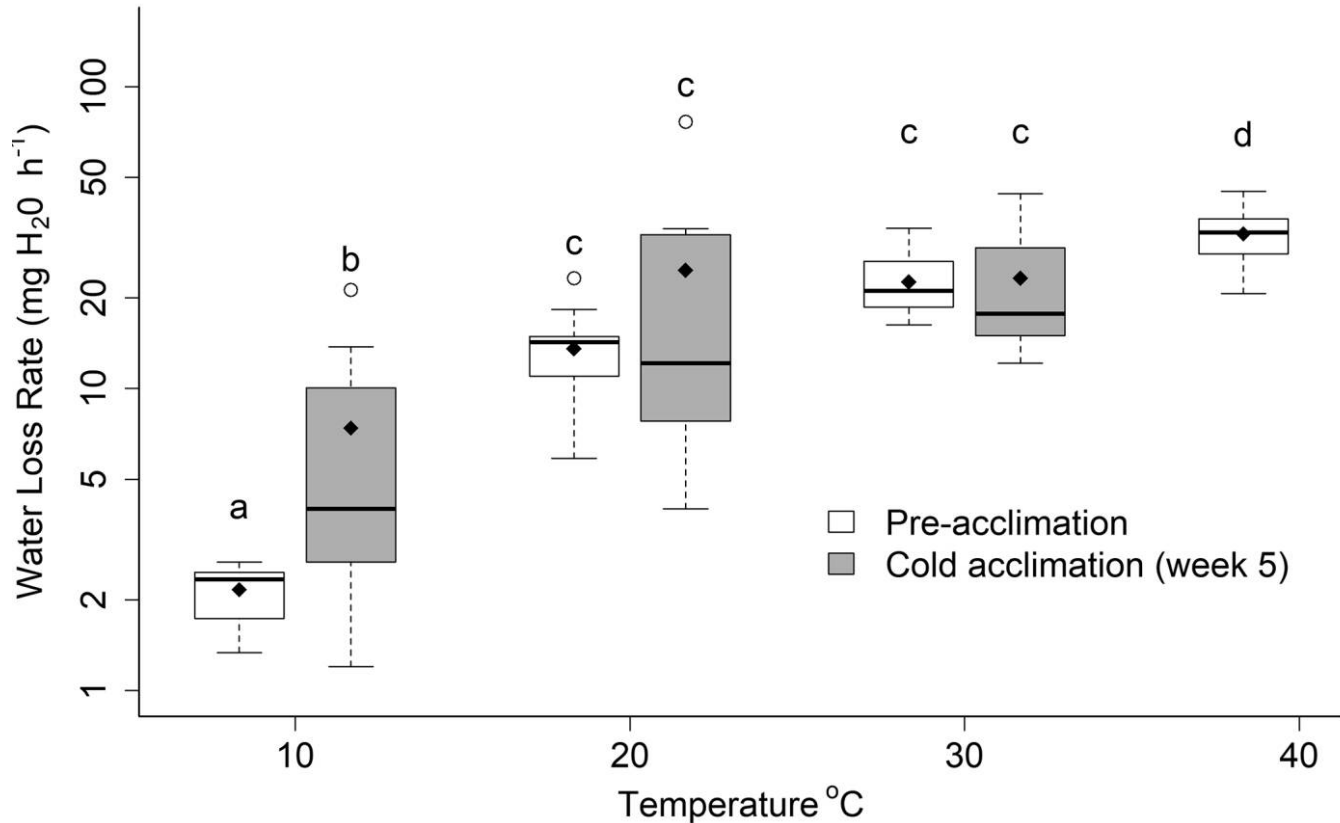


Figure 4. Evaporative water loss ( $\text{mg H}_2\text{O h}^{-1}$ ) for *Podarcis siculus* before a cold acclimation treatment (white boxplots,  $n = 11$  at  $20^\circ\text{C}$ ,  $n = 12$  at all other temperatures) and at week 5 of a cold acclimation treatment (gray boxplots,  $n = 7$ ) across temperatures ( $^\circ\text{C}$ ). The y-axis is shown on a log scale. The horizontal line within each box indicates the median value, and the box boundaries indicate the lower quartile (lower boundary) and the upper quartile (upper boundary). Whiskers indicate minimum and maximum values within a quartile  $\pm 1.5$  times the interquartile range; individual points are outliers that occurred outside of this range. Diamonds within boxplots indicate the mean values. Lowercase letters above boxes represent statistically different groups, as determined by Tukey's all-pair comparisons; groups not sharing letters are significantly different from each other at the  $P < 0.05$  level.

to sublethal conditions in lizards seeking suitable winter refugia when sublethal temperatures are common on the surface. It is important to note that surface temperatures during winter are unlikely to be experienced by lizards unless they are disturbed from their refugia. Should disturbance occur, however, increased cold tolerance may assist lizards in searching for new refugia and escaping from stressful conditions (fig. 3). If we assume that steady exposure to  $20^\circ\text{C}$  or colder temperatures stimulates the acclimation response of  $\text{CT}_{\min}$  in *P. siculus* and if we expect average temperatures to drop below  $20^\circ\text{C}$  on September 12 each year (as predicted using the WorldClim mean temperature variable), lizards would have 26 d until low surface temperatures drop below the unacclimated  $\text{CT}_{\min}$  ( $10.94^\circ\text{C}$ ) on October 8.  $\text{CT}_{\min}$  plasticity would increase the number of days before low surface temperatures drop below  $\text{CT}_{\min}$  ( $6.61^\circ\text{C}$ ) to 46 d, up to October 29. In line with these predictions, surveys suggest that *P. siculus* in Long Island, New York, retreat to refugia between mid-October and mid-November and that they are not observed again until mid-March (Burke and Ner 2005).

The rate at which  $\text{CT}_{\min}$  changes appears to be suitable for the rate at which environmental temperatures decrease during

winter in Hempstead. Additionally, because *P. siculus* occupies primarily urban habitats in the United States, it likely experiences warmer temperatures than populations in nearby forested areas because of the urban heat island effect (Campbell-Staton et al. 2020), further suggesting that the rate of acclimation is suitable for finding winter refugia. If the cold acclimation response of *P. siculus* is indeed relatively fast compared with other reptile species and if this response allows lizards to be active for more days during fall, winter, and early spring, then *P. siculus* may be better suited to cold climates than sympatric species as a result of having more days to find suitable winter refugia and a greater ability to escape stressful conditions during winter. *Podarcis siculus* populations currently exist across the northern United States, including New York, New Jersey, Kansas, and Massachusetts (Kolbe et al. 2013; Donihue 2017), where few other lizard species exist. In Massachusetts, for example, *P. siculus* is the only extant lizard species. However, cold tolerance has also been studied in populations of a closely related lizard species that also experiences winter climates in the United States. A study of  $\text{CT}_{\min}$  in *Podarcis muralis* from Kentucky and Ohio suggests that lizards from expanding populations may have higher variability in  $\text{CT}_{\min}$  than



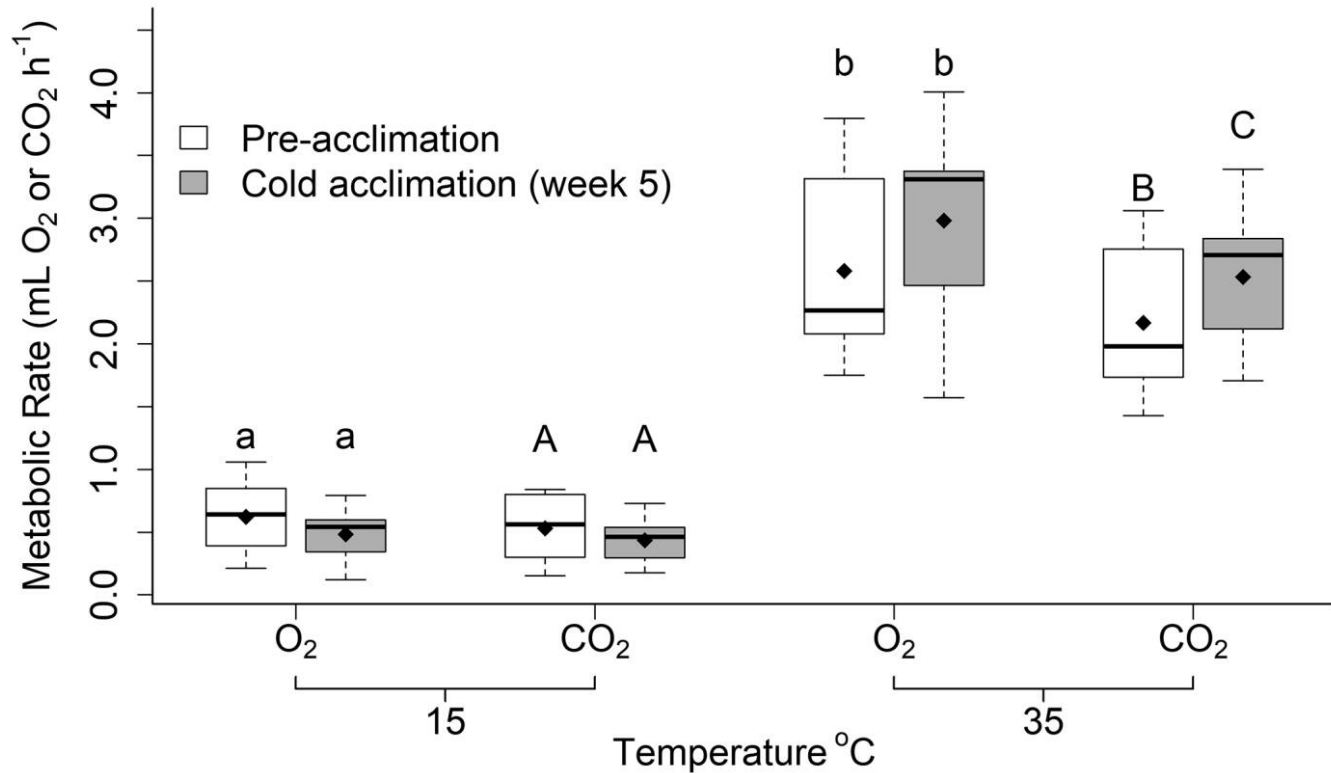


Figure 5. Resting metabolic rate ( $\text{mL O}_2 \text{ h}^{-1}$  or  $\text{mL CO}_2 \text{ h}^{-1}$ ) for *Podarcis siculus* before a cold acclimation treatment (white boxplots,  $n = 12$ ) and at week 5 of a cold acclimation treatment (gray boxplots,  $n = 11$ ) across temperatures ( $15^\circ\text{C}$  and  $35^\circ\text{C}$ ). The horizontal line within each box indicates the median value, and the box boundaries indicate the lower quartile (lower boundary) and the upper quartile (upper boundary). Whiskers indicate minimum and maximum values within a quartile  $\pm 1.5$  times the interquartile range. Diamonds within boxplots indicate the mean values. Letters (lowercase for  $\text{O}_2$ , uppercase for  $\text{CO}_2$ ) above boxes represent statistically different groups, as determined by Tukey's all-pair comparisons; groups not sharing letters are significantly different from each other at the  $P < 0.05$  level.

source populations, suggesting a greater capacity for adaptation to novel environments in expanding populations (Litmer and Murray 2019), although the authors did not assess  $\text{CT}_{\min}$  plasticity. Whether *P. muralis* shows plasticity of  $\text{CT}_{\min}$  is unknown, as  $\text{CT}_{\min}$  was measured once during summer in that study (Litmer and Murray 2019). An interesting area for future research to consider is whether populations of lizards from colder climates are more likely to have lower fixed  $\text{CT}_{\min}$  values or whether they have a greater plasticity of  $\text{CT}_{\min}$  than populations from warmer and/or more stable environments. For US lizards currently limited in range because of cold boundaries, it appears that suitable habitats may be increasing in the near future. In the past decades, there has been a reduction in the number of days with extremely low  $T_{\min}$ 's and a reduction in the length of winter across the United States (Kunkel et al. 2004; IPCC 2013). Across the globe, poleward range expansion has been observed in various taxa as cold regions have become suitable to more species (Parmesan and Yohe 2003). Consequently, we can expect *P. siculus* to be able to expand its range northward, allowing this species to continue establishing populations across the northern range boundary of lizards in the United States.

$\text{CT}_{\max}$  also decreased as part of the cold acclimation response of *P. siculus* (fig. 2).  $\text{CT}_{\max}$  plasticity is typically studied in the

context of increasing global temperatures, with few studies investigating how cold acclimation affects  $\text{CT}_{\max}$ . Biological systems tend to combat the effects of cold by increasing fluidity (e.g., enzyme orthologs, cell membranes; Hochachka and Somero 2002; Somero et al. 2017). If  $\text{CT}_{\max}$  is partly determined by such systems, the increased fluidity in response to cold acclimation may explain why  $\text{CT}_{\max}$  decreased with cold acclimation; the system would have been able to allow a righting response in lizards at  $43.0^\circ\text{C} \pm 0.4^\circ\text{C}$  before becoming more fluid, but this was likely too fluid to allow a righting response at  $41.1^\circ\text{C} \pm 0.5^\circ\text{C}$  after cold acclimation. Interestingly, we found that  $T_{\text{pref}}$  also decreased with cold acclimation, with values occurring within the  $28.4^\circ\text{C}$ – $38.8^\circ\text{C}$  range of laboratory-determined  $T_{\text{pref}}$  for *P. siculus* in previous studies (preacclimation  $T_{\text{pref}}$ :  $35.0^\circ\text{C} \pm 0.6^\circ\text{C}$ ; postacclimation  $T_{\text{pref}}$ :  $32.3^\circ\text{C} \pm 1.0^\circ\text{C}$ ; Avery 1978; Ortega et al. 2016). If  $T_{\text{pref}}$  is reflective of an optimal temperature for maintenance, the decrease in  $T_{\text{pref}}$  may be the result of maintenance systems becoming better able to function at lower temperatures after acclimation. Researchers have previously hypothesized (Huey and Kingsolver 1993; Angilletta et al. 2002) and observed (Glanville and Seebacher 2006) that a shift in mean  $T_b$  leads to a compensatory shift in thermal tolerances and optimal temperatures for a given function after some degree of acclimation.

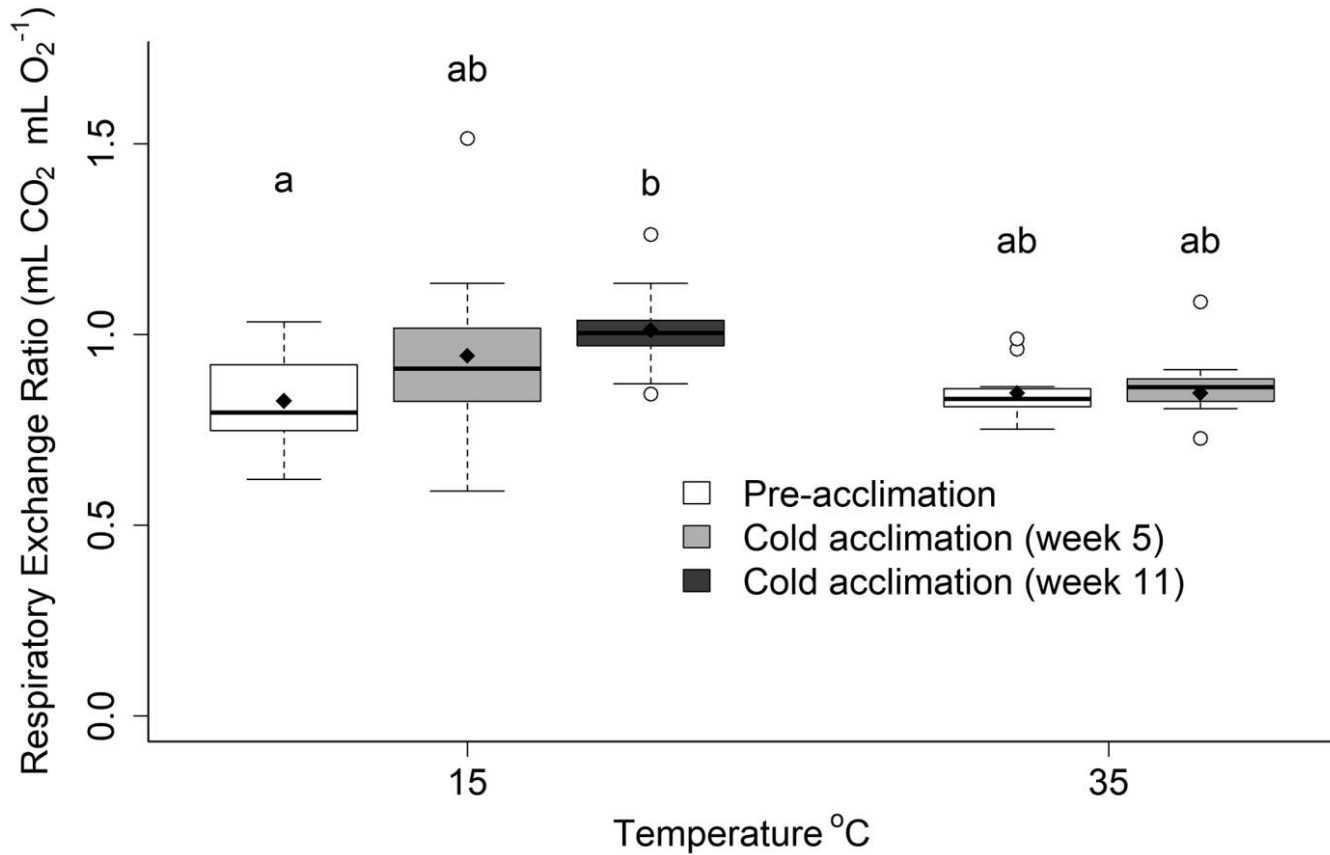


Figure 6. Respiratory exchange ratio ( $\text{mL CO}_2 \text{ mL O}_2^{-1}$ ) for *Podarcis siculus* before a cold acclimation treatment (white boxplots,  $n = 12$ ), at week 5 of a cold acclimation treatment (light gray boxplots,  $n = 11$ ), and at week 11 of a cold acclimation treatment (dark gray boxplot,  $n = 13$ ) across temperatures ( $15^\circ\text{C}$  and  $35^\circ\text{C}$ ). The horizontal line within each box indicates the median value, and the box boundaries indicate the lower quartile (lower boundary) and the upper quartile (upper boundary). Whiskers indicate minimum and maximum values within a quartile  $\pm 1.5$  times the interquartile range; individual points are outliers that occurred outside of this range. Diamonds within boxplots indicate the mean values. Lowercase letters above boxes represent statistically different groups, as determined by Tukey's all-pair comparisons; groups not sharing letters are significantly different from each other at the  $P < 0.05$  level.

The mechanisms that determine thermal tolerances are not fully understood, but several hypotheses exist (e.g., Robertson 2004; Pörtner et al. 2017; Bowler 2018; Gangloff and Telemeco 2018). Although the exact mechanisms and interactions that determine  $CT_{\min}$ ,  $CT_{\max}$ , and  $T_{\text{pref}}$  are likely complex, our current understanding and results suggest that the processes that determine these traits are indeed linked, which can result in a trade-off between increasing performance at one temperature extreme and increasing performance at the other.

Additional evidence for trade-offs associated with cold acclimation includes an increase in logEWL following cold acclimation and the death of four lizards 1.5 h into the post-acclimation  $40^\circ\text{C}$  EWL trial. We found that logEWL significantly increased after cold acclimation, as we expected, and that the effect was primarily driven by an increase in logEWL at  $10^\circ\text{C}$  (fig. 4). Water loss can occur through cutaneous or respiratory means, with cutaneous water loss being the primary means, especially in relatively small lizards (Le Gaillard et al. 2021). The increase in logEWL at  $10^\circ\text{C}$  is likely not due to respiratory means,

because such a low temperature is unlikely to elicit any panting response and because we did not observe a concomitant increase in metabolic rate at low temperature. The observed difference is therefore likely due to a change in cutaneous water loss. The effect of aridity on cutaneous water loss is well studied in lizards, with lizards from more arid environments typically showing lower water loss rates and decreased sensitivity of water loss to changes in temperature (Dmi'el 2001). However, the effects of temperature acclimation on water loss are not well known. Because water loss increases with temperature, it is reasonable to expect that warm-acclimated lizards would compensate by altering their integument, such that EWL is reduced during warm conditions. Warm-acclimated lizards would thus have lower EWL rates than cold-acclimated lizards. If this were the case, an increase in water loss should be observed across all temperatures. Although we did not find this to be the case, there was weak evidence for a potential difference in the effect of temperature between acclimation periods in our model ( $P = 0.053$ ), suggesting that increased sampling may be necessary to determine whether logEWL

at higher temperatures also increases following cold acclimation. The untransformed values of water loss rate are much higher and have a greater variability at warmer temperatures (fig. 4), further suggesting that a larger sample size is needed to discern differences at warmer temperatures. Additionally, the biological relevance of a difference of 5 mg H<sub>2</sub>O h<sup>-1</sup> in mean water loss rate at 10°C may be less meaningful than a difference of approximately 11 mg H<sub>2</sub>O h<sup>-1</sup> in mean water loss rate at 20°C. Presumably, lizards in the wild will be gradually exposed to warmer temperatures after a long exposure to cold, further buffering them from the effects of acclimation on water loss that are observed in this study. This is especially true when considering the effect of rapid exposure to a 40°C trial, which resulted in the death of four lizards. The risk of death was unintentional and unanticipated, and our measurement temperatures should serve as a warning to future researchers to avoid measurement temperatures that may approach lethal limits after acclimation in their research designs.

As initially predicted, we found that RMR<sub>CO<sub>2</sub></sub> increased after cold acclimation but only at 35°C (fig. 5). We found weak evidence for an increase in RMR<sub>CO<sub>2</sub></sub> as well ( $P = 0.050$ ), which appears to also be driven by metabolic rate measured at 35°C (fig. 5). As an increase in metabolic rate at a given temperature after cold acclimation is common among ectotherms (Seebacher et al. 2015), it is likely that an underlying physiological change is partially responsible for the significant increase in RMR<sub>CO<sub>2</sub></sub> at 35°C that we observed. Plasticity of metabolic rate in response to a shift in temperature is thought to occur as a compensatory response that aims to match ATP production with use of ATP by energy-consuming processes (Hulbert and Else 2000). Metabolic rate is ultimately determined by how transcriptional mechanisms and cell membrane composition interact with temperature and energy substrates (Seebacher et al. 2010). Future work would need to examine whether key enzyme orthologs or fatty acid composition of mitochondrial membranes change in response to cold acclimation in *P. siculus* to determine the mechanisms of its metabolic rate plasticity.

Although we controlled for available diet, a change in energy substrates available to lizards may also explain some of our results. A decrease in lipid stores during winter dormancy, particularly in fat bodies, has been documented in various reptile species (Price 2017). For example, the proportion of dry body weight attributed to lipids in wild viviparous lizards (*Zootoca vivipara*) decreased from one-third of dry body weight in August and September to one-sixth of dry body weight in March and April following the Northern Hemisphere winter (Avery 1970). RER indicates the primary fuel sources used to support metabolism; values close to 1 suggest a higher proportional use of carbohydrates as a fuel source, values close to 0.7 suggest a higher proportional use of lipids as a fuel source, and values between 0.7 and 1 suggest a mixed use of substrates or a higher proportional use of protein as a fuel source (Schmidt-Nielsen 1997). RER values exceeding 1 indicate anaerobic metabolism, lactate accumulation, and/or measurement error (Solberg et al. 2005). Our estimation of RER at 15°C showed an increase in values approaching 1 as the cold acclimation treatment progressed (fig. 6), which may reflect increased relative use of carbohydrates because

of loss of lipids. A similar increase in RER has been observed in *P. siculus* after tail autotomy, possibly because of loss of lipids stored at the base of the tail (Eberle et al. 2022). RER values are notoriously difficult to interpret, however, as studies frequently estimate values below 0.7 or above 1. Error is partially introduced through a greater relative ability for organisms to store CO<sub>2</sub> within tissues, especially in mildly hypoxic environments (Malte et al. 2016). Further studies of the effect of cold acclimation on RER for *P. siculus* should examine the proportion of energy substrates available before and after cold acclimation, preferably using open-system respirometry to better estimate RER.

In summary, we found that thermal tolerance of *P. siculus* decreased in response to a cold acclimation treatment. The decrease in cold tolerance during acclimation to cooler temperatures appears to happen in a suitable time frame to allow lizards to find winter refugia before conditions become potentially lethal. By investigating the rate of change and extent to which cold tolerance changed during a cold acclimation treatment, we were better able to investigate how a physiological trait might explain why this lizard species has been so successful at persisting in the seasonally cold climate of Hempstead, New York. Future studies investigating the plasticity of physiological traits may also benefit from measuring traits at regular time periods during acclimation. We also found that cold acclimation affects heat tolerance,  $T_{pref}$  and to a lesser extent metabolism and EWL of *P. siculus*, suggesting that cold acclimation involves changes to biological systems that affect more than cold tolerance. More generally, our findings highlight the relevance of thermal physiology and plasticity to invasive species research. With increasing global connectivity, introductions of nonnative animals through various means are likely to increase in frequency, making an understanding of the mechanisms that aid persistence in novel stressful environments a priority for ecologists and conservation biologists.

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