

## Geographic variation of resistance to water loss within two species of lungless salamanders: implications for activity

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**Citation:** Riddell, E. A., and M. W. Sears. 2015. Geographic variation of resistance to water loss within two species of lungless salamanders: implications for activity. *Ecosphere* 6(5):86. <http://dx.doi.org/10.1890/ES14-00360.1>

**Abstract.** For many organisms, constraints on activity increase energetic costs, which ultimately reduce the suitability of a particular habitat. Mechanistic species distribution models often use estimates of activity to predict how organisms will respond to climate change. These models couple physiology and morphology with climatic data to estimate potential activity. In turn, the duration of activity is used to estimate the energetic balance of individuals at a given location. Whether individuals remain in positive net energetic balance determines if a given location is suitable for the species. However, because these models often assume that physiology does not vary across the species range, estimates of activity (and consequently energetics) are potentially misleading. To test the consequence of this assumption, we measured total resistance to water loss ( $R$ ) within two species of lungless salamanders (*Plethodon metcalfi* and *P. teyahalee*) collected from locations along their elevational extent in southwestern North Carolina. Because hydration state constrains the activity of salamanders, increasing  $R$  would increase potential activity. Here, we leveraged the natural changes in environmental conditions along an elevational gradient to determine if salamanders modify  $R$  in different environments. We predicted that salamanders collected from low elevations would have higher  $R$  to compensate for the warmer, drier conditions at low elevations that may limit activity. We determined  $R$  in the laboratory using a flow-through system at two temperatures (12°C, 18°C) and at three vapor pressure deficits (0.2 kPa, 0.35 kPa, 0.5 kPa). For *P. metcalfi*, individuals collected from low elevations exhibited the highest  $R$ , suggesting either acclimatization or adaptation to local conditions. For *P. teyahalee*, individuals collected from high elevations exhibited the highest  $R$ , but these results may reflect alternative pressures due to differences in behavior. The results also suggest that salamanders might use temperature as a cue to increase  $R$ , but the capacity to do so depends upon the temperatures experienced in nature. Moreover, we show that variation in  $R$  has the potential to alter the duration of activity over the elevational ranges of these species, illustrating the importance of incorporating geographic variation of physiological traits for predicting a species' response to climate.

**Key words:** activity; climate change; cutaneous water loss; elevational gradient; geographic variation of physiology; *Plethodon metcalfi*; *Plethodon teyahalee*; resistance to water loss.

**Received** 25 September 2014; revised 15 January 2015; accepted 21 January 2015; final version received 19 March 2015; **published** 29 May 2015. Corresponding Editor: D. P. C. Peters.

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### INTRODUCTION

Environmental conditions influence habitat

suitability by limiting the duration of activity of an organism (Huey 1991). For instance, constraints on activity reduce opportunities to

forage, increasing the energetic costs of a particular habitat. Mechanistic species distribution models estimate habitat suitability based upon energetic costs; therefore, understanding the conditions that limit activity of an organism may improve predictions of habitat suitability. Several models have taken this approach to predict extensive shifts of suitable habitat for many taxa over the next century (Porter et al. 2002, Morin and Thuiller 2009, Buckley et al. 2010). To predict these shifts, species distribution models often link the physiological performance of organisms with relevant climatic factors and topographical features (Porter et al. 2000, Kearney and Porter 2004, Buckley 2008). However, this approach requires detailed information about an organism's physiological traits, often limiting these predictions to well-studied organisms (Kearney and Porter 2009). Often empirical evidence for variation of physiology is lacking, and models assume that physiological traits do not vary over a species' range (Gifford and Kozak 2012). However, there is ample evidence that physiological traits change over a species' range. For example, water loss rates of an the brown anole (*Anolis sagrei*) were lower at higher latitudes (Kolbe et al. 2013), and water loss rates decreased at lower elevations of a lungless salamander (*Plethodon monantus*) (Winters and Gifford 2013). Also, metabolic rates of lizard populations (*Sceloporus* spp.) varied with latitude (Angilletta 2001) and elevation (Sears 2005). Similar patterns of geographic variation occur for thermal tolerances of many marine and terrestrial ectotherms (Sunday et al. 2012). Due to the prevalence of these patterns, estimates of activity may be improved by incorporating known geographic variation of physiological traits.

Of particular importance to predicting species ranges are traits that influence the activity budgets of individuals. Many models based on energetics (e.g., Buckley et al. 2010) use estimates for the duration of activity to determine energetic intakes and expenditures. Clearly, animals require specific thermal or hydric conditions for activity, and often make physiological or behavioral adjustments to prolong activity in those environments. For example, Grigg and Seebacher (1999) demonstrated physiological control of heart rate prevented overheating in bearded

dragons (*Pogona barbata*) thereby increasing activity in hot environments. In birds, a decrease in abdominal temperature of the Gentoo penguin (*Pygoscelis papua*) was linked to reduced energetic costs while diving for prey (Bevan et al. 2002). Also, deer mice (*Peromyscus maniculatus*) that consumed more food exhibited higher maximal metabolic rates that resulted in higher rates of activity in cold conditions, illustrating how behavioral decisions and physiological capacities influence the potential for energetic gain (Sears et al. 2009). Thus, any variation in physiological traits that influence activity has direct implications for predicting the relationship between an organism and its environment.

To date, relatively few studies have incorporated physiological, morphological, and behavioral variation into physiologically-structured, species distribution models. Buckley (2008) demonstrated population-specific life history and morphological traits influence the extent of predicted range shifts of *Sceloporus undulatus* in response to warming climates. Recent models have also identified acclimation of critical minimum temperatures within individuals of *Bufo marinus* as a potential mechanism for range expansion (Kolbe et al. 2010). Incorporating behavioral plasticity of individuals into models has also been shown to reduce the skewing of temperature-dependent sex ratios in response to climate change in *Sphenodon guntheri* (Mitchell et al. 2008). Because of the known geographic variation of physiological traits (Stillman 2003) and its direct link to energetics (Porter et al. 2000), mechanistic models should develop predictions from documented variation of physiological traits (Kearney 2012). From these data, mechanistic models may provide better estimates of growth rates, energetic costs, and duration of activity throughout the range of a species.

Here, we consider how geographic variation of total resistance to water loss,  $R$  (s/cm), within a high elevation species (*Plethodon metcalfi*) and a low elevation species (*Plethodon teyahalee*) of plethodontid salamanders might influence activity. We examined a low and high elevation species to determine if geographic variation of  $R$  was consistent between species. Lacking lungs, plethodontid salamanders rely on wet skin for cutaneous gas exchange (Gatz et al. 1975), limiting both the habitats and conditions in

which these salamanders can be active. By maintaining moist skin, salamanders lose water to their environment. Consequently, activity on the surface of the forest floor is largely determined by hydration state (Feder and Londos 1984). Surface activity is a relevant behavior to estimate because longer durations of surface activity increase the total time available to forage, grow, and find mates (Feder 1983). For example, a fully hydrated salamander may emerge from moist microhabitat to forage or reproduce, dehydrate while moving across the forest floor, and subsequently return to the moist microhabitat to rehydrate. For some plethodontids, surface activity is abandoned upon losing just 3.8% of their body mass due to water loss (Feder and Londos 1984). With these constraints, the total duration of activity can be predicted given rates of water loss, initial mass of the salamander, physiological tolerances to dehydration, environmental and body temperature, and humidity.

For terrestrial salamanders, rates of water loss are largely dependent upon the thermal and hydric environment. For these species, the lowest rates of water loss should occur while the environment is cool, moist, and wind-free. By limiting activity to nighttime conditions, they reduce exposure to warm, dry conditions that occur during the day, which minimizes water loss rates. We predicted that along the elevational gradient, populations would experience microclimates ranging from cooler, wetter conditions on mountaintops to warmer, drier conditions in the lowlands. This prediction was developed from first principles due to the increasing saturation vapor pressure with warmer temperatures. Because warm air has the capacity to hold more vapor, lower elevations potentially increase rates of cutaneous water loss (CWL) due to higher vapor pressure deficits (VPD), the difference between how much vapor the air can hold and how much vapor exists in the air. In response, salamanders might acclimatize by increasing total resistance to water loss to reduce CWL, thereby increasing potential surface activity and net energetic gain. To test how activity changed with the environment, we measured  $R$  under three different VPDs under a cool and warm temperature that were representative of the range of values found in natural environments. We then predicted potential activity times

by coupling CWL with climates that occur over the elevational gradient. Our results suggest that incorporating geographic variation in physiological traits influences estimates of activity. Consequently, mechanistic species distribution models that are dependent on estimates of activity have the potential to make better predictions.

## METHODS

### Study area

To determine geographic variation of total resistance to water loss ( $R$ ), we collected southern gray-cheeked salamander (*P. metcalfi*) and Southern Appalachian salamander (*P. teyahalee*) over their elevational range near Cullowhee, NC in the Nantahala National Forest (35°20' N, 83°4' W). The location of the study area was selected based upon road access that spanned a suitable elevational gradient and upon the high abundance of the two species of interest. The forest within the study area was predominately mixed hardwood with an abundance of streams. Using Quantum QGIS 1.8.0 (QGIS Development Team, <http://qgis.org>), we created a polygon within 600 m of the road with a total area of 10.1 km<sup>2</sup> along an elevational gradient (700–1600 m). We randomly generated coordinates for collection sites within the 10.1 km<sup>2</sup> polygon. Points within 35 m of the road were excluded to reduce potential effects of the road on microclimate and salamander abundance (Semlitsch et al. 2007). We sorted the coordinates into groups for every 100 m section of altitude along the mountain to select coordinates in a stratified random design. We randomly selected each coordinate from these groups to ensure the elevational gradient would be covered. The order that the sites were visited was randomized with respect to elevation across the sampling period to ensure that time of collection and elevation were not confounded. We collected salamanders between 2100 and 0600 during July and August 2013. On a given night, collection was limited to a single species, either *P. metcalfi* or *P. teyahalee*. While sampling, we searched for salamanders within a 20 m radius of the random collection point by searching on the forest floor and under objects commonly associated with salamanders, such as logs, stumps, rocks, and leaf litter. We captured the first two individuals of the specific species and

transported them to the laboratory in plastic resealable bags (17.7 cm × 19.5 cm) with wet leaves to maintain a fully hydrated state and mimic their natural habitat. Furthermore, we only collected adult salamanders (>1 g) and individuals in healthy conditions (i.e., complete tails and limbs). In the laboratory, we housed salamanders in individual plastic containers (2000 cm<sup>2</sup>) containing moist sphagnum moss to ensure a fully hydrated state. We stored containers in a temperature-controlled incubator (MIR-154; Panasonic) at 15°C at the Highlands Biological Station in Highlands, NC for three days. We assessed the health of each salamander daily by visual inspection and the limb withdrawal reflex. We collected environmental data along the elevational gradient by placing iButton Hygrochrons (DS1023; Maxim Integrated) at randomly generated coordinates (n = 17). The Hygrochrons collected temperature and relative humidity every 10 minutes for the months of July and August 2013.

#### Activity transects

The thermal and hydric environments impose limits on the potential for salamanders to be active. To identify how activity varied with temperature and *VPD*, we determined air temperatures and *VPDs* during times when salamanders were active on the surface of the forest floor. We selected coordinates for the activity transects using the same method that was used for collection sites. We used the selected coordinates as the start location for the surface activity transects. Each transect was conducted at the same elevation. We chose the direction of each transect to minimize changes in slope and aspect. The observer counted the number of surface-active salamanders within a 2-m distance on either side of a 100-m linear transect (*P. teyahalee*: number of transects (n) = 110; *P. metcalfei*: n = 115) once per hour from 1900 to 2300. To collect environmental conditions during the transect, we placed three Hygrochron iButtons at random locations along each transect to record temperature and relative humidity. From these data, we calculated *VPDs* by:

$$VPD = e_s - e_a$$

where  $e_s$  is the saturation vapor pressure (kPa) at a given temperature and  $e_a$  is the vapor pressure

of ambient air (kPa). To calculate  $e_s$ , we used the Clausius-Clapeyron equation (Stull 2000):

$$e_s = e_o \cdot \exp \left[ \frac{L}{R_v} \left( \frac{1}{T_o} - \frac{1}{T} \right) \right]$$

where  $e_o = 0.611$  kPa and  $T_o = 273$ K are constant parameters, and  $R_v = 461.5$  J·K<sup>-1</sup>·kg<sup>-1</sup> and  $L = 2.5 \times 10^6$  J/kg are the gas constant for water vapor and the latent heat of vaporization, respectively. To calculate  $e_a$ , we used:

$$e_a = e_s - VPD$$

where *VPD* was the desired vapor pressure deficit for the experiment.

#### Flow-through system and experimental design

To determine geographic variation of *R*, we exposed salamanders to a combination of temperature and *VPD* in the laboratory using a flow-through system. After collection, we held salamanders at 15°C for three days to clear their gut, reducing the chance of defecation during the experiment. Six hours prior to measuring *CWL*, we transported individual containers to an environmental chamber at an experimental temperature (12°C, 18°C). In the environmental chamber, we used a flow-through system starting with a sub-sampler (SS-4; Sable Systems) to push air through a dewpoint generator (DG-4; Sable Systems) to control *VPD*. The air stream was then divided into separate air streams with a manifold (MF-8; Sable Systems), each at a flow rate of 180 mL/min. Next, the airstream passed into the cylindrical acrylic chamber (16 cm × 3.5 cm; volume ~ 153 mL) containing the salamander. The chambers were lined with hardware mesh to expose the entire surface area of the salamander to the air stream, which simulated posture during activity. After passing through the chamber, the air was sampled via a vapor analyzer (RH-300; SSI) to measure the change in water vapor pressure (kPa). We measured *R* of a single salamander at a combination of one temperature (12°C, 18°C) and one *VPD* (0.2 kPa, 0.35 kPa, 0.5 kPa). We randomized the order of temperature and *VPD* treatments to avoid any ordering effects. Each dehydration experiment lasted three hours and occurred between 1900 and 500 EST. We switched between each chamber three times every ten minutes using a multiplexer (RM-8; SSI) to provide sufficient time to reach a steady-

state (Lighton and Halsey 2011). Salamanders that defecated or urinated during the experiment were not included in the analysis due to the moisture that feces and urine contribute to the airstream. We removed four *P. metcalfi* and ten *P. teyahalee* from the analyses due to defecation.

#### Calculation of total resistance to water loss

Calculating total resistance to water loss requires specific knowledge on water loss rates of the organism and environmental conditions. The water vapor pressure ( $e$ ; kPa) flowing from the chamber was measured by the vapor analyzer. We calculated the difference of the incurrent and excurrent  $e$ , which was converted to water vapor density ( $\rho_v$ ; g/m<sup>3</sup>) using:

$$\rho_v = \frac{e}{(T \cdot R_v)}$$

where  $T$  is temperature in Kelvin (K) and  $R_v$  is the gas constant for water vapor (461.5 J·K<sup>-1</sup>·kg<sup>-1</sup>). To calculate total mass lost from evaporative water loss (EWL; mg/hr), we used the following equation:

$$EWL = \rho_v \cdot FR \cdot 0.001$$

where  $FR$  is the flow rate of the air stream (mL/hr) and 0.001 is a conversion factor for mg/min (Lighton 2008). To calculate CWL (mg·hr<sup>-1</sup>·cm<sup>-2</sup>), we divided the rate of water loss by the estimated surface area of each salamander. The surface area (cm<sup>2</sup>) was estimated by an empirically derived formula for the Family *Plethodontidae*, where surface area = 8.42 × mass (g)<sup>0.694</sup> (Whitford and Hutchison 1967). Total resistance was calculated by:

$$R = \frac{\rho}{CWL}$$

where  $\rho$  is the vapor density gradient (g/cm<sup>3</sup>) and  $CWL$  is the cutaneous water loss rate (g·cm<sup>-2</sup>·s<sup>-1</sup>) (Feder and Burggren 1992).

#### STATISTICAL ANALYSIS

Statistical analyses of  $R$ , body size, environmental data, and environmental conditions during activity transects were conducted separately for each species. Statistical analyses were conducted in R v. 3.0.3 (R Development Core Team 2013). For the environmental data, we used a

linear regression to determine the effect of elevation on temperature and  $VPD$  along the elevational gradient. To analyze the activity transect data, we used a logistic regression to determine the effect of  $VPD$  on the probability of finding a surface-active *P. metcalfi* and *P. teyahalee*. Analyses of activity included transects conducted within the elevational range of the respective species (*P. teyahalee*: 800–1300 m; *P. metcalfi* 1200–1700 m). We calculated effect sizes for environmental and body size data using:

$$\omega^2 = \frac{SS_{treatment} - df_{treatment} \times MS_{error}}{SS_{total} + MS_{error}}$$

where  $SS_{treatment}$  is the sum of squares for a given parameter,  $df_{treatment}$  is the degrees of freedom for that parameter,  $MS_{error}$  is the mean square error, and  $SS_{total}$  is the total sum of squares. These values were calculated from an analysis of variance to determine the proportion of explained variance of each parameter (Olejnik and Algina 2003). To determine the best regression model to predict  $R$ , we used an information-theoretic approach using Akaike's information criterion (AIC) (Burnham and Anderson 1998) for *P. metcalfi* ( $n=65$ ) and *P. teyahalee* ( $n=49$ ). The approach weighs models by their complexity and the amount of variance explained by the model. We used AIC corrected (AIC<sub>c</sub>) values to reduce bias and improve model selection in small sample sizes (Sugiure 1978). The level of support for an AIC<sub>c</sub> value was evaluated by  $\Delta AIC_c$  (i.e.,  $AIC_c = AIC_i - AIC_{min}$ ) and Akaike weights, the relative likelihood of the model given the data (Burnham and Anderson 1998). We performed all-possible subsets regression by competing models containing every possible combination of the parameters (i.e., temperature,  $VPD$ , elevation, surface area) and interactions. We then averaged parameter estimates of all models (weighted by each model weight) for all models with a cumulative weight of 0.95, resulting in a 95% chance that the averaged model contained the true predictors of the response variable. To determine relative variable importance, we calculated the sum of all the Akaike weights for a given predictor variable (including interactions) across the models within the cumulative weight of 0.95.

To determine potential activity, we calculated time to dehydration ( $D_t$ ) using:

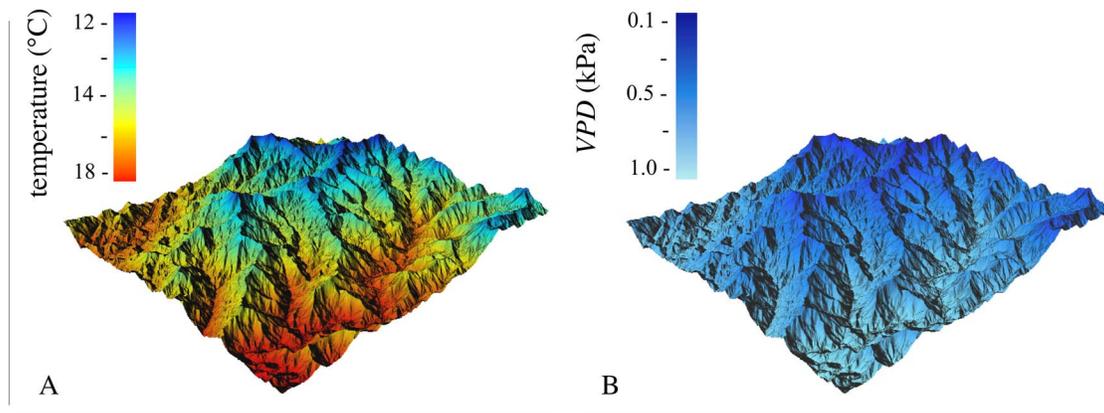


Fig. 1. A 3-D representation of the average changes in temperature ( $^{\circ}\text{C}$ ) and  $VPD$  (kPa) in July 2013 interpolated from ibutton data along an elevational gradient (700–1600 m) at the field site located near Cullowhee, NC. The colors represent values for (A) temperature (blue represents the cooler temperatures and red represents warmer temperatures) and (B)  $VPD$  (dark blue represents the wetter conditions and light blue represents the drier conditions). Along the elevational gradient, environmental conditions are typically cool and wet on the mountaintops and, in the lowlands, the conditions are typically warm and dry during this study.

$$D_t = \frac{\mu}{CWL \cdot SA}$$

where  $\mu$  is the average body mass lost (3.8% of total body mass) due to water loss at which a terrestrial salamander retreats to microhabitat (Feder and Londos 1984),  $CWL$  is the cutaneous water loss rates ( $\text{mg}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ ), and  $SA$  is the empirically determined average surface area ( $\text{cm}^2$ ) for an adult salamander (*P. metcalfi*:  $\sim 4$  g; *P. teyahalee*:  $\sim 7$  g) in this study. To illustrate the consequences of incorporating geographic variation of  $R$ , we predicted activity for each species based upon average values of  $R$  and the variation of  $R$  along the elevational gradient. In the model without acclimation, we calculated  $CWL$  based upon average values of  $R$  for each respective species. In the model with acclimation, we calculated  $CWL$  based upon the variation of  $R$  exhibited along the elevational gradient for each species. In both models (with and without acclimation),  $CWL$  were calculated based upon the changes in temperature and  $VPD$  that occurred along the elevational gradient during the same time period in July–August 2013 in which the salamanders were collected and  $R$  was measured.

## RESULTS

Temperatures became cooler as elevation increased (regression coefficient ( $\beta$ ) =  $-0.0034 \pm 0.000041$  (SE),  $\omega^2 = 0.42$ ), and  $VPDs$  decreased as elevation increased ( $\beta$  =  $-0.00021 \pm 0.0000073$  (SE),  $\omega^2 = 0.09$ ), confirming that mountaintops are typically cooler and wetter than the lowlands during the study (Fig. 1). The activity transects indicated that high  $VPDs$  reduced the probability of finding a surface-active salamander for both *P. metcalfi* ( $\chi_1^2 = 18.9$ ; Fig. 2A) and *P. teyahalee* ( $\chi_1^2 = 15.6$ ; Fig. 2B). For *P. metcalfi*, model selection using AIC indicated that temperature,  $VPD$ , elevation, surface area, and the interaction between temperature and  $VPD$  were included in all top models with a cumulative weight of 0.95 as indicated by the importance (Appendix: Table A1). Furthermore, the interaction between elevation and temperature was included in 89% of the top models (Appendix: Table A1). After model-averaging, temperature,  $VPD$ , and their interaction had the greatest influence on  $R$  (Table 1). For this species,  $R$  increased when environmental conditions were warm and dry, and  $CWL$  decreased at higher temperatures (Fig. 3). The interaction between temperature,  $VPD$ , and elevation was largely responsible for the change in  $R$  along the elevational gradient (Table 1).

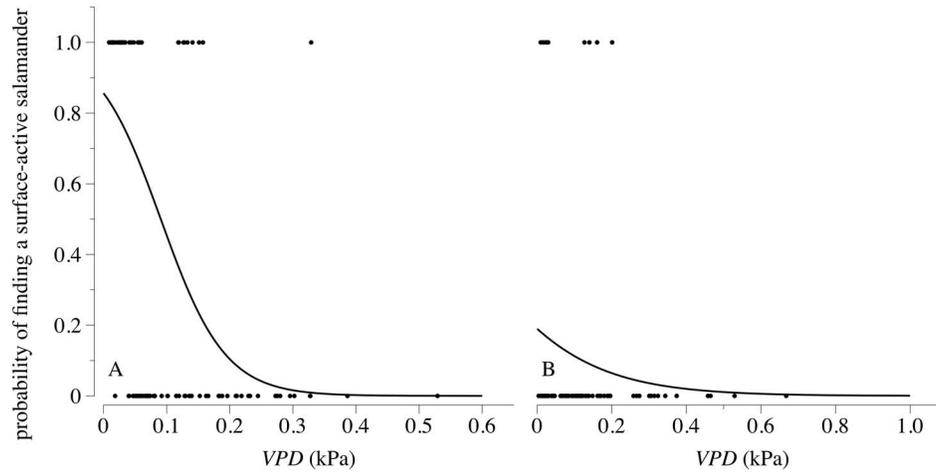


Fig. 2. As VPD (kPa) increased, the probability of finding a surface-active salamander decreased for (A) *P. metcalfi* and (B) *P. teyahalee*. Points represent presences and absences for surface-active salamanders observed during the activity transects.

With this interaction, individuals from low elevations exhibited the highest *R* when under warm, dry conditions (Fig. 4). For *P. teyahalee*, model selection using AIC indicated that temperature, VPD, elevation, surface area, and the interaction between temperature and VPD were included in all top models with a cumulative weight of 0.95 as indicated by the importance (Appendix: Table A2). After model-averaging, the interaction between temperature and VPD

Table 1. The averaged model of *R* for *P. metcalfi* with coefficients and standard errors of parameters.

Independent variable	Coefficient	SE
(Intercept)	7.23E+00	5.64E-01
elev	-3.48E-03	2.80E-03
SA	2.74E-01	1.07E-01
TEMP 18	-1.87E+00	8.05E-01
VPD 0.35	-6.97E-01	8.05E-01
VPD 0.5	1.66E+00	8.28E-01
elev:TEMP 18	-8.44E-03	3.54E-03
TEMP 18:VPD 0.35	5.69E+00	1.14E+00
TEMP 18:VPD 0.5	3.54E+00	1.15E+00
SA:VPD 0.35	1.8E-01	1.45E-01
SA:VPD 0.5	2.38E-01	1.3E-01
SA:TEMP 18	9.61E-02	1.18E-01
elev:VPD 0.35	3.77E-03	4.33E-03
elev:VPD 0.5	6.43E-03	3.75E-03
elev:SA	-5.07E-05	4.52E-04
elev:SA:TEMP 18	-1.20E-03	8.43E-04
elev:TEMP 18:VPD 0.35	-1.08E-02	8.79E-03
elev:TEMP 18:VPD 0.5	-5.92E-03	7.79E-03

Note: The parameters are elevation (elev), surface area (SA), temperature (TEMP), vapor pressure deficit (VPD), and the interactions between the parameters.

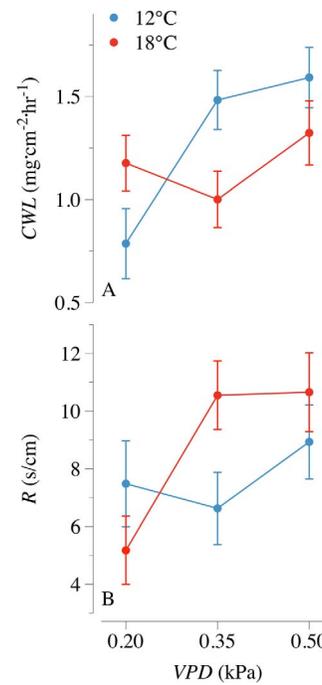


Fig. 3. The figure demonstrates (A) CWL ( $\text{mg}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ ) decreased at high VPD (kPa) and warm temperatures ( $^{\circ}\text{C}$ ), and (B) *R* (s/cm) increased at high VPDs and warm temperatures for *P. metcalfi*. Each color schemes represents the temperature treatment. The means with 95% confidence intervals are displayed for the interaction between VPD and temperature.

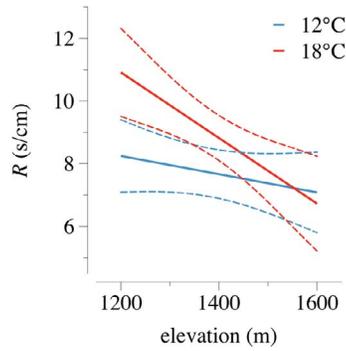


Fig. 4. The figure demonstrates  $R$  (s/cm) increased with temperature with individuals collected from low elevations for *P. metcalfi*. The different temperature treatments are blue and red for 12°C and 18°C, respectively. The 95% confidence intervals are also plotted for each temperature treatment in the respective colors.

had the greatest influence on  $R$  (Table 2). For this species,  $R$  remained constant under the different  $VPD$  treatments at cool temperatures, but under warm temperatures,  $R$  increased with drier air (Fig. 5). Similarly,  $CWL$  increased as  $VPD$  increased; however, under warm, dry conditions,  $CWL$  decreased (Fig. 5). The interaction between

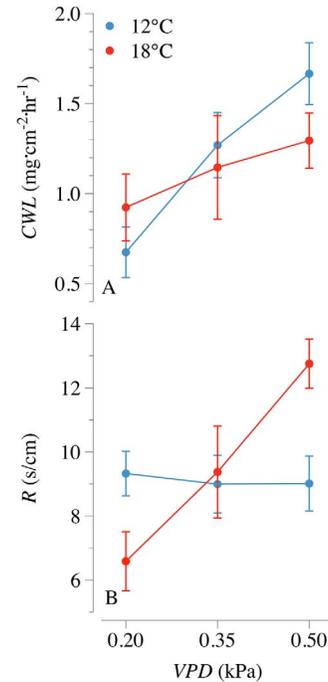


Fig. 5. The figure demonstrates that (A)  $CWL$  ( $\text{mg}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ ) decreased at high  $VPDs$  (kPa) and warm temperatures ( $^{\circ}\text{C}$ ), and (B)  $R$  (s/cm) increased at the high  $VPDs$  and warm temperature treatments for *P. teyahalee*. Each color scheme represents the temperature treatment. The means with 95% confidence intervals are displayed for the interaction between  $VPD$  and temperature.

Table 2. The averaged model of  $R$  for *P. teyahalee* with coefficients and standard errors of parameters.

Independent variable	Coefficient	SE
(Intercept)	9.36E+00	3.68E-01
elev	-2.43E-03	3.08E-03
SA	3.11E-01	3.75E-02
TEMP 18	-2.89E+00	5.82E-01
VPD 0.35	-4.03E-01	6.04E-01
VPD 0.5	-3.88E-01	5.83E-01
elev:SA	1.92E-04	1.81E-04
elev:TEMP 18	7.79E-03	4.36E-03
elev:VPD 0.35	4.37E-03	4.71E-03
elev:VPD 0.5	3.90E-03	4.29E-03
SA:TEMP 18	-7.07E-02	6.12E-02
SA:VPD 0.35	5.25E-03	6.08E-02
SA:VPD 0.5	2.49E-02	7.65E-02
TEMP 18:VPD 0.35	3.15E+00	1.04E+00
TEMP 18:VPD 0.5	6.67E+00	8.43E-01
SA:TEMP 18:VPD 0.35	-1.57E-01	1.12E-01
SA:TEMP 18:VPD 0.5	2.28E-01	9.09E-02
elev:SA:TEMP 18	3.67E-04	2.40E-04
elev:TEMP 18:VPD 0.35	1.21E-02	5.40E-03
elev:TEMP 18:VPD 0.5	9.97E-03	4.63E-03
elev:SA:VPD 0.35	-1.84E-04	3.95E-04
elev:SA:WVPD0.5	3.67E-04	2.55E-04

Note: The parameters are elevation (elev), surface area (SA), temperature (TEMP), vapor pressure deficit ( $VPD$ ), and the interactions between the parameters.

temperature,  $VPD$ , and elevation was largely responsible for the change in  $R$  along the elevational gradient (Table 2). Due to this interaction, individuals from high elevations exhibited the highest  $R$  when under dry conditions (Fig. 6). From the estimates of  $D_t$  without acclimation, the highest estimates of potential activity occurred at high elevations within the elevational range of each species (Fig. 7), suggesting that only high elevation salamanders could be active for the duration of the night (~10 hr). Upon including acclimation, activity estimates increased to nearly the entire night along the elevational gradient for both species. Although body size largely determines rates of water loss, body size did not change with respect to elevation for either species (Fig. 8; *P. metcalfi*:  $\beta = -0.00040 \pm 0.00076$  (SE),  $\omega^2 = 0.01$ ; *P. teyahalee*:  $\beta = 0.0018 \pm 0.0016$  (SE),  $\omega^2 = 0.002$ ).

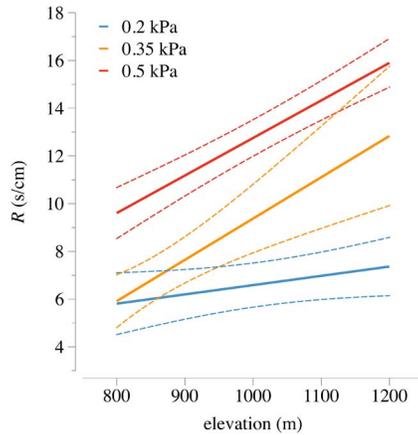


Fig. 6. The figure demonstrates that  $R$  (s/cm) of *P. teyahalee* increased at the high VPD (kPa) treatments for individuals collected from high elevations.  $R$  increased across all elevational ranges at higher VPDs. The different VPD treatments are illustrated in blue, orange, and red for the 0.2 kPa, 0.35 kPa, and 0.5 kPa treatments, respectively.

## DISCUSSION

This study uncovered geographic variation of  $R$  within two species of salamanders along an elevational gradient. The variation in  $R$  also resulted in both species doubling their potential activity at low elevations, potentially offsetting the warm, dry conditions that may limit activity. For *P. metcalfi*, individuals collected from low elevations exhibited higher  $R$  than individuals from high elevations under warm conditions. These results suggest that salamanders from low elevations might use temperature as a cue to regulate skin resistance to water loss. Because the saturation vapor pressure increases exponentially with warmer temperatures, warm air has a greater potential to be drier than cool air given the same amount of water vapor in the air. By exposing salamanders to the same VPDs at warm and cool temperatures, we controlled for different drying potential. Thus, the experiment was designed to determine the capacity of salamanders to respond to temperature by modifying physiology. During our study, low elevations

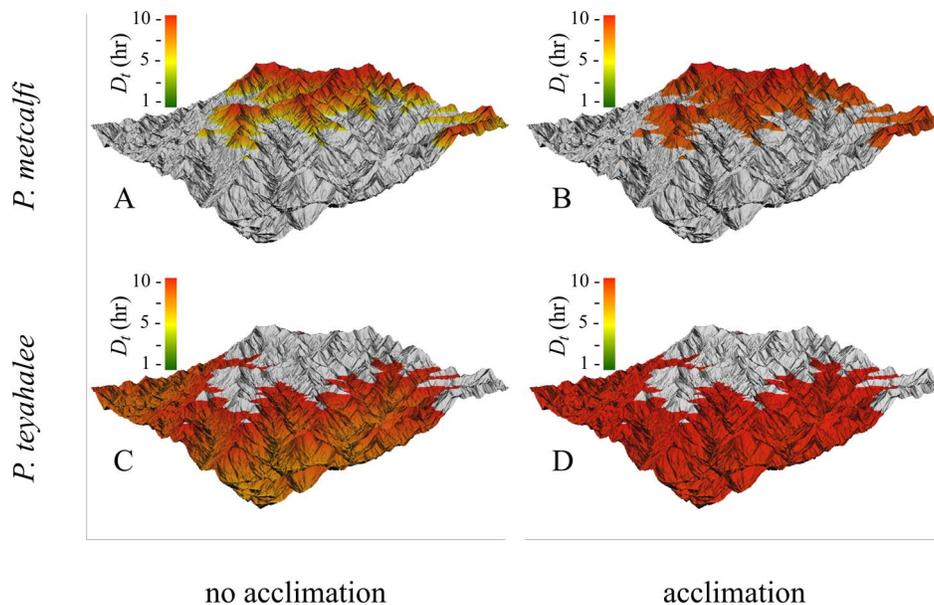


Fig. 7. A 3D representation of the collection area (13.2 km  $\times$  14.8 km at a resolution of 1/3 arc sec) with  $D_t$  (hr) for an adult *P. metcalfi* and *P. teyahalee* interpolated over their elevational range near Cullowhee, NC. The figure compares potential hours of activity using average values of  $R$  (s/cm) (A, C) and incorporating the capacity to acclimate by modifying  $R$  (B, D) for each species (*P. metcalfi*: A, B; *P. teyahalee*: C, D). Locations in gray are beyond the elevational range of the species of interest. The figure demonstrates that acclimation increased the duration of potential activity time.

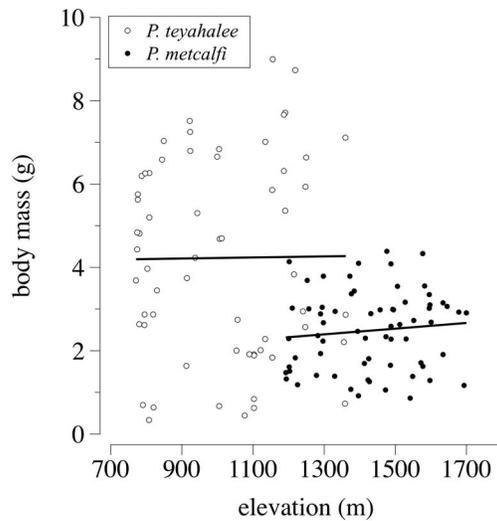


Fig. 8. Body size (g) does not vary along the elevational range for either species (*P. metcalfi*,  $n = 69$ ; *P. teyahalee*,  $n = 58$ ). The figure demonstrates the distinct elevational ranges of the two species as well as the difference in body sizes between the species.

were typically warmer and drier than high elevations. Therefore, salamanders may have anticipated dry air by modifying  $R$  to increase potential duration of activity. This conclusion is reinforced due to the interaction between temperature and  $VPD$ . When temperatures were warm, salamanders increased their resistance to water loss as  $VPD$  increased; conversely, the lowest values of  $R$  were observed under cool temperatures. In addition to the thermal and hydric environment, body size greatly influenced water balance in salamanders.

Surface area of the individual influenced  $R$  for both species, and consequently, the largest individuals exhibited the highest  $R$ . These results suggest a greater pressure for small individuals to respond to the warm, dry conditions compared to larger individuals due to the high surface area to volume ratio of small salamanders. Several studies have demonstrated that large salamanders have lower  $CWL$  compared to small salamanders within a species (Spight 1968, Spotila 1972), which increases the time to dehydration threshold and, thus, provides a substantial ecological advantage for large salamanders (Feder 1983). Because these estimates of water loss control for differences in surface area,

future research on mechanisms that control skin resistance to water loss may reveal the mechanism underlying variation of  $R$  among body sizes. Interestingly, body size did not vary systematically along the elevational gradient within either species, suggesting that the variation in  $R$  within our study is due to underlying physiological mechanisms. However, making any conclusions about physiological variation requires specific knowledge on the ecology, behavior, and life history of each species.

Current species range models ignore physiological variation and assign similar physiological trait values to related species of salamanders (Gifford and Kozak 2012), yet the observed patterns of  $R$  from this study suggest species-specific responses to environmental conditions. For *P. teyahalee*, individuals collected from high elevations exhibited higher  $R$  compared to individuals collected from low elevations, particularly under the warm, dry conditions. The direction of the effect of elevation is not intuitive based on the environmental conditions at the surface, but future research on the influence of behavior and microhabitat selection of *P. teyahalee* might provide an ecological explanation for this phenomenon. For instance, *P. teyahalee* is known to have small home range sizes on the surface and appears to be more fossorial (Nishikawa 1990). Conversely, our activity transects indicated that *P. metcalfi* appears to be much more active on the surface, implying that this species may be more closely tied to temperature and humidity of the air. Therefore, the combination of behavior and microhabitat selection during activity of *P. teyahalee* may explain the different patterns of  $R$  along the elevation gradient between these two species. More importantly, these considerations may reveal how different species acclimatize to environmental conditions in their habitats.

With only two species, we cannot determine whether interspecific differences are due to adaptation or are plastic responses to the environment (Garland and Adolph 1994). However, we suspect that physiological differences along the elevational gradient are due to acclimatization because suitable habitats are continuous across the elevational range (but see Peterman et al. 2014). However, the variation of  $R$  along elevational gradients has important implications for modeling activity. The time to

dehydration ( $D_t$ ) is a critical factor to estimate potential activity (Feder 1983) because salamanders forage on the surface and retreat to moist microhabitat upon losing 3.8% of their body mass due to water loss on average (Feder and Londos 1984). In our estimates of activity, both species increased  $D_t$  at low elevations, resulting in estimates of activity that last almost the entire night along the elevational gradient. However, the responses between species appeared to occur due to different environmental cues. In respect to changes in  $R$  along the elevational gradient, *P. metcalfi* appears to respond to warm temperatures by increasing  $R$ , whereas *P. teyahalee* appears to be increasing  $R$  in response to high VPDs. Thus, accurate estimates of activity must consider species-specific responses to their environment even between closely related species (Wiens et al. 2006). Equally as important, measuring physiological traits under relevant environmental conditions can identify cues that drive physiological responses.

Organisms adopt strategies to cope with the range of environmental conditions experienced in nature, underscoring the importance of using ecologically relevant conditions in the laboratory. In this experiment, we measured  $R$  under humidities and temperatures associated with salamander activity. The experimental conditions also identified potential drivers and cues for modifying  $R$ . For instance, as VPD increased, CWL increased across experimental conditions, demonstrating the difference between the vapor pressure at the surface of the salamander and ambient air drives evaporation rates. For both species,  $R$  increased during the warm, dry conditions, suggesting that these species use temperature as a cue to modify  $R$ , regardless of their elevational origin. Similarly, Rosenthal (1957) demonstrated that plethodontids selected cooler temperatures as the environments became drier. More recent research has indicated that warmer temperatures increased CWL, but VPDs were confounded with temperature (Spotila 1972). In contrast, the thermal and hydric conditions for this experiment were determined from conditions over which salamanders are active. By exposing field-fresh salamanders to ecologically relevant conditions, this experiment unveiled novel physiological responses to the thermal environment. However, the mechanism

underlying these responses remains unknown for many amphibians.

Uncovering the mechanism behind increasing  $R$  may improve our understanding of how amphibians acclimatize to the thermal and hydric environment. Amphibians have several strategies to cope with environmental conditions, but the specific strategies of salamanders are less understood. Salamanders can limit water loss behaviorally by curling onto themselves to reduce surface area of exposed skin (Heatwole 1960). Brown (1972) suggested plethodontids reduce CWL through perfusion, which reduces blood flow to capillaries near the skin. This mechanism may be useful in acute responses to warm, dry environments, but limiting blood flow to the skin would be harmful over long periods due to the need for cutaneous gas exchange. In anurans, there has been extensive research on differential expression of the aquaporin family on certain tissues (Suzuki and Tanaka 2009), but the literature lacks sufficient information of the role these water-transporting proteins in urodeles despite our knowledge of their presence (Brunelli et al. 2007, Suzuki et al. 2007). Because of the critical role these proteins play in water homeostasis for anurans, modifications of these pathways may aid plethodontids in regulating water loss. More likely, interactions between membrane-spanning proteins and the lipid bilayer govern water permeability (Lillywhite 2006). Modification of the skin resistance may also aid in regulating CWL for plethodontids, but to date, most of the research has focused on anuran mucus (Young et al. 2006) and lipid secretions (Shoemaker et al. 1972, McClanahan et al. 1978). Skin secretions are common in many plethodontids, but the role that these secretions play in limiting water permeability are not well understood. Further research on the composition of these secretions (Hamning et al. 2000) and mucus will improve our understanding of the specific physiological mechanisms underlying acclimatization.

To date, nearly 32% of amphibians are threatened by pollution, habitat alteration, disease, and overexploitation (IUCN 2014). With the additional threat of rapidly changing climates, the importance of understanding the capacity to acclimatize is clear and urgent. Currently, plethodontids are predicted to experience significant

declines in suitable habitat by 2020 (Milanovich et al. 2010). To make matters worse, recent research suggests that some species of lowland semi-aquatic plethodontids currently live at the edge of their thermal tolerances (Bernardo and Spotila 2006). Future research that identifies the mechanism behind acclimatization will improve our knowledge of the threat that climate change poses to many organisms (Somero 2010). For example, local adaptation to thermal conditions may attenuate stress related to warming conditions (Kuo and Sanford 2009), and the inability to acclimatize may reveal genetic constraints on the capacity to tolerate changing environmental conditions (Gunderson et al. 2011). For amphibians, the literature lacks direct evidence for evolution in response to anthropogenic climate change (Urban et al. 2013); however, there are several examples of adaptations in response to climate along spatial gradients (Skelly and Freidenburg 2000, Gibbs and Karraker 2006, Orizaola et al. 2010). Moreover, recent research suggests that reduced activity due to warming temperatures are linked to rapid reductions of body sizes in several species of plethodontids (Caruso et al. 2014). By uncovering the geographic variation of  $R$  in these species, we demonstrated a previously unknown capacity of salamanders to modify physiology in response to changes in temperature. Furthermore, the observed geographic variation of  $R$  within these species influenced estimates of the potential duration of activity along elevational gradients. Therefore, mechanistic species distribution models that use activity to estimate habitat suitability may make better predictions by incorporating geographic variation of physiological traits.

## ACKNOWLEDGMENTS

Funding for this project was provided by the Grant-in-Aid program and the Bruce Family Scholarship in Herpetology from the Highlands Biological Station and the Grant-in-Aid of Research program by Sigma Xi: The Scientific Research Society. I also thank the North Carolina Wildlife Commission (License #: 13-SC00746) and the Nantahala National Forest for permission to conduct this study. Experiments were sanctioned by the Institute for Animal Care and Use Committee at the Highlands Biological Station and Clemson University. I also thank Michael Carlo, Josh VanEe, Carrie Miller, Walid Naseri, Chris O'Bryan, Nikki Roach, and Jeff Duck for their assistance in the

field and laboratory. We would also like to thank Grant Connette for letting me pick his brain about salamanders in the early hours of the morning.

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SUPPLEMENTAL MATERIAL

APPENDIX

Table A1. Top models from the all-possible subsets regression that include models within a cumulative weight of 0.95 for *P. metcalfi*.

Parameters	df	logLik	AICc	delta	weight	R <sup>2</sup>
elev + SA + temp + VPD + elev × temp + temp × VPD	10	-131.5	287	0	0.276	0.703
elev + SA + temp + VPD + elev × temp + SA × VPD + temp × VPD	12	-129.5	288.9	1.9	0.107	0.688
elev + SA + temp + VPD + elev × temp + SA × temp + temp × VPD	11	-131.2	289.3	2.31	0.087	0.701
elev + SA + temp + VPD + elev × temp + elev × VPD + temp × VPD	12	-129.7	289.4	2.4	0.083	0.686
elev + SA + temp + VPD + elev × temp + elev × SA + temp × VPD	11	-131.4	289.8	2.82	0.068	0.711
elev + SA + temp + VPD + elev × temp + SA × temp + SA × VPD + temp × VPD	13	-128.6	290.2	3.28	0.054	0.652
elev + SA + temp + VPD + temp × VPD	9	-134.9	291	4.04	0.037	0.718
elev + SA + temp + VPD + elev × temp + elev × VPD + SA × VPD + temp × VPD	14	-127.8	291.7	4.75	0.026	0.704
elev + SA + temp + VPD + elev × temp + elev × SA + SA × VPD + temp × VPD	13	-129.4	291.7	4.78	0.025	0.703
elev + SA + temp + VPD + elev × temp + elev × VPD + SA × temp + temp × VPD	13	-129.6	292.1	5.13	0.021	0.688
elev + SA + temp + VPD + elev × temp + elev × SA + SA × temp + temp × VPD	12	-131.2	292.2	5.23	0.02	0.702
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + temp × VPD	13	-129.7	292.3	5.33	0.019	0.672
elev + SA + temp + VPD elev × VPD + temp × VPD	11	-132.8	292.5	5.58	0.017	0.699
elev + SA + temp + VPD + elev × temp + elev × SA + SA × temp + temp × VPD + elev × SA × temp	13	-129.9	292.8	5.79	0.015	0.655
elev + SA + temp + VPD + SA × temp + temp × VPD + elev × temp × VPD	10	-134.5	293.1	6.12	0.013	0.669
elev + SA + temp + VPD + SA × VPD + temp × VPD	11	-133.1	293.1	6.18	0.013	0.712
elev + SA + temp + VPD + elev × temp + elev × SA + SA × temp + SA × VPD + temp × VPD	14	-128.5	293.2	6.22	0.012	0.652
elev + SA + temp + VPD + elev × SA + temp × VPD	10	-134.8	293.7	6.72	0.01	0.724
elev + SA + temp + VPD + elev × temp + elev × VPD + SA × temp + SA × VPD + temp × VPD	15	-127.1	293.7	6.73	0.01	0.709
elev + SA + temp + VPD + elev × temp + elev × VPD + temp × VPD	14	-128.8	293.7	6.77	0.009	0.722
elev + SA + temp + VPD + elev × temp + elev × SA + SA × temp + SA × VPD + temp × VPD + elev × SA × temp	15	-127.4	294.2	7.27	0.007	0.678
elev + SA + temp + VPD + SA × temp + SA × VPD + temp × VPD	12	-132.2	294.3	7.35	0.007	0.719
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × VPD + temp × VPD	15	-127.6	294.7	7.69	0.006	0.689
elev + SA + temp + VPD + elev × VPD + SA × VPD + temp × VPD	13	-131.1	295	8.04	0.005	0.674
elev + SA + temp + VPD + elev × VPD + SA × temp + temp × VPD	12	-132.6	295.1	8.11	0.005	0.703

Note: The parameters are elev (elevation), surface area (SA), temperature (temp), vapor pressure deficit (VPD), and the interactions between the parameters. The sum of all the Akaike weight for a given predictor were: elev = 1.0, SA = 1.0, temp = 1.0, VPD = 1.0, elev × temp = 0.89, elev × SA = 0.19, elev × VPD = 0.21, SA × temp = 0.26, SA × VPD = 0.29, temp × VPD = 1.0, elev × SA × temp = 0.02, and elev × temp × VPD = 0.009.

Table A2. Top models from the all-possible subsets regression that include models within a cumulative weight of 0.95 for *P. teyahalee*.

Parameters	df	logLik	AICc	delta	weight	R <sup>2</sup>
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + temp × VPD + SA × temp × VPD	18	-82.065	218.1	0	0.261	0.945
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × SA × temp + elev × VPD + SA × temp × VPD	21	-75.565	219.5	1.4	0.129	0.939
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × VPD + SA × temp × VPD	20	-78.308	219.9	1.82	0.105	0.922
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp × VPD + temp × VPD + elev × VPD + SA × temp × VPD	17	-85.259	220.2	2.08	0.092	0.922
elev + SA + temp + VPD + elev × temp + elev × VPD + SA × temp + SA × VPD + temp × VPD + SA × temp × VPD	17	-85.349	220.4	2.26	0.084	0.932
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × SA × temp + SA × temp × VPD	19	-81.289	221.1	2.99	0.058	0.931
elev + SA + temp + VPD + elev × temp + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × temp × VPD + SA × temp × VPD	19	-81.613	221.8	3.64	0.042	0.908
elev + SA + temp + VPD + elev × temp + SA × temp + SA × VPD + temp × VPD + SA × temp × VPD	15	-90.117	221.9	3.81	0.039	0.907
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × VPD + temp × VPD + elev × SA × VPD	15	-90.197	222.1	3.97	0.036	0.931
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × VPD + temp × VPD + elev × temp × VPD	19	-81.936	222.4	4.28	0.031	0.905
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + temp × VPD + elev × temp:VPD	15	-90.902	223.5	5.38	0.018	0.911
elev + SA + temp + VPD + elev × temp + elev × SA + SA × temp + SA × VPD + temp × VPD + elev × SA × VPD + SA × temp × VPD	16	-89.158	223.9	5.79	0.014	0.934
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + SA × VPD + temp × VPD + SA × temp × VPD	20	-80.469	224.3	6.14	0.012	0.923
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × temp × VPD	18	-85.194	224.4	6.26	0.011	0.910
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × SA × VPD	16	-89.578	224.8	6.63	0.009	0.944
elev + SA + temp + VPD + elev × temp + elev × SA + elev × VPD + SA × VPD + temp × VPD + elev × temp × VPD + SA × temp × VPD	22	-75.796	225.4	7.23	0.007	0.908
elev + SA + temp + VPD + elev × temp + elev × VPD + SA × temp + SA × VPD + temp × VPD + elev × SA × temp + elev × temp × VPD	16	-90.146	225.9	7.76	0.005	0.945

Note: The parameters are elev (elevation), surface area (SA), temperature (temp), vapor pressure deficit (VPD), and the interactions between the parameters. The bolded row represents the sum of all the Akaike weight for a given predictor. The sum of all the Akaike weight for a given predictor were: elev = 1.0, SA = 1.0, temp = 1.0, VPD = 1.0, elev × temp = 0.89, elev × SA = 0.83, elev × VPD = 0.94, SA × temp = 0.81, SA × VPD = 0.98, temp × VPD = 1.0, elev × SA × temp = 0.2, elev × SA × VPD = 0.05, elev × temp × VPD = .46, and SA × temp × VPD = 0.79.