Measuring the acclimatory potential of thermal physiology in *Ensatina eschscholtzii*

0. Abstract

I set out to determine how Ensatina eschscholtzii would be affected by climate change. Ensatina is a species of plethodontidae, the largest family of salamanders in the world. While the ensatina is not an important species on its own, plethodontid salamanders serve as key indicator species of ecosystem health. The goal of this study was to determine how ensatina extinction risk and geographic range will be affected by climate change. I particularly looked at how ensatina are able to acclimate their thermal preferences and thermal performance under different long-term temperatures. I also looked at differences in both thermoregulation and skin resistance between several subspecies, to understand the effects of local adaptations. To determine thermal preference, all ensatina were run on metal tracks with thermal gradients of approximately 5 to 25 degrees celsius. Thermal performance was estimated by measuring the latencies of anti-predator behaviors at different temperatures. Preference and performance were each measured for all ensatina before and after long-term acclimation. Skin resistance was approximated by calculating average water loss rates. Results suggested significant differences in thermal preference and performance between subspecies. Evidence also supports the idea that ensatina are able to acclimate their thermal physiology, although the details on when and how they do so are still unclear. These experiments should be repeated on more subspecies of ensatina to determine any possible differences between subspecies, and to increase the reliability of the data.

1. Introduction

Most amphibians have adaptations for surviving climatic variability and suboptimal temperatures. Traits such as skin resistance, thermoregulation, and regulatory chemical production allow them to tolerate conditions that would otherwise be fatal (Kearney, Shine, & Porter, 2009; Mark L. Wygoda, 1984; Vitt & Caldwell, 2014). Salamanders of the family *Plethodontidae* are thought not to have any of these things. They don’t thermoregulate, they don’t produce resistant chemicals, and they don’t have much skin resistance. How then, do they tolerate periods of high temperature and low humidity if they have so few adaptations to resistance? Simple: They wait. When conditions become very unfavorable, plethodontids forego foraging and aestivate in their moist microhabitats until the environment around them becomes more suitable. In particular, It's been hypothesized that when environmental temperatures exceed their preferred temperatures, they cease activity (Sinervo et al., 2018). Rather than spending energy on costly adaptations to resistance, they use their extraordinarily low metabolism to forego foraging for potentially very long periods of time (Feder, 1983).

Although they can aestivate, eventually they must leave their microhabitats and resume foraging, risking death given a low enough humidity or high enough temperatures. Because of their sensitivity to climatic conditions, site fidelity, and low fecundity, it's been suggested that plethodontids may be used as indicator species for forest ecosystem health. Unfortunately, these same traits make them highly vulnerable to long term climate change, in particular the current wave of human-caused global climate change (Hartwell H. Welsh Jr. & Sam Droege, 2001; Joseph R. Milanovich, Stanley E. Trauth, David A. Saugey, & Robyn R. Jordan, 2006).

To understand just how plethodontids, and consequently their ecosystems, will be affected by climate change, we studied the thermal physiology of *Ensatina eschscholtzii*, a plethodontid ring species endemic to Western North America. Although categorized as one species, seven morphologically and genetically distinct subspecies of ensatina are currently recognized, and can be found along a geographic ring in Central California (Fig. 1). Their range stretches from the northern edge of Baja California up through British Columbia (Wake, 1997). By running identical experiments with morphologically and geographically distinct subspecies, we tried to gauge the capability and effects of localized adaptations in plethodontids.

The broadest question we asked was “How will ensatina range be affected by climate change?” To address this question, we primarily looked at three factors: thermal preference, thermal performance, and acclimation potential. These traits were measured across five subspecies, and will be used to build a predictive species-distribution model indicating which geographical areas will become high risk for plethodontids. Once that information is known, we'll be able to design conservation plans to save the most at-risk ecosystems. This study is based on very similar, previous study that I previously conducted. That study and its results will hereafter be referred to as preliminary.

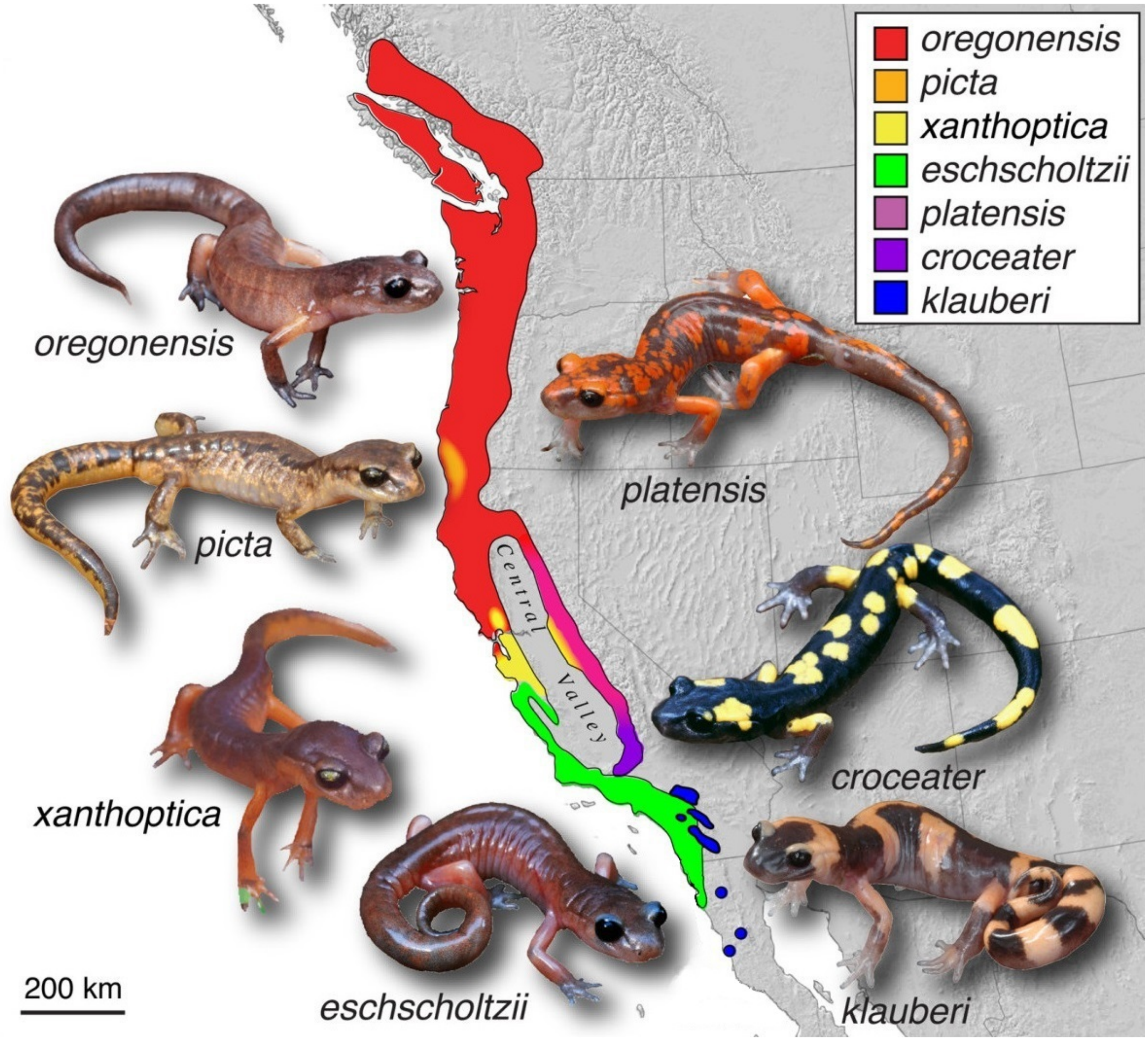


Fig. 1. The range of the seven subspecies of E. eschscholtzii. Note that ranges in this map are approximate, and don't show all overlapping between subspecies. (Thanukos, Anna & Devitt, Tom, n.d.).

2. Materials and Methods

This study was performed at the Sinervo Lab at the University of California, Santa Cruz. To determine how *Ensatina eschscholtzii* adjust their thermal physiology in response to long-term temperature change, 67 ensatina were captured from the wild and placed in controlled laboratory conditions. Five subspecies were collected to look for a difference in acclimatory potential between different subspecies. Since there are seven subspecies total, with fairly large and distinct ranges, it's important to verify a lack of difference between subspecies before drawing any conclusions about the species as a whole. 26 individuals of the e. *xanthoptica* subspecies were collected from a region of redwood forest on the UCSC campus. 17 individuals of the *e. oregonensis* subspecies were collected from Gazos Creek, a mixed-evergreen region located in San Mateo, California. The *e. oregonensis* were collected five days after the *e. xanthoptica*. Additionally, 10 *e. platensis*, 10 *e. picta*, and 4 *e. eschscholtzii* were collected.

2.1 Sampling design

When an ensatina was found, it was assigned an ID and had a corresponding GPS coordinate saved to ensure its proper return after the experiment. During a three-minute observation period, the latencies of any anti-predator behavior were observed and recorded (See section 2.3 for details on observation procedure). Body temperature (Tb), substrate temperature (Tsub), air temperature (Tair), and the temperature of the ground immediately outside the microclimate in which the ensatina was found (Tout) were recorded. All temperatures except Tair were recorded with an Amprobe IR-750 infrared thermometer. Tair was recorded with an Omega thermometer. Once temperatures and behavioral latencies were recorded, each ensatina was placed in an appropriately labeled plastic container lined with moist paper towels. The collected ensatina were moved to a 15 degree CER for pre-acclimation trials. All individuals were weighed and measured (SVL and total length) later on the same day they were collected.

Once captured, ensatina had their thermal preferences (Tpref) and performance (TPC) measured (Pre-acclimation period). They were then divided into three different temperature treatments (15, 18, and 21 degrees), and placed in appropriate controlled environment rooms (CERs) for three or more weeks. At the end of this period, each salamander had its tpref and TPC measured once more (post-acclimation period). In between these periods, each salamander had its skin resistance to water loss measured as well. These experiments used behavior to estimate thermal performance at different temperatures.

2.2 Measuring thermal preference and its acclimation potential

All individual ensatina had their thermal preference tested within three nights of collection, to ensure measurements were taken before any acclimation to lab conditions had taken place. Ensatina are crepuscular, so to emulate behavior during periods of activity, trials were run between 5 and 11 PM. Only one subspecies was run at a time, and it was randomized which night each ensatina went on. Eight to nine individuals were run each night for the three nights both following collection, and after a month of acclimation.

Trials were run in metal tracks surrounding by heated water on one end and ice on the other. This created a smooth temperature gradient of 2-6 to 24-28 degrees celsius from one end to the other. The tracks were lined with moist paper towels to ensure no desiccation during trials. Once prepared, one ensatina was placed in each track, and the tracks were covered to sufficiently block light from the lab and better emulate the natural environment (as well as to hide the giant, terrifying researchers). Every five minutes, for 65 minutes, the covers were removed and body temperatures were read and recorded for each ensatina by using Amprobe IR-750 infrared thermometer on their backs. Anyone who attempted to climb out of their track was gently moved back down to its base.

This test was designed to gauge what the preferred temperature of each individual ensatina would be in the wild. An underlying assumption of this experiment is that amphibians will not actively forage when temperatures outside their microclimates exceed their preferred temperature. Holding that assumption to be true, information about ensatina thermal preference helps us estimate when they'll be active under certain climates. By testing acclimatory potential, we can gauge how well ensatina will be able to adapt to climate change.

2.3 Measuring thermal performance and its acclimation potential

All ensatina had their thermal performance tested within five days of collection, as to be done before any long-term acclimation. To avoid affecting thermal preference results, thermal performance tests were always performed after thermal preference. Only one subspecies was run at a time. All ensatina were tested at 10, 15, 18, 21, and 24 degrees Celsius, and most were run at 5 degrees as well. Their critical maximum and minimum temperatures were already known, so extreme temperatures were unnecessary (Brattstrom, 1963). Prior to being tested, individuals were acclimated in their experimental temperature for two to four hours.

Trials were run on large metal counters covered with moist paper towels. Counters were located in the same CERs in which the ensatina were acclimated. To simulate escape routes (ensatina often run into underground tunnels when threatened), four PVC half-pipes were lined with paper towels and placed on the north, south, east, and west regions of the counter. For each trial, one ensatina was dropped roughly six inches onto the center of the counter to simulate a predator encounter. It was then observed for two minutes (initial observation period). The latencies (how long it took for them to occur, in seconds) of any anti-predator behaviors were recorded. Behaviors included initial immobility, running away, seeking shelter, standing on toes, arching the tail, rocking back and forth, and emitting noxious chemicals from the tail or head. After the initial observation period, it was then forcibly bopped on the head five times by the researcher, meant to simulate a predator attack. The ensatina was then observed for thirty seconds, after which it was forcibly bopped five more times and observed for another thirty seconds. After this, observation ended and the ensatina was returned to its enclosure. The total observation time for each ensatina was three minutes.

The latencies of anti-predator behaviors are used to estimate physiological performance capability. Slower latencies suggest less ability, while faster ones suggest more. By collecting data across five different temperatures, and drawing from literature maximum and minimum values, we used this data to assemble a thermal performance curve. The main purpose of this curve is to inform the thermal preference. Since ensatina seem to remain inactive above their preferred temperatures, does it mean that they can't, or simply choose not to? This information is critical to understanding how adaptable ensatina foraging behavior may be.

2.4 Measuring skin resistance

There's a widely known assumption that amphibian skin is a free evaporative surface, meaning that it has no more resistance to water loss than a direct surface of water would. When tested, extensive evidence has suggested otherwise (Riddell, McPhail, Damm, & Sears, 2018; Mark L. Wygoda, 1984; Vitt & Caldwell, 2014). Nonetheless, many species distribution models completely ignored skin resistance when calculating predicted habitats under climate change. To build the most accurate model we could, we measured the skin resistance to water loss of all ensatina after they had been acclimated for three weeks. This was done by measuring desiccation rates from both the ensatina and a comparable agar model (an actual free-evaporative surface).

The setup for this experiment was fairly simple. An air pump was connected to a sealed container of drierite, an agent to absorb all moisture from the air. The container was then attached to three sealed PVC tubes containing HOBO RH and temperature data loggers. These loggers were each attached to three sealed containers. The first container held an ensatina, which was placed on a layer of hardwire mesh to ensure air flow across its entire body. The second held a cylindrical agar model of comparable surface area and mass, also placed on a layer of hardwire mesh. The third was empty, and was used simply to read control conditions of the system. Each container was attached to a flow meter, used both to read air flow rates and adjust pressure to ensure equal rates across all three containers.

For each trial, an ensatina was weighed and had 97% of its body weight calculated. This was the target, losing 3% of water by body weight for each ensatina. The ensatina chamber was then placed onto the scale, which was zeroed. This was so we could observe weight change without unsealing any chambers and disrupting the air pressure of the system. The ensatina was placed in this chamber, and an agar model was placed in the agar chamber. All three were then sealed, and the air pump was turned on. The weight of the ensatina was periodically observed, and after losing 3% of its body weight, the time it took was recorded and the ensatina was removed from the system. The agar model's final weight was recorded, and it was removed from the system to be used later.

Resistance to water loss in amphibians has to components: skin resistance and boundary layer resistance. Boundary layer resistance is how much the layer of air immediately surrounding the individual can resist evaporation. Skin resistance is how the individual's physical properties can resist evaporation, and it's the useful measure we were trying to calculate. Total resistance is R_T equals boundary layer resistance R_B plus skin resistance R_S, where . Since agar models are free-evaporative surfaces, they have no skin resistance, so their total resistance to evaporation is only boundary layer resistance $R_T = R_B + 0$. Ensatina have both, so we only measure $R_T$despite needing to find R_S. With simple substitution, we can then solve for the ensatina's skin resistance. Although we did not take pre-acclimation values, we compared skin resistance across subspecies and to pre-acclimatory values from the literature for comparable species.

2.5 Analysis and Statistical Design

JMP Pro 14 was used to run statistical analysis on the data, primarily comparative analysis and model visualization. R was used for additional data analysis.

MaxEnt 3.4.1, a modeling program that employs machine learning, was used to perform sensitivity analysis on how well 19 bioclimatic variables serve as predictors of ensatina range. This model takes geographic presence data of a species, calculate 'non-presence' values for areas where it isn't found, and compares properties of the two regions. It then uses input variables to predict the species' range, and estimate each variable's importance in doing so, indicating what climatic variables may be most important to the species. Climate projection and geographic data were taken from WorldClim 1.4 (Hijmans, Cameron, Parra, Jones, & Jarvis, 2005). ArcMAP 10.5.1 was used to prepare mapping data for use in MaxEnt. Ensatina range data was compiled from www.vertnet.org.

A species distribution model will be used to generate predictions of ensatina geographic range under expected climate change (Sinervo et al. 2010; Caetano et al. 2017). This model uses the difference between projected environmental temperature values and measured Tprefs to estimate hours of restriction, the times at which temperatures are too high for a species to remain active, for a given geographic population. It estimates extinction risk by estimating how hours of restriction limit essential foraging and activity times. (Sinervo et al., n.d.). This model will be improved upon by adding skin resistance and acclimatory potential to its predictive variables.

3. Results

3.1 Is there a difference in field body temperature between subspecies?

I used a bar graph to compare mean field body temperatures between *e. oregonensis, e. picta,* and *e. xanthoptica* (Fig. 2). At the time of writing, I lack field data for other subspecies, so they're excluded from this and all other field data analysis. The *e. oregonensis, e. picta,* and *e. xanthoptica* represented here were collected from Gazos creek, Crescent City, and UCSC, respectively. Their mean body temperatures are 13.64, 9.86, and 14.91 degrees. An ANOVA test found them to be significantly different (p = 0.0039).

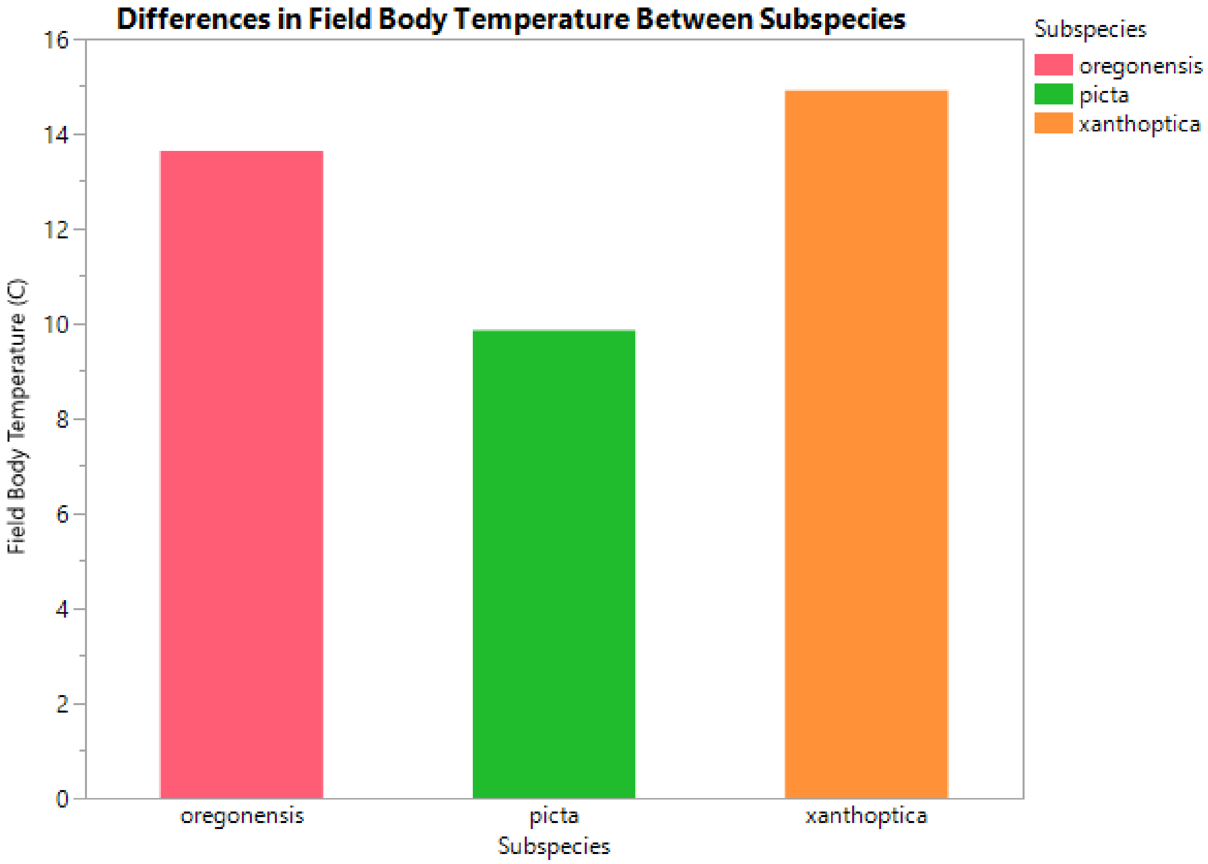


Fig. 2. A bar graph comparing field body temperatures of *e. oregonensis, e. picta,* and *e. xanthoptica*.

3.2 Do ensatina cease activity above their preferred temperatures?

A key assumption of this study is that ensatina will cease activity at temperatures above their preference. To test this assumption, I created a two boundary plots, each comparing each animal's pre-acclimation thermal preference to environmental temperatures it was found at. I combined results from both this study and the preliminary study where possible. Only *e. oregonensis, e. xanthoptica,* and *e. picta* are represented, as field data for the other subspecies is unavailable to me at the time of writing.

The first boundary plot compares air temperature to thermal preference (Fig. 3). 5 of the 49 individuals were found above their preferred temperature, about 10%.

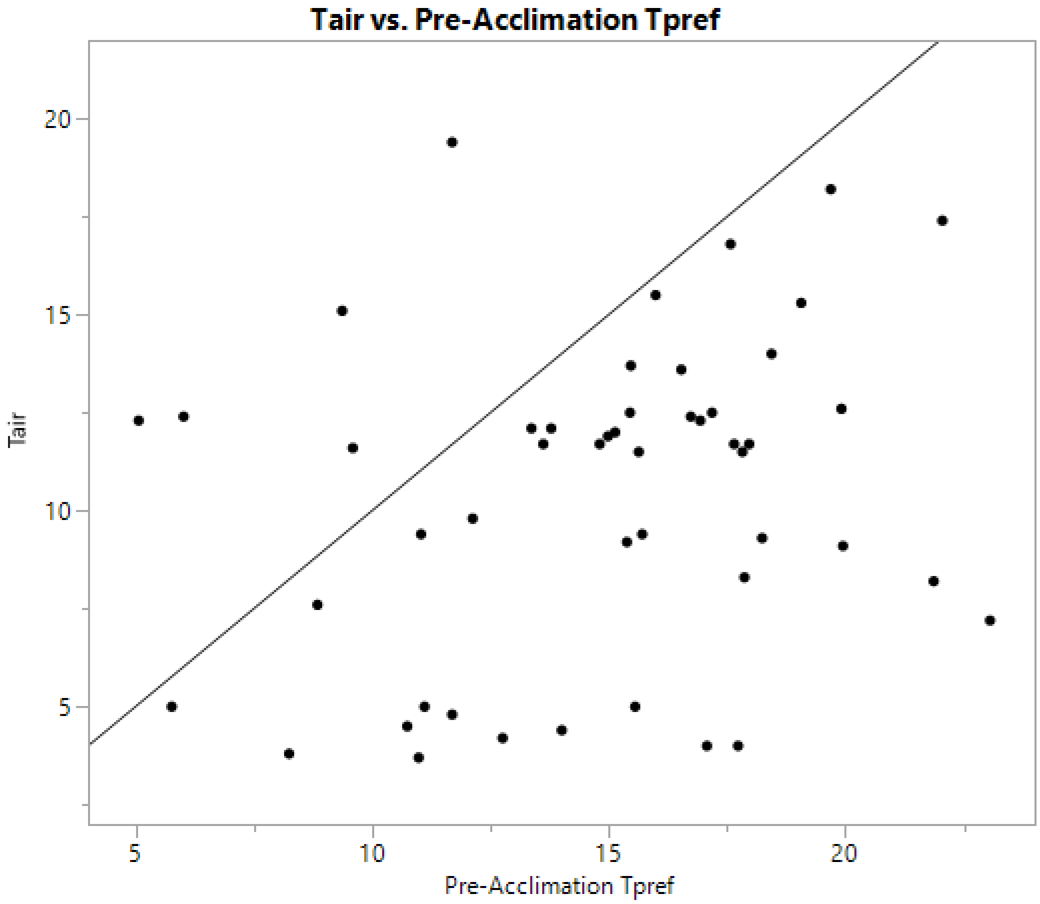


Fig. 3. A boundary plot comparing each animal's field air temperature to its thermal preference.

The second boundary plot compares non-substrate ground temperature (Tout, the ground immediately outside of each animal's microclimate) to thermal preference (Fig. 4). 11 of the 36 individuals were found above their preferred temperature, about 31%.

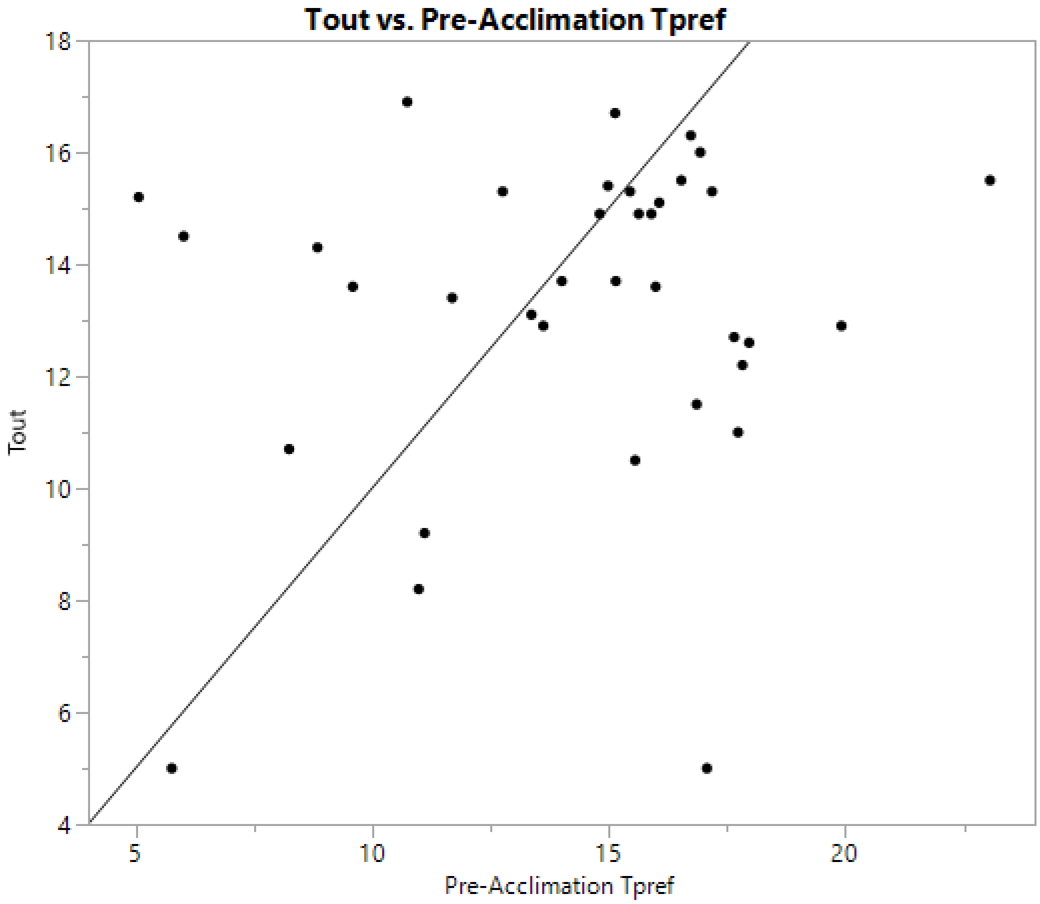


Fig. 4. A boundary plot comparing each animal’s immediate ground temperature to its thermal preference.

3.3 How does ensatina body temperature relate to environmental conditions?

To determine how air temperature functions as a predictor of ensatina body temperature in the wild, I performed a linear regression. Data from both this and the preliminary experiment was used for this analysis. A quadratic model seems to be a good fit, with normally distributed residuals (Fig. 5). The resulting equation is Tb = 9.4129592 + 0.206897*Tair + 0.0569741*(Tair - 11.2957)^2

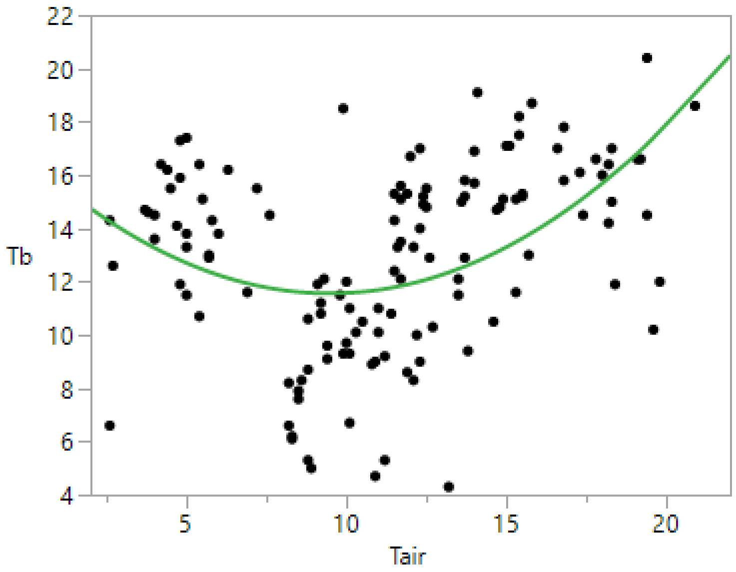


Fig. 5. A quadratic model fitting the relationship between field air and body temperatures ($p < 0.0001$).

I performed a linear regression on how ground temperature functions as a predictor of body temperature as well. A linear model seemed to be a good fit with normally distributed residuals (Fig. 6). The resulting equation is Tb = 11.839464 + 0.2395378*Tout.

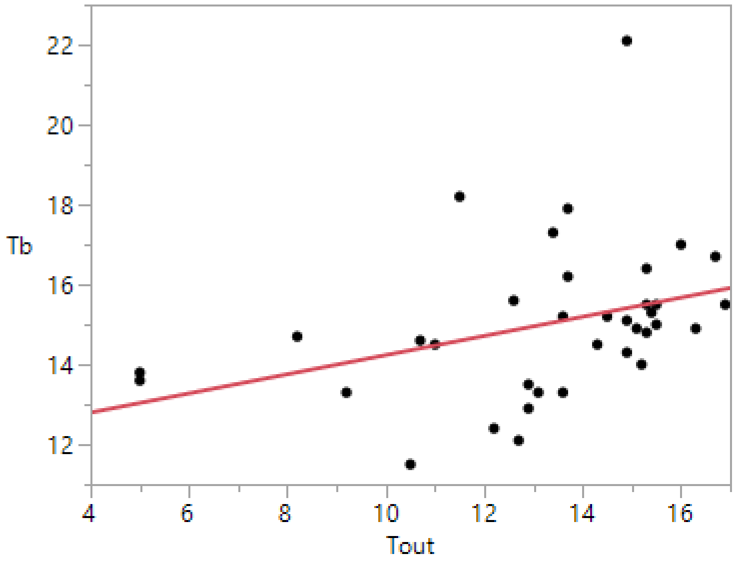
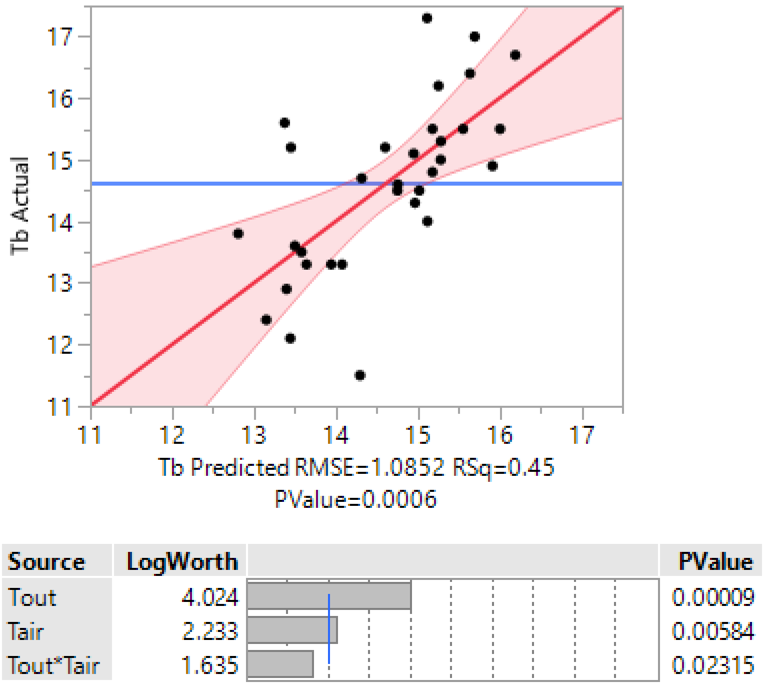


Fig. 6. A linear model fitting the relationship between field ground and body temperatures ($p = 0.0352$).

A leverage plot indicated that air temperature, immediate ground temperature, and their interaction, are all strong predictors of body temperature (Fig. 7). Immediate ground temperature is by far the most important predictor, while air temperature is second and their interaction is least important.



Source	LogWorth		PValue
Tout	4.024		0.00009
Tair	2.233		0.00584
Tout*Tair	1.635		0.02315

Fig. 7. A leverage plot and corresponding effect analysis indicating that immediate ground temperature, air temperature, and their interaction are all strong predictors of field body temperature in ensatina (p = 0.0006).

I've thus far excluded substrate temperature from analysis. Data on air temperature is readily available, and ground temperature can often be found or estimated. That isn't the case for microhabitats, as their buffering effect seems to be poorly understood in quantitative terms. Although I think it's significantly less important for building a predictive model, I'll now go through how substrate relates to field body temperature.

Unsurprisingly, there's an extremely strong positive relationship between substrate and body temperature (Fig. 8). The line is almost one-to-one, save for some outliers. A leverage plot indicates that substrate is a much more important predictor of body temperature than air temperature, immediate ground temperature, or any interactions between any of them (Fig. 9).

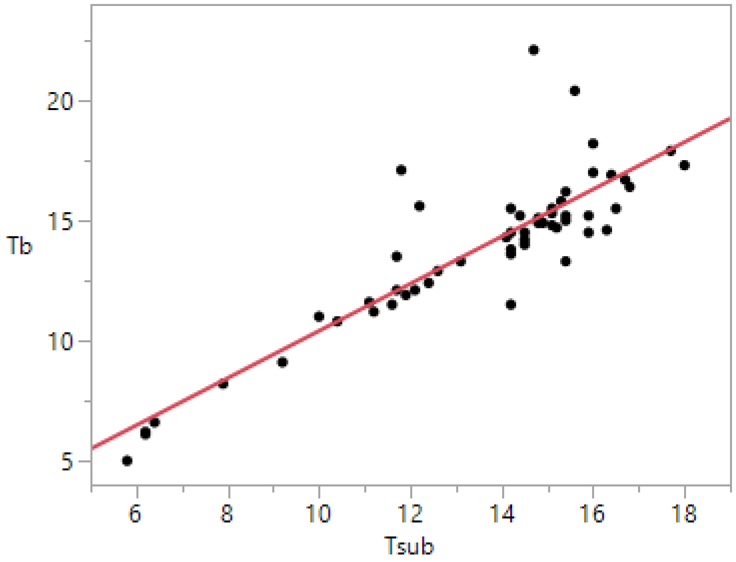
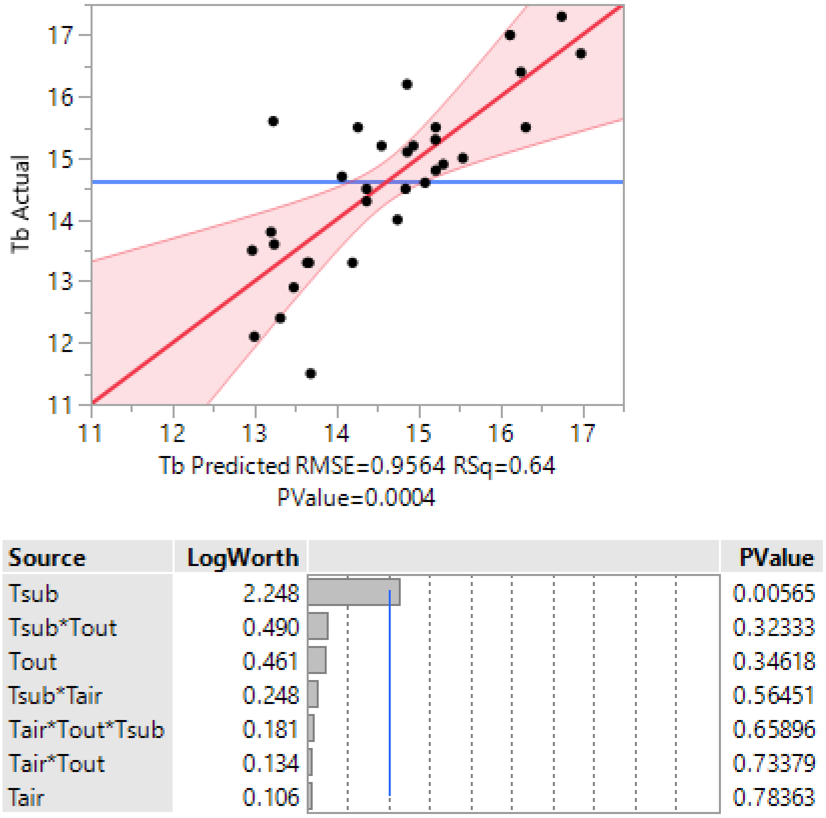


Fig. 8. A linear model fitting the relationship between field substrate and body temperatures ($p < 0.0001$).



Source	LogWorth	PValue
Tsub	2.248	0.00565
Tsub*Tout	0.490	0.32333
Tout	0.461	0.34618
Tsub*Tair	0.248	0.56451
Tair*Tout*Tsub	0.181	0.65896
Tair*Tout	0.134	0.73379
Tair	0.106	0.78363

Fig. 9. A leverage plot and corresponding effect analysis indicating that substrate temperature is by far the most important predictor of field body temperature ($p = 0.0004$).

3.4 What's the buffering effect of ensatina microhabitats?

I performed a matched-pair t-test to compare the substrate (microhabitat) and immediate ground temperature (outside of microhabitat) of each ensatina (Fig. 10). I used no data from the preliminary experiment, as immediate ground temperature was not measured for it. Substrate temperatures were typically either greater than immediate ground temperatures, around the same value, or very slightly lower. Altogether, substrate temperatures ranged from 11.7 to 17.7 degrees (6 degree range). Immediate ground temperature ranged from 5 to 16.9 degrees (11.9 degree range).

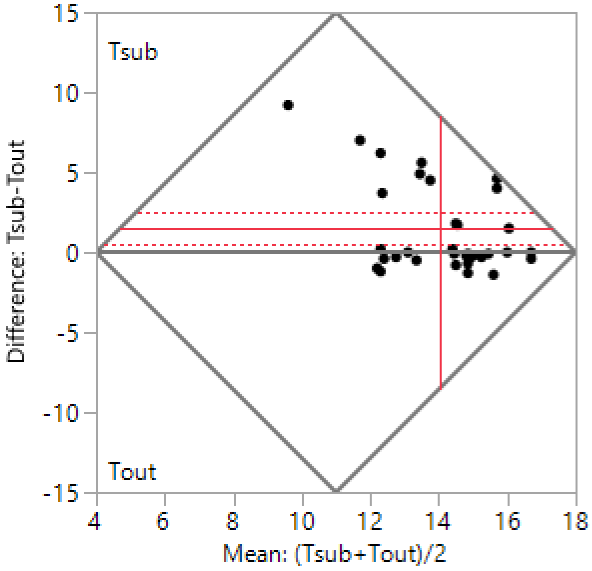
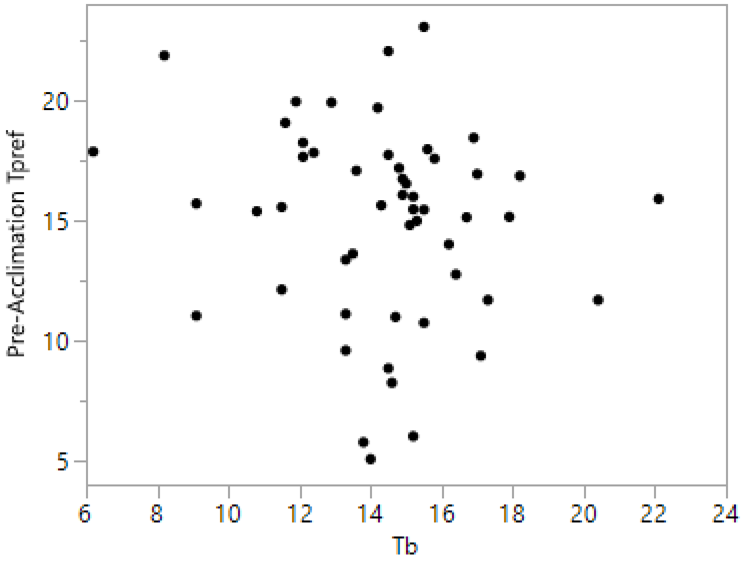


Fig. 10. A Tukey mean difference plot comparing substrate and immediate ground temperature for each ensatina (p = 0.0045)

3.5 Does thermal preference relate to environmental conditions?

I compared pre-acclimation thermal preference to field body temperature, substrate temperature, immediate ground temperature, and substrate temperature. There appears to be no correlation between any of these variables and thermal preference.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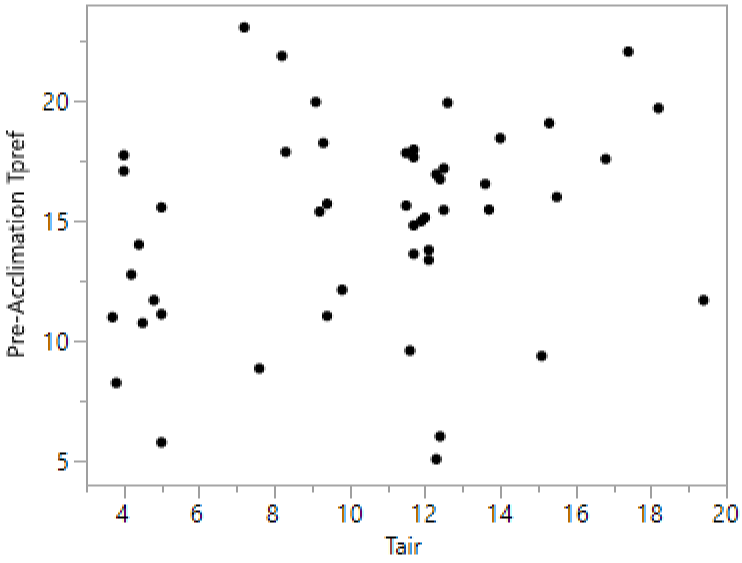
Pre-Acclimation Tpref
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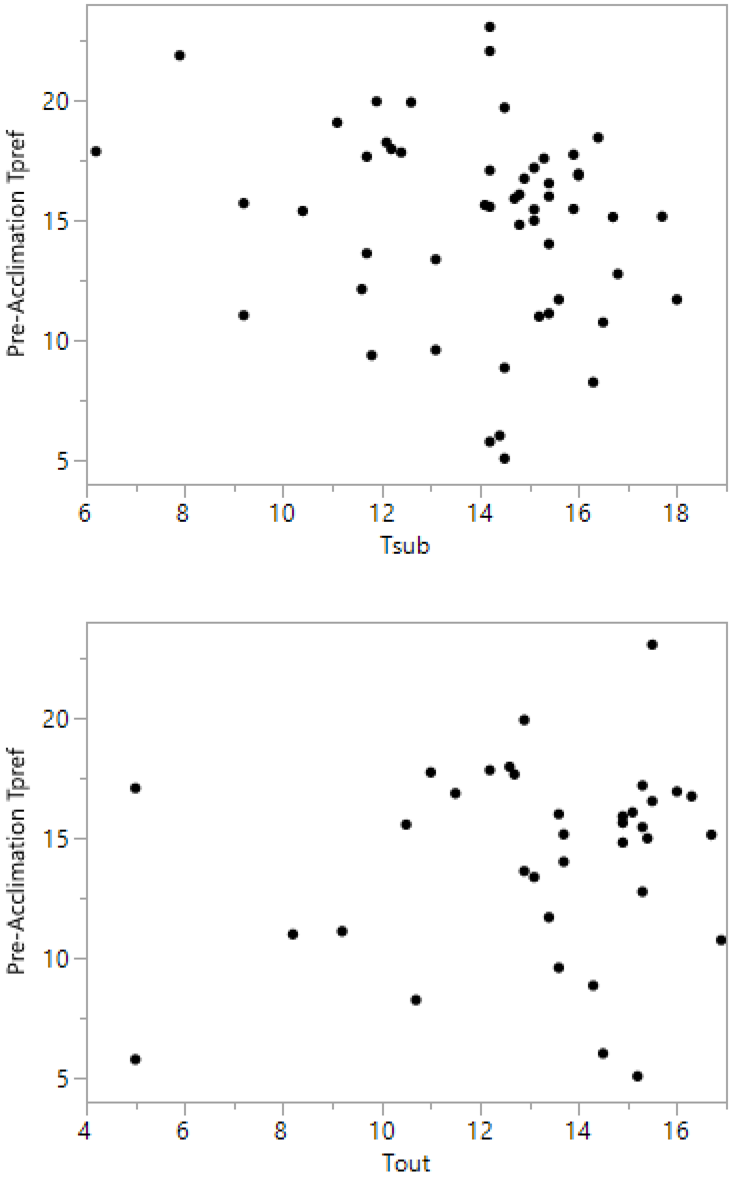


Fig. 8. Four dot plots comparing pre-acclimation thermal preference to field body, substrate, air, and immediate ground temperatures.

3.6 Is there a difference in thermal preference between track types?

I used a series of ANOVA tests to compare mean thermal preference values between the metal track trials used in this experiment and the wooden track trials used in the preliminary experiment. Only *e. picta* had post-acclimation thermal preference measured for the preliminary experiment, so only that subspecies was compared for post-acclimation trials. *e. picta*, *e. xanthoptica*, and *e. eschscholtzii* had pre-acclimation thermal preference measured in the preliminary experiment, but I've excluded *e. eschscholtzii* from this analysis on account of only having two subjects.

For pre-acclimation comparisons, differences between track types for both *e. xanthoptica* and *e. picta* were negligible, although average preference was slightly higher for *e. xanthoptica* on the wooden tracks (Fig. 9, 10). For the post-acclimation comparison, the difference was significant, with the wood track average being about 2.01 degrees lower than the metal track average (Fig. 11).

Due to time constraints and uncertainty about differences in acclimation results between this and the preliminary study, all preliminary thermal preference results are excluded from the following data analysis.

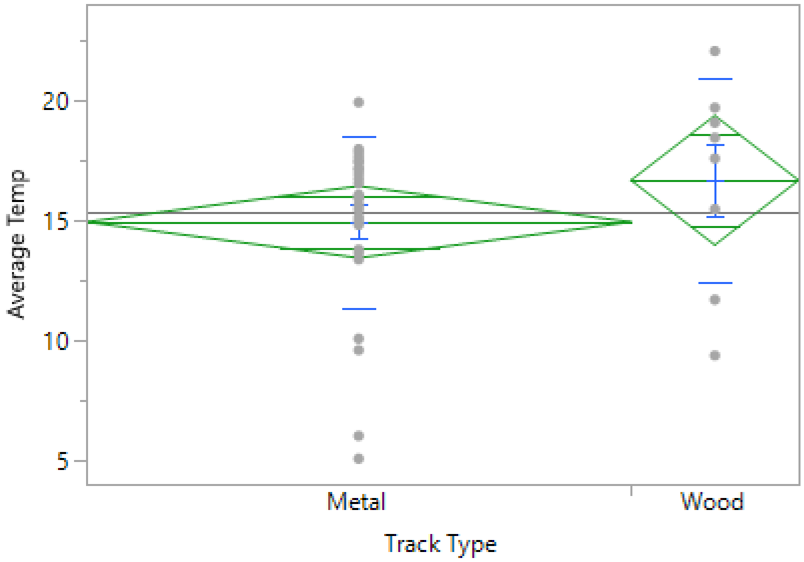


Fig. 9. A graph comparing pre-acclimation thermal preference values between track types for *e. xanthoptica* (p = 0.2594).

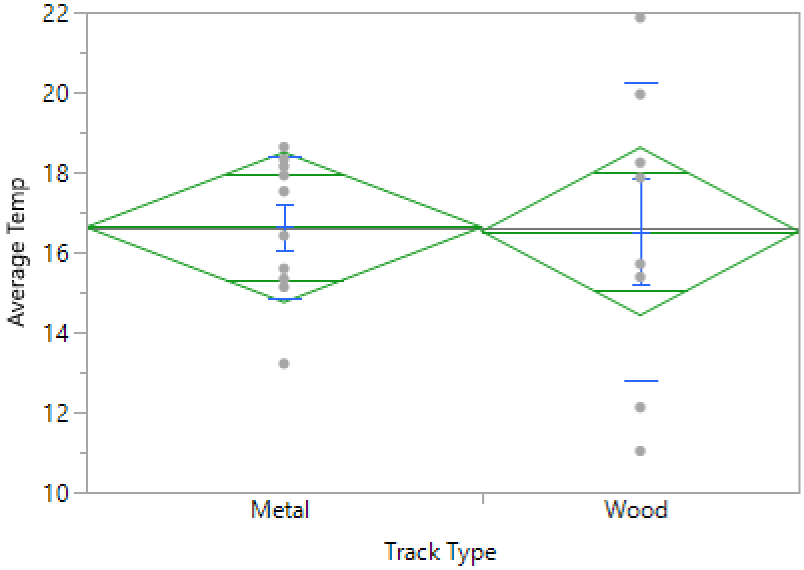


Fig. 10. A graph comparing pre-acclimation thermal preference values between track types for *e. picta* (p = 0.9384).

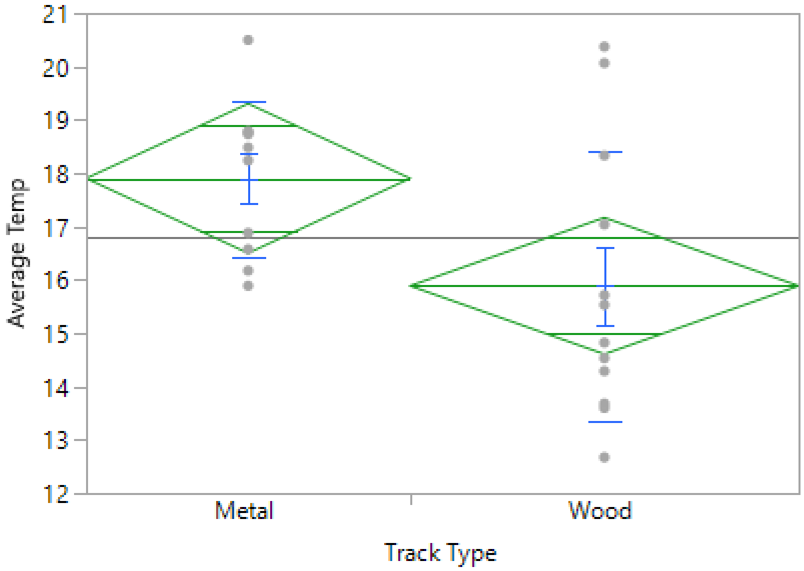


Fig. 11. A graph comparing post-acclimation thermal preference values between track types for *e. picta* (p = .0385).

3.7 Thermal preference across all subspecies

ANOVA tests indicated significant differences between subspecies for pre-acclimation thermal preference (p = 0.0006). *e. eschscholtzii* and *e. platensis* had very similar averages: 18.37 and 18.10 degrees, respectively. *e. picta* was a little lower at 16.63 degrees, while *e. xanthoptica* and *e. oregonensis* were substantially lower, at 14.93 and 12.34 degrees, respectively.

For post-acclimation thermal preference, differences were found to be insignificant overall and with all individual t-tests (p = 0.1514). Mean preferences for *e. oregonensis* and *e. xanthoptica* increased, while *e. eschscholtzii* and *e. picta* barely changed from pre-acclimation trials. *e. platensis* was excluded, as its post-acclimation thermal preference trials have not been completed at the time of writing. Note that this comparison does not take acclimation groups into account; this analysis is performed in section 3.2.

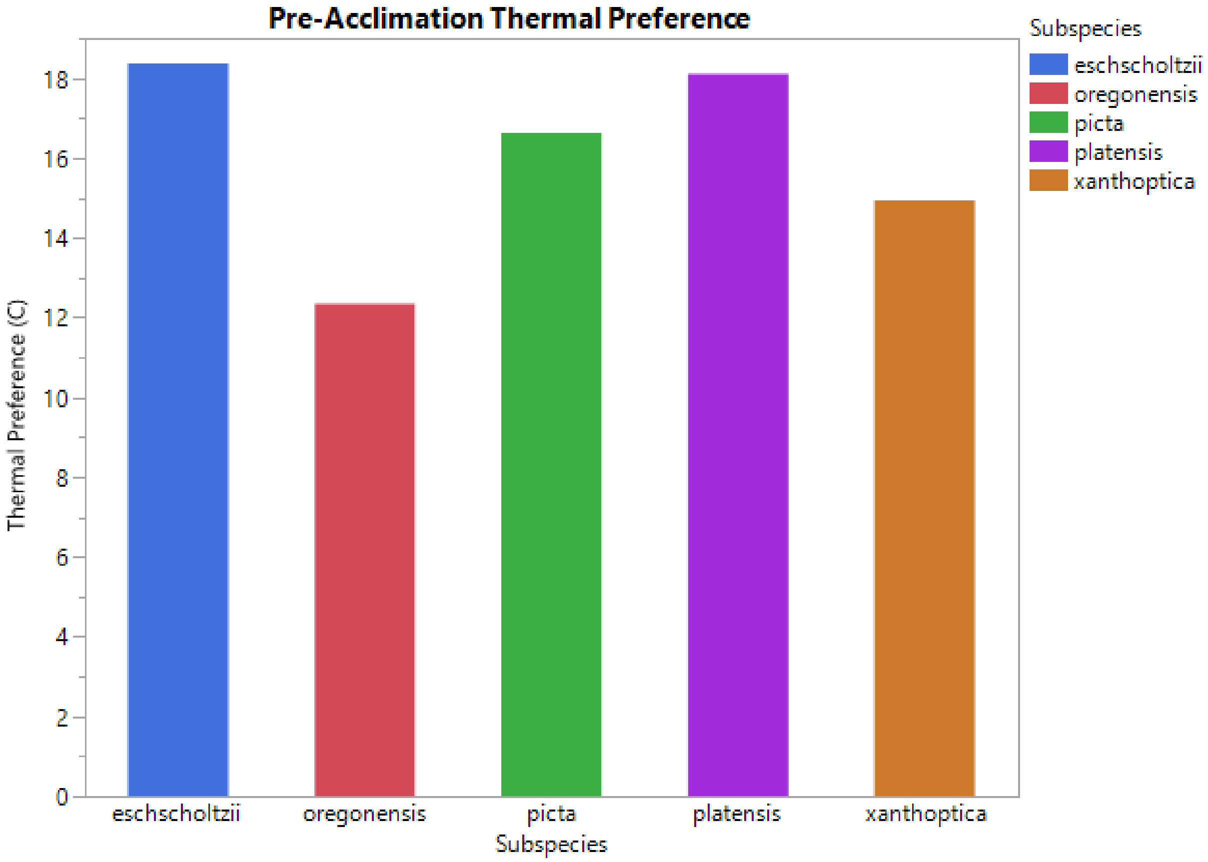


Fig. 12. A bar graph comparing mean pre-acclimation thermal preference values across all five subspecies.

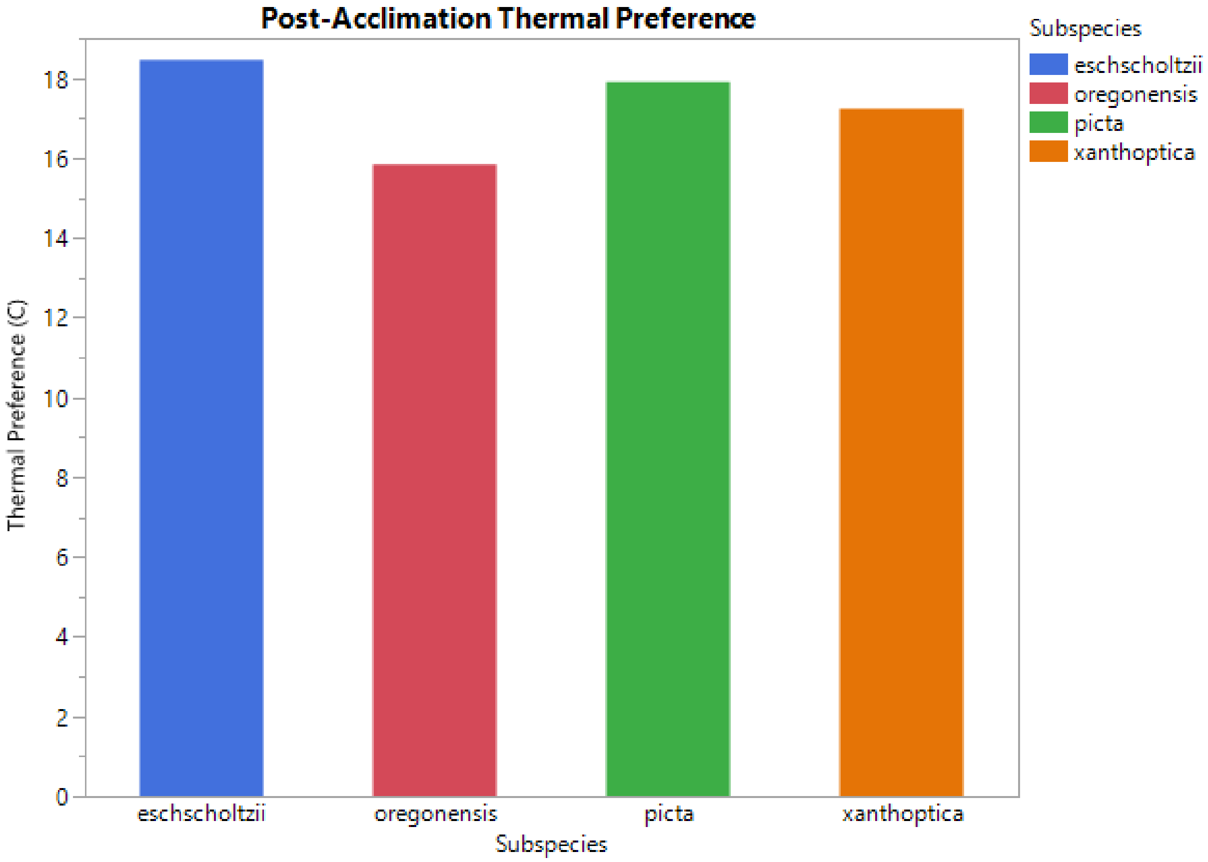


Fig. 13. A bar graph comparing mean post-acclimation thermal preference values across all subspecies except *e. platensis*.

3.8 Do ensatina acclimate their thermal preference?

All subspecies except *e. eschscholtzii* were acclimated in a 15, 18, and 21-degree long-term acclimation temperature. *e. eschscholtzii* was only acclimated at 15 and 21 degrees due to an error. Patterns of acclimation varied greatly between subspecies (Fig. 14). At the time of writing *e. platensis* has not had post-acclimation preference measured, so it's excluded from this analysis.

All subspecies had a mean increase of preference across all treatments. *e. eschscholtzii* increased preference slightly more in the 15 degree treatment than in the 21-degree treatment, but the increase, being less than half a degree, was negligible. *e. picta* increased preference more as treatment temperature increased. *e. xanthoptica* increased preference less as treatment temperature increased. *e. oregonensis* had the highest increase of all in its 18-degree group, close to 5 degrees. It also increased significantly in the 21 and 15-degree treatments.

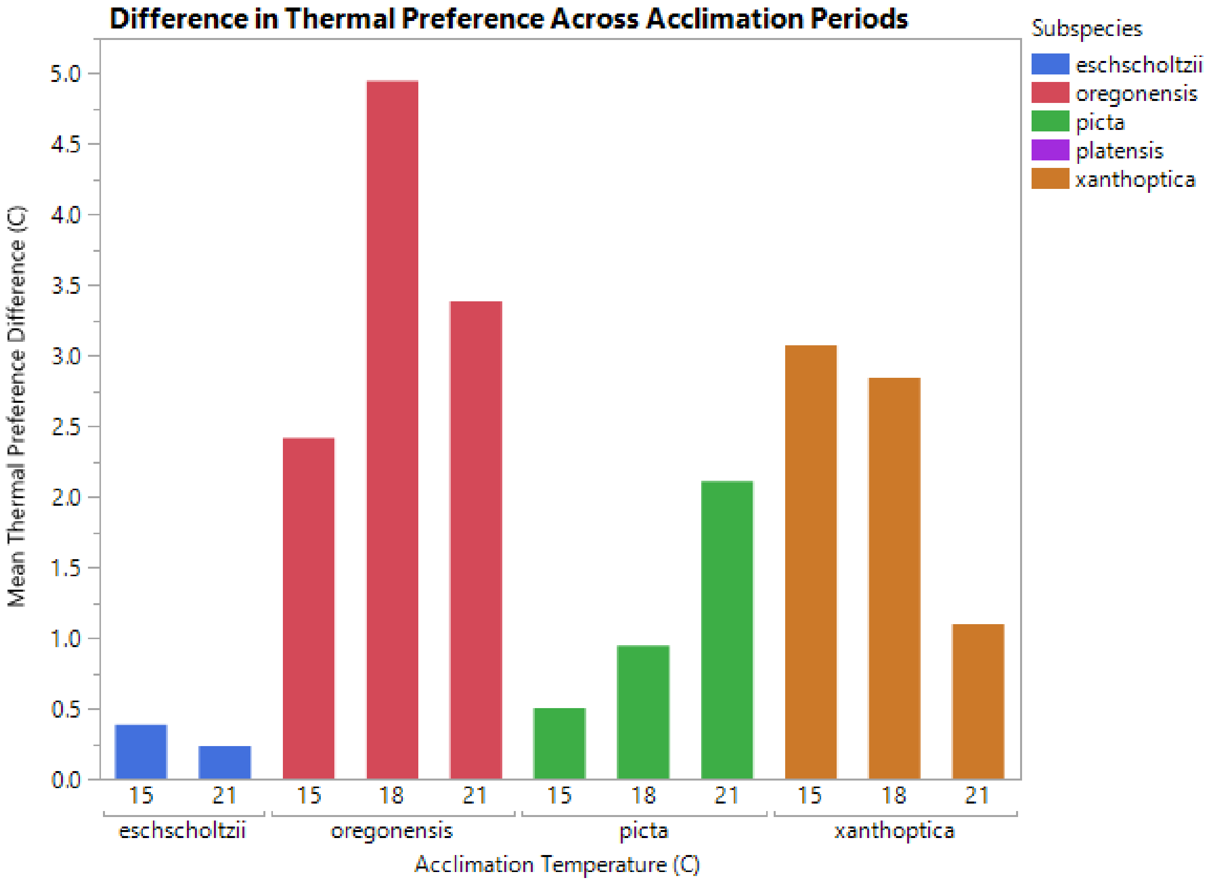
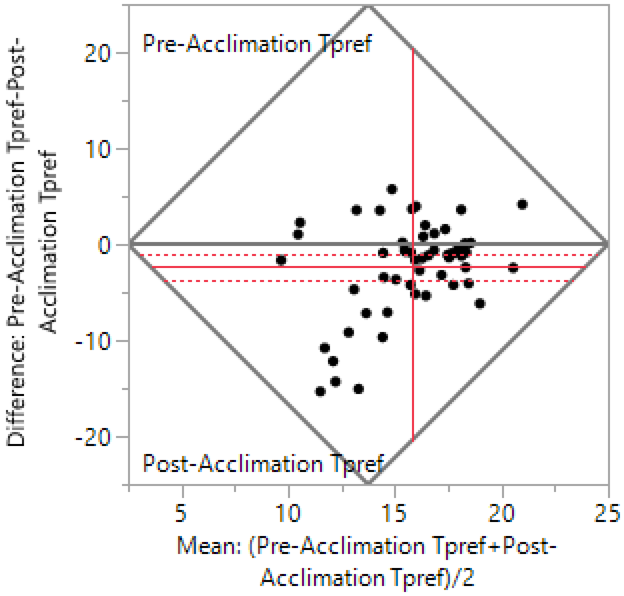


Fig. 14. A bar graph comparing mean differences between post and pre-acclimation thermal preference across all subspecies except *e. platensis*.

3.8.1 Thermal preference acclimation breakdown

This section is a more specific breakdown of acclimation in the different subspecies/treatments. All differences were found to be statistically significant except in eschscholtzii and picta. The differences are post-acclimation subtracted from pre-acclimation, so negative differences signify increased thermal preference. It's kind of confusing, I know.

All subspecies

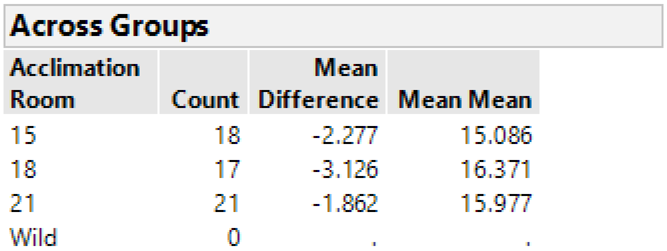


Pre-Acclimation Tpref	14.6204	t-Ratio	-3.70538
Post-Acclimation Tpref	16.9996	DF	55
Mean Difference	-2.3792	Prob > |t|	0.0005*
Std Error	0.64209	Prob > t	0.9998
Upper 95%	-1.0924	Prob < t	0.0002*
Lower 95%	-3.666		
N	56		
Correlation	0.04218		

Across Groups

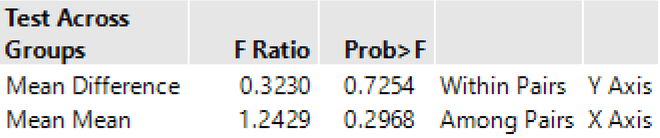
Subspecies	Count	Mean Difference	Mean Mean
eschscholtzii	3	-0.283	18.314
oregonensis	17	-3.502	14.089
picta	10	-1.278	17.265
platensis	0	.	.
xanthoptica	26	-2.31	16.087

Test Across Groups	F Ratio	Prob>F		
Mean Difference	0.6641	0.5779	Within Pairs	Y Axis
Mean Mean	6.3813	0.0009*	Among Pairs	X Axis



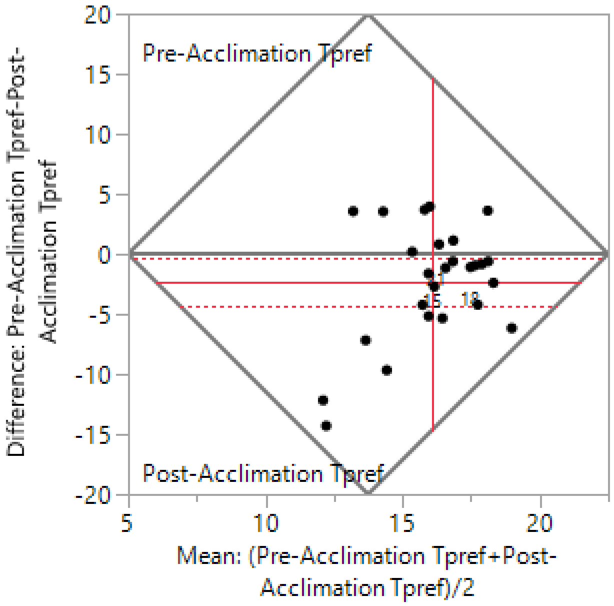
Across Groups

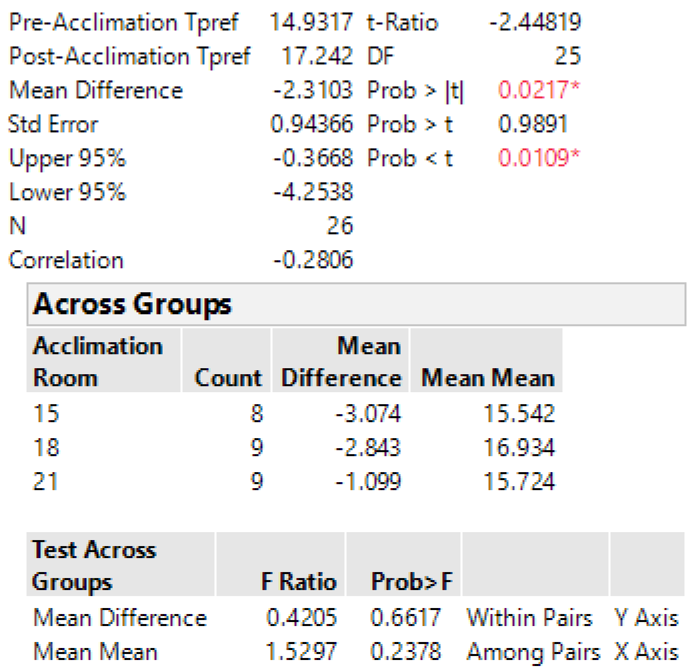
Acclimation Room	Count	Mean Difference	Mean Mean
15	18	-2.277	15.086
18	17	-3.126	16.371
21	21	-1.862	15.977
Wild	0	.	.



Test Across Groups	F Ratio	Prob>F		
Mean Difference	0.3230	0.7254	Within Pairs	Y Axis
Mean Mean	1.2429	0.2968	Among Pairs	X Axis

Xanthoptica





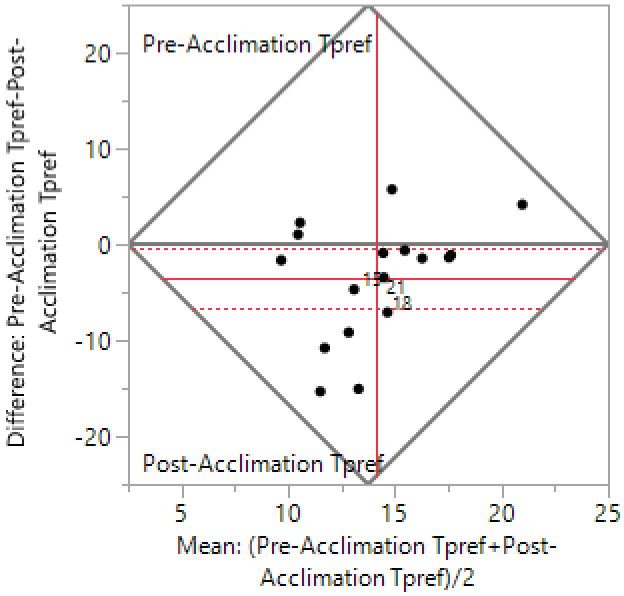
Pre-Acclimation Tpref	14.9317	t-Ratio	-2.44819
Post-Acclimation Tpref	17.242	DF	25
Mean Difference	-2.3103	Prob > |t|	0.0217*
Std Error	0.94366	Prob > t	0.9891
Upper 95%	-0.3668	Prob < t	0.0109*
Lower 95%	-4.2538		
N	26		
Correlation	-0.2806		

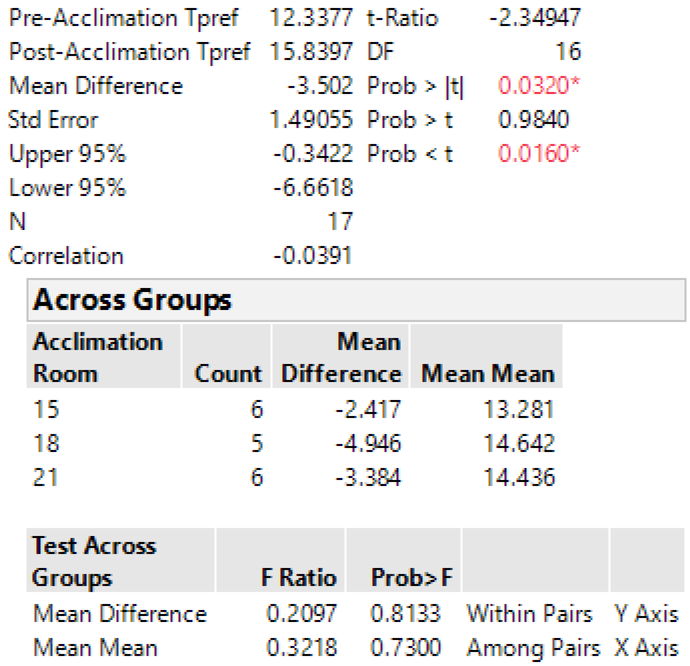
Across Groups

Acclimation Room	Count	Mean Difference	Mean Mean
15	8	-3.074	15.542
18	9	-2.843	16.934
21	9	-1.099	15.724

Test Across Groups	F Ratio	Prob>F		
Mean Difference	0.4205	0.6617	Within Pairs	Y Axis
Mean Mean	1.5297	0.2378	Among Pairs	X Axis

Oregonensis





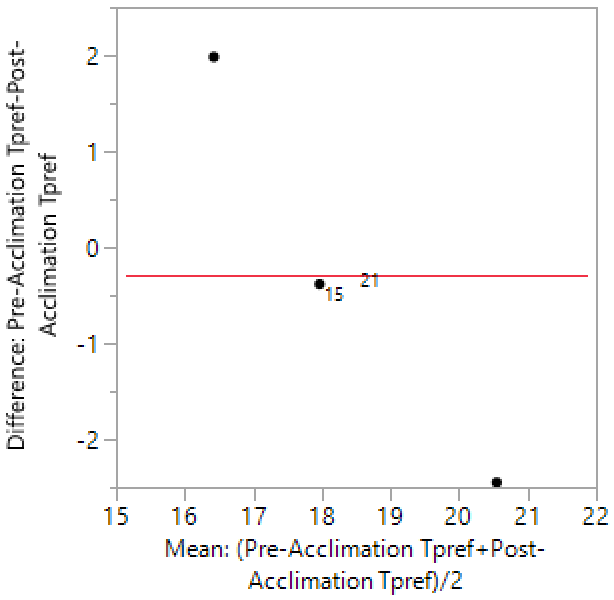
Pre-Acclimation Tpref	12.3377	t-Ratio	-2.34947
Post-Acclimation Tpref	15.8397	DF	16
Mean Difference	-3.502	Prob > |t|	0.0320*
Std Error	1.49055	Prob > t	0.9840
Upper 95%	-0.3422	Prob < t	0.0160*
Lower 95%	-6.6618		
N	17		
Correlation	-0.0391		

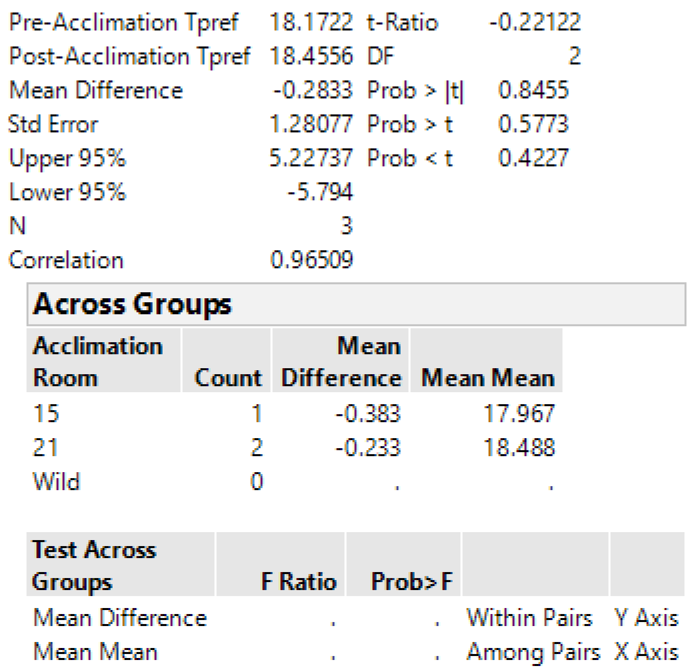
Across Groups

Acclimation Room	Count	Mean Difference	Mean Mean
15	6	-2.417	13.281
18	5	-4.946	14.642
21	6	-3.384	14.436

Test Across Groups	F Ratio	Prob> F		
Mean Difference	0.2097	0.8133	Within Pairs	Y Axis
Mean Mean	0.3218	0.7300	Among Pairs	X Axis

Eschscholtzii





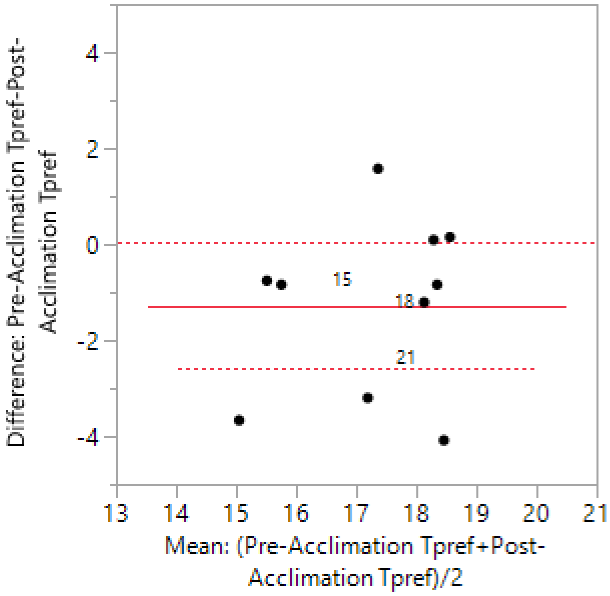
Pre-Acclimation Tpref	18.1722	t-Ratio	-0.22122
Post-Acclimation Tpref	18.4556	DF	2
Mean Difference	-0.2833	Prob > |t|	0.8455
Std Error	1.28077	Prob > t	0.5773
Upper 95%	5.22737	Prob < t	0.4227
Lower 95%	-5.794		
N	3		
Correlation	0.96509		

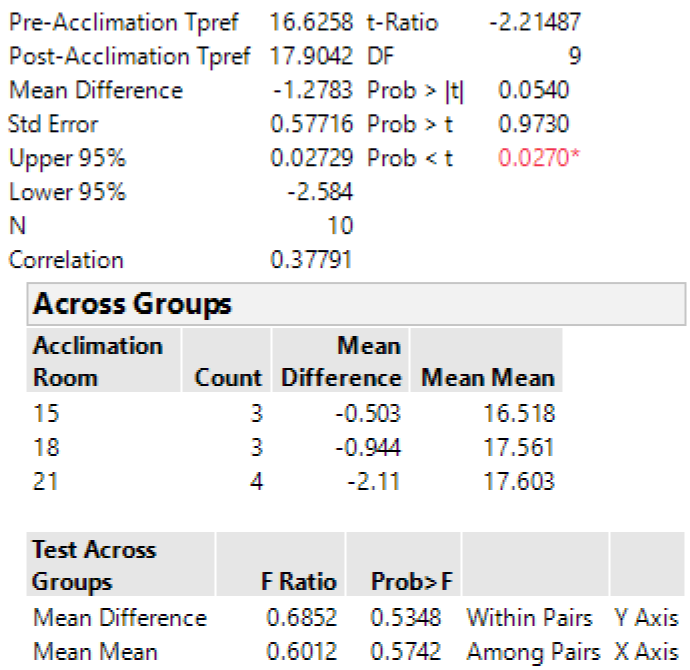
Across Groups

Acclimation Room	Count	Mean Difference	Mean Mean
15	1	-0.383	17.967
21	2	-0.233	18.488
Wild	0	.	.

Test Across Groups	F Ratio	Prob>F		
Mean Difference	.	.	Within Pairs	Y Axis
Mean Mean	.	.	Among Pairs	X Axis

Picta





Pre-Acclimation Tpref	16.6258	t-Ratio	-2.21487
Post-Acclimation Tpref	17.9042	DF	9
Mean Difference	-1.2783	Prob > |t|	0.0540
Std Error	0.57716	Prob > t	0.9730
Upper 95%	0.02729	Prob < t	0.0270*
Lower 95%	-2.584		
N	10		
Correlation	0.37791		

Across Groups

Acclimation Room	Count	Mean Difference	Mean Mean
15	3	-0.503	16.518
18	3	-0.944	17.561
21	4	-2.11	17.603

Test Across Groups	F Ratio	Prob>F		
Mean Difference	0.6852	0.5348	Within Pairs	Y Axis
Mean Mean	0.6012	0.5742	Among Pairs	X Axis

3.9 Differences in thermal performance across subspecies (Pre-acclimation)

This and the following section (3.10) detail specific, individual differences between subspecies for different anti-predator behaviors. The purpose of these sections is to provide details (and counterexamples) underlying a key assumption used in section 3.11, namely that differences in thermal performance between subspecies are negligible.

I compared behavioral latencies across all five subspecies using a series of ANOVA tests.

For 10, 15, and 18 degrees, there were no significant differences between any subspecies for any behavior.

For 24 degrees, some notable differences were found. *e. picta* had a significantly lower average run latency at 69 seconds. This is more than 52 seconds less than the next lowest average. *e. xanthoptica* had a significantly lower average seek shelter latency, 56.69 seconds. For toe stand, rocking, and tail arch, *e. xanthoptica* and *e. picta* had below average latencies, with their averages ranging from 64.5 to 93.89 seconds. No subspecies other than *e. xanthoptica* ever emitted noxious chemicals in pre-acclimation trials, so the *xanthoptica*'s average was predictably lower than all other subspecies at 142.19 seconds.

For 21 degrees, all ANOVA tests showed an insignificant difference except for run latency, which was significantly lower in *e. xanthoptica* (98.208 seconds) than all other subspecies.

Only *e. eschscholtzii*, *e. picta*, and *e. platensis* were run at 5 degrees in pre-acclimation trials. Their averages were very high, at or close to 180 seconds for all latencies. The only latency for which a significant difference was found was tail arch latency, with *e. eschscholtzii* at a significantly lower average (129 seconds) than *picta* and *platensis* (180 and 172.8 seconds, respectively).

3.10 Differences in thermal performance across subspecies (Post-acclimation)

When this section was written, only *e. oregonensis* and *e. xanthoptica* had been run through post-acclimation thermal performance trials, so only they are compared. A more complete analysis would of course be better, but seeing as the purpose of this section is simply to inform the assumption of another, I'm focusing my efforts on other sections.

Noxious chemicals were never emitted by individuals of either subspecies except at 24 degrees, when only individual *xanthoptica* emitted them. That latency is equal between the two for all other temperatures.

For 5 degrees, ANOVA tests indicated that *e. oregonensis* and *e. xanthoptica* were found to be significantly different for all behaviors except running and tail arch. *oregonensis* and *xanthoptica* have running latencies of 133.77 and 175.44 seconds, respectively. For tail arch latency, their averages are 180 and 153.28 seconds, respectively.

For 10 degrees, differences were found to be insignificant across all behaviors.

For 15 degrees, differences between were found to be insignificant for all behaviors except for running and seek shelter latencies, for which *xanthoptica* had lower latencies.

For 18 degrees, differences were found to be insignificant for all behaviors except tail arch, toe stand, and running. *e. xanthoptica* had lower latencies for all three.

For 21 degrees, *e. xanthoptica* had significantly lower latencies for all behaviors, except noxious chemicals, which was never exhibited by either.

For 24 degrees, differences were found to be insignificant except for rocking and noxious chemicals. For both the latencies, *e. xanthoptica* had a significantly lower latency than *e. oregonensis*. For noxious chemicals, only *e. xanthoptica* ever emitted them.

3.11 Thermal performance across all subspecies

Although ANOVA tests indicated some individual differences between subspecies (See sections 3.9 and 3.10), I'm grouping all subspecies together for TPC analysis. Most differences between subspecies were small and hyperspecific, and analyzing unique differences between individual behaviors, temperatures, subspecies, and trial period would be incredibly time-consuming.

Behavioral performance was calculated from each latency by subtracting measured latencies from 180, the maximum possible latency. The performances, separated by behavior, were graphed in response to temperature to create a thermal performance curve (Fig. 15). Generally, performance increased at a steady rate as the temperature increased from 5 to 24 degrees.

I performed a multivariate correlation analysis to see if any behaviors were correlated. Tail arch, rocking, and toe stand were strongly positively correlated. Running and seeking shelter were weakly positively correlated. For further analysis, I group these together into evasion (run, seek shelter) and intimidation (tail arch, rocking, toe stand) behaviors.

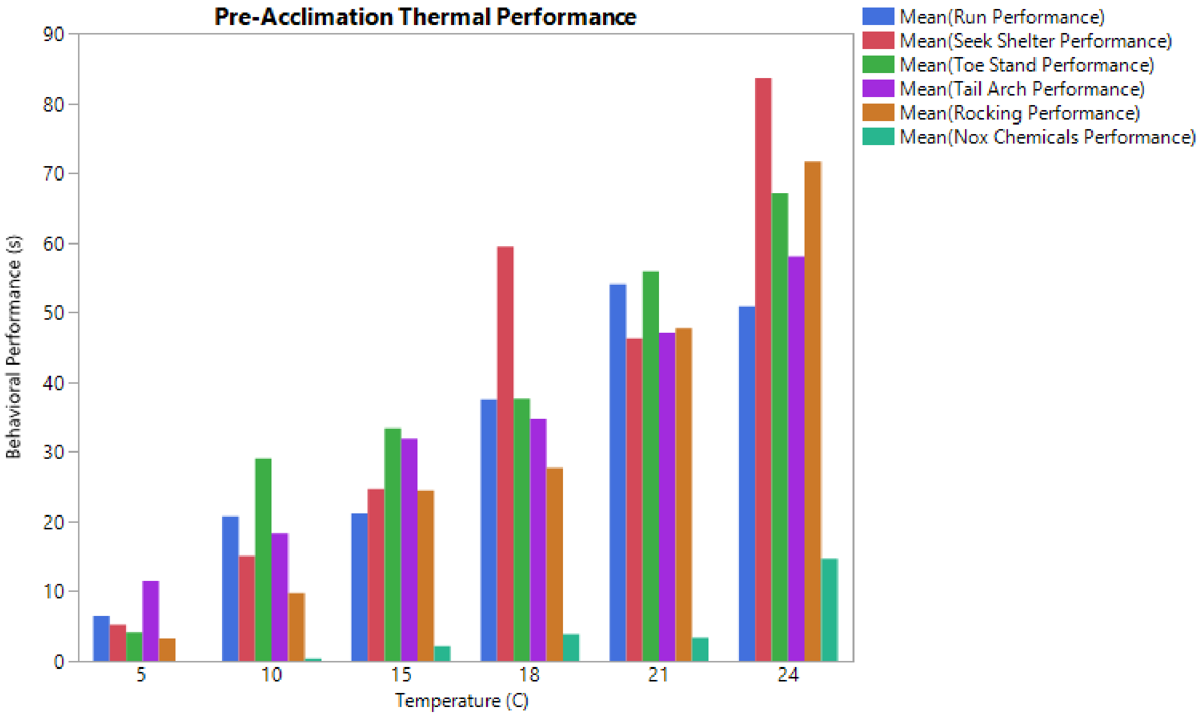
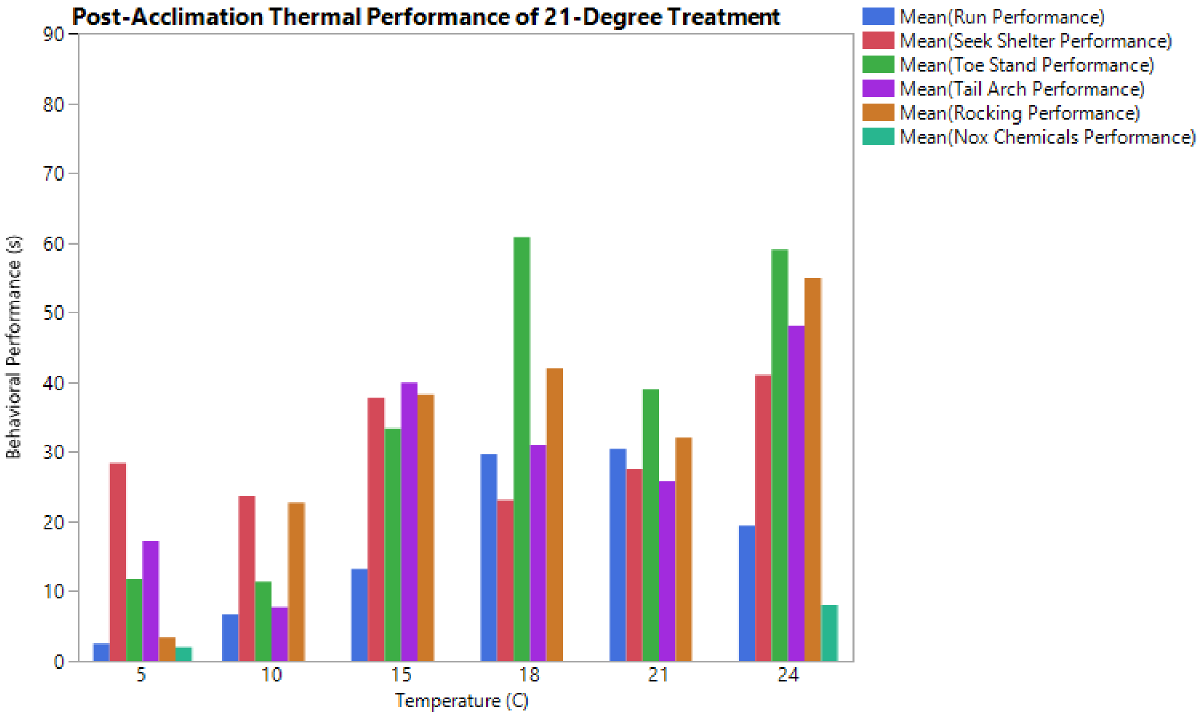


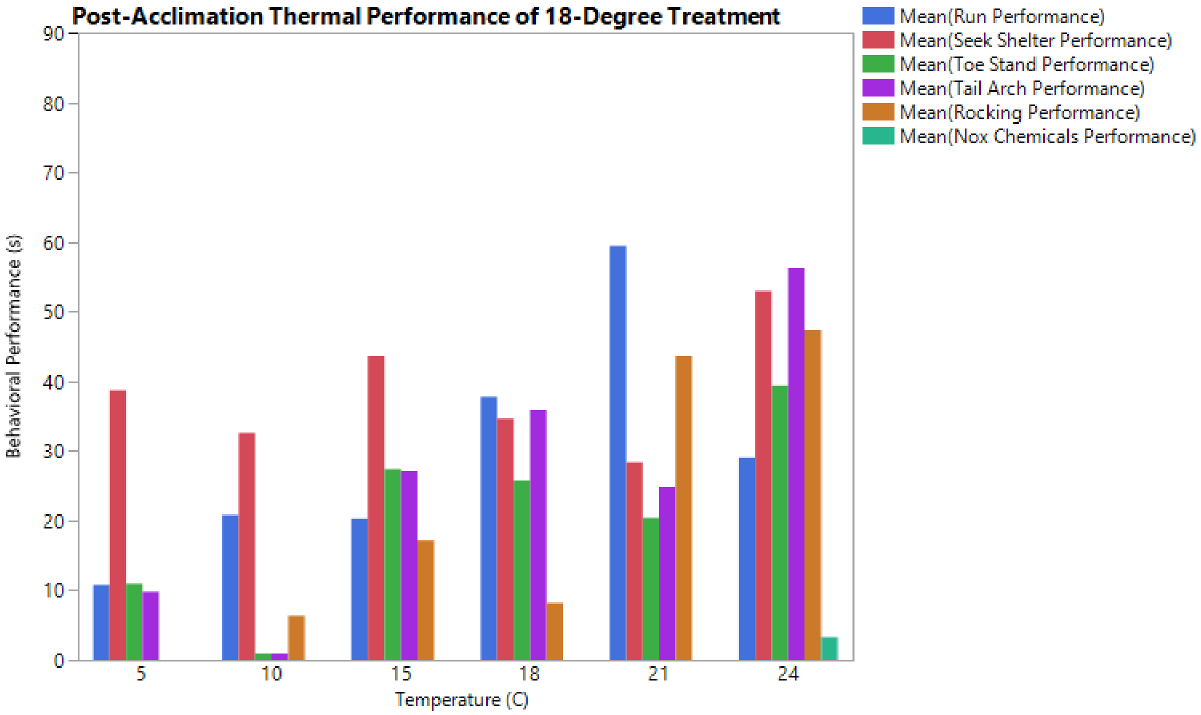
Fig. 15. A bar graph comparing the mean thermal performance of each behavioral latency at each trial temperature. This includes all subspecies, and is an approximation of a continuous thermal performance curve for ensatina.

3.12 Acclimation of thermal performance

I created a separate post-acclimation TPC curve for each long-term acclimation temperature (15, 18, and 21 degrees) (Fig. 16). Generally, performance increased at lower temperatures and decreased at higher temperatures. The general trend of increased performance seems to be present in all three treatments, but the amount of variation in this pattern for individual behaviors is substantially, especially in the 21-degree treatment.

Notably, seek shelter performance increased at lower temperatures for all three treatments. Evasive behavior had a significantly higher overall performance in the 21-degree treatment.


Post-Acclimation Thermal Performance of 21-Degree Treatment
Behavioral Performance (s)
0
10
20
30
40
50
60
70
80
90
5
10
15
18
21
24
Temperature (C)
Mean(Run Performance)
Mean(Seek Shelter Performance)
Mean(Toe Stand Performance)
Mean(Tail Arch Performance)
Mean(Rocking Performance)
Mean(Nox Chemicals Performance)


Post-Acclimation Thermal Performance of 18-Degree Treatment
Behavioral Performance (s)
0
10
20
30
40
50
60
70
80
90
5
10
15
18
21
24
Temperature (C)
Mean(Run Performance)
Mean(Seek Shelter Performance)
Mean(Toe Stand Performance)
Mean(Tail Arch Performance)
Mean(Rocking Performance)
Mean(Nox Chemicals Performance)

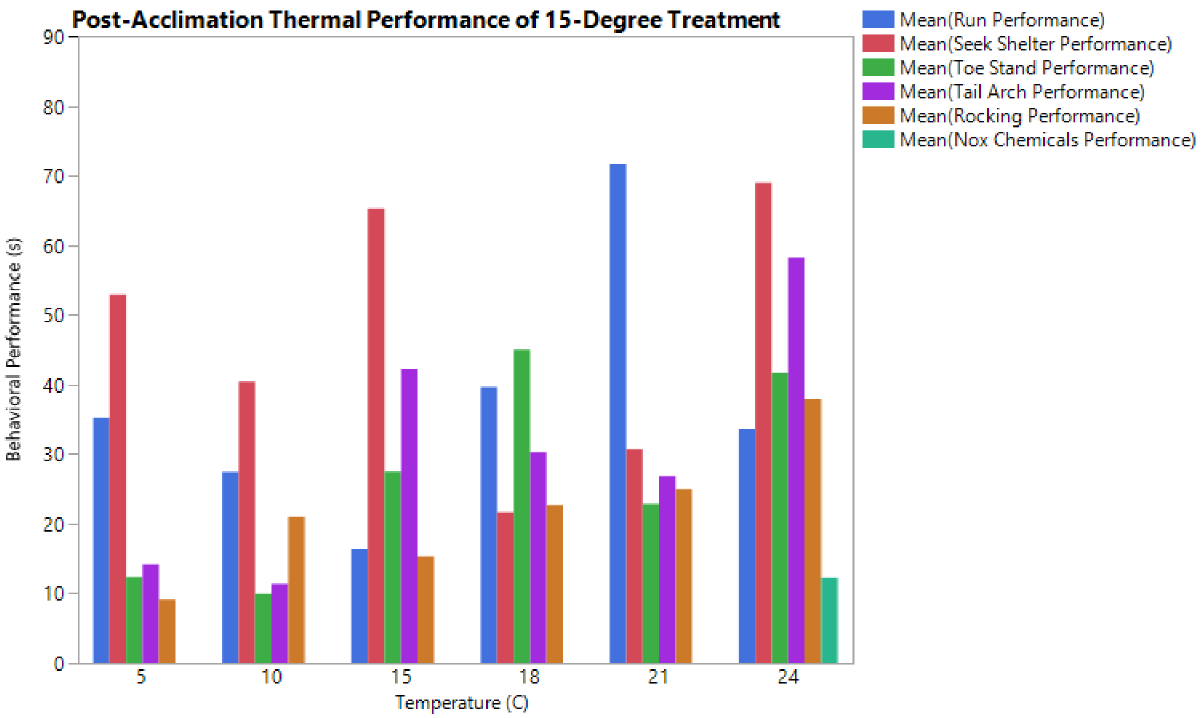
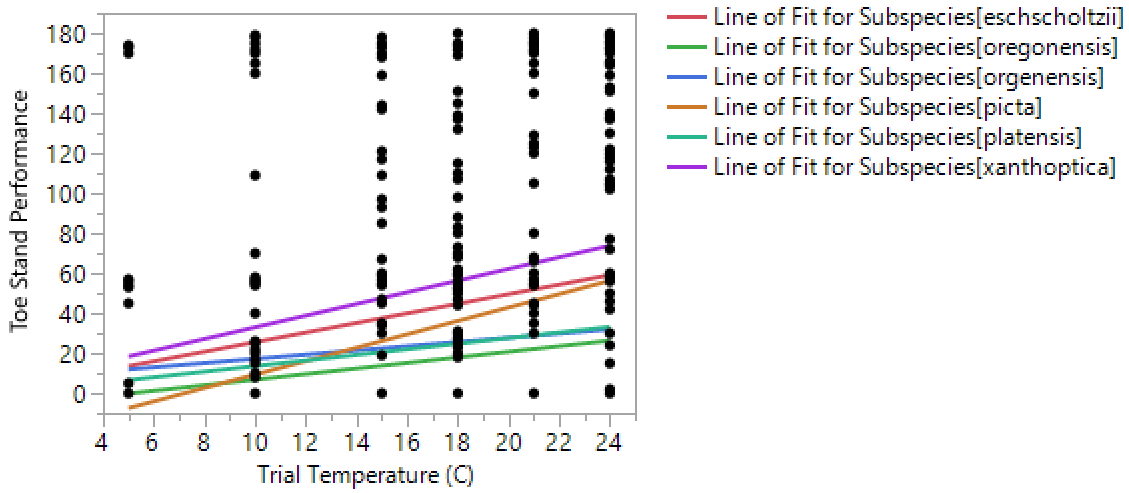
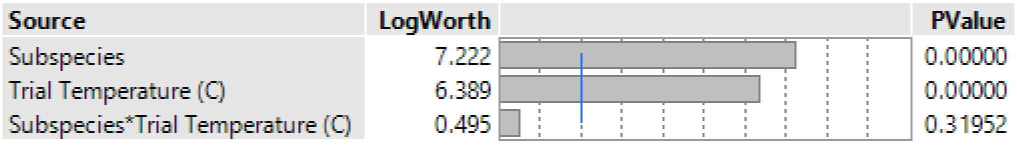


Fig. 16. Three thermal performance curves, one for each long-term acclimation treatment.

3.13 Which factors are most important in predicting thermal performance?

I ran an ANCOVA covariance test to determine which variables are important predictors of thermal performance. Results were highly consistent between pre and post-acclimation periods, so they're grouped for this section. Trial temperature and subspecies were both found to be strong predictors of behavioral latencies/performance. Their interaction was significant for most behaviors, but was a much weaker effect than subspecies and temperature (except for running and noxious chemicals). Results were similar across all latencies, so I will focus on toe stand for the sake of simplicity. The model predicts an increase in performance as temperature increases (Fig. 17). As seen in the boundary plot, the fit of these lines seems to diverge greatly from the actual distribution of data (Fig. 18). The model predicts only relatively low performances, and this is consistent across all behaviors.





Source	LogWorth		PValue
Subspecies	7.222		0.00000
Trial Temperature (C)	6.389		0.00000
Subspecies*Trial Temperature (C)	0.495		0.31952

Fig. 17. A graph showing the lines of fit for each subspecies generated by the ANCOVA model for the run latency (p = 0.0002).

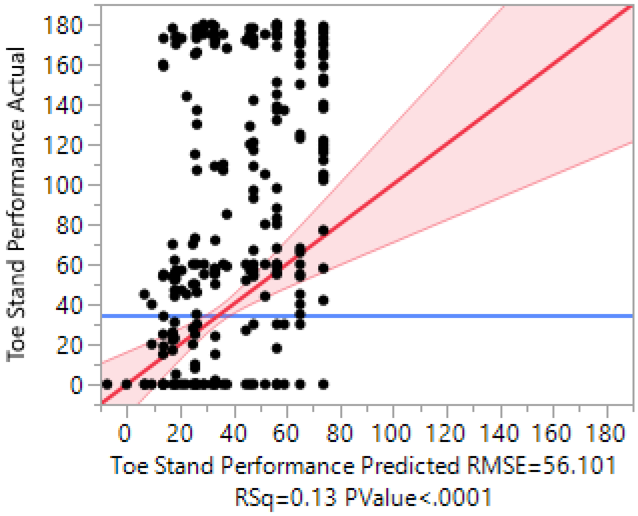
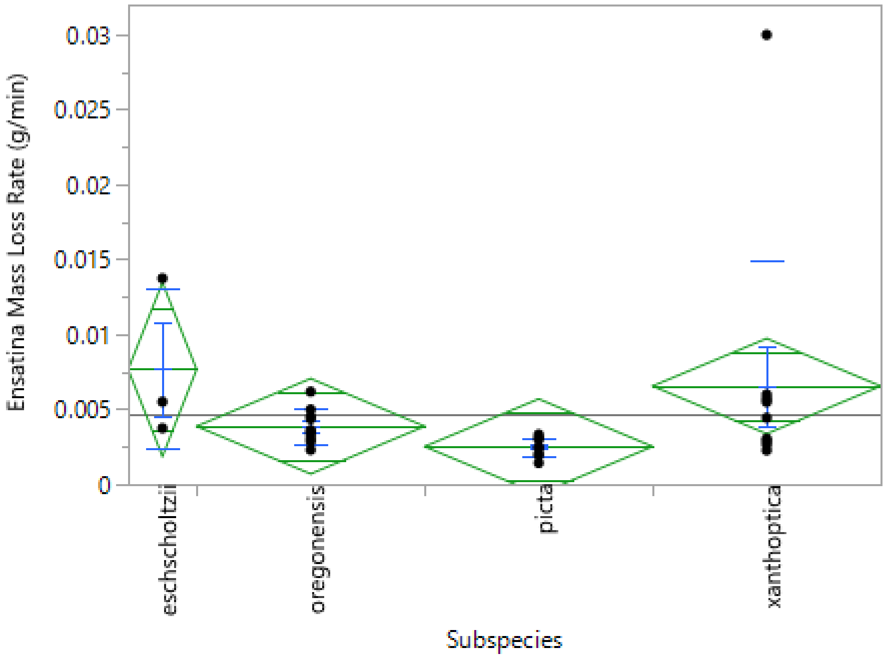


Fig. 18. A boundary plot comparing actual and predicted latencies. Note the lack of any predicted points below 90 seconds.

3.14 Does skin resistance vary between subspecies?

I performed ANOVA tests to see if the rate of water loss differed between subspecies. The RH in our desiccation setup never stabilized, and proper skin resistance values can’t be calculated as a consequence, so these results should be taken with a grain of salt. Since skin resistance values couldn’t be calculated, I’m using average water loss rates to approximate relative skin resistance. *e. platensis* was not included, as it has not been run in desiccation trials at the time of writing. The tests, which compared mass loss rates, logmass loss rates, and percent body mass loss rates found differences to be insignificant across subspecies ($0.2147 \leq p \leq 0.4563$). Individual t-tests found no differences between any two subspecies as well.



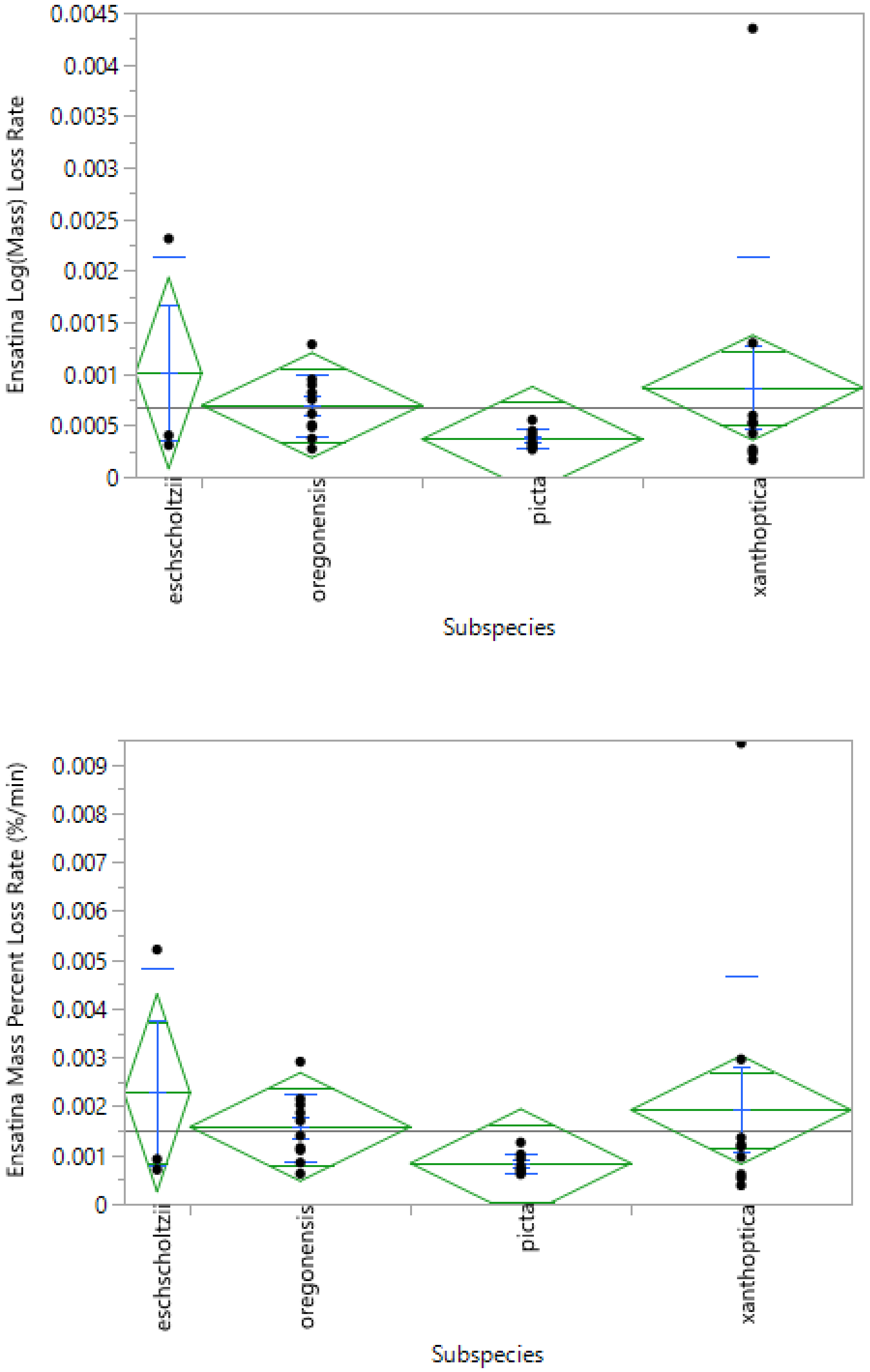
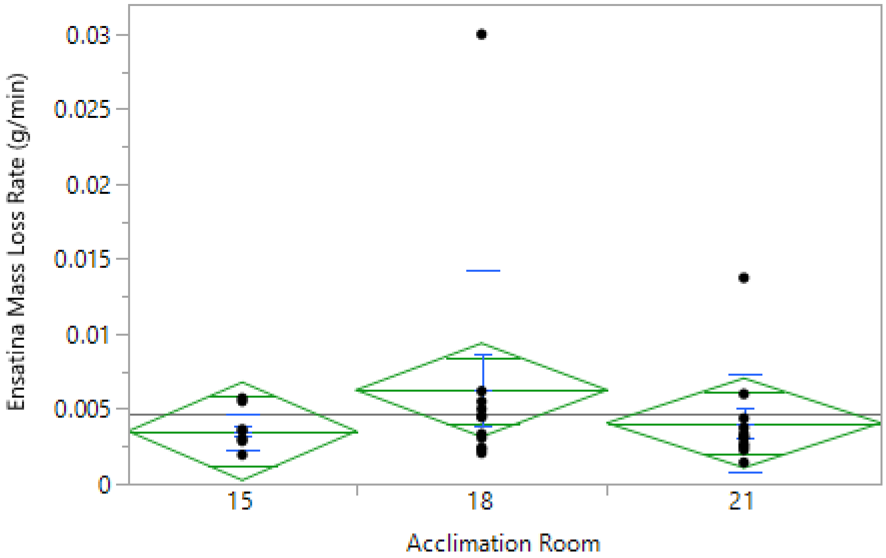
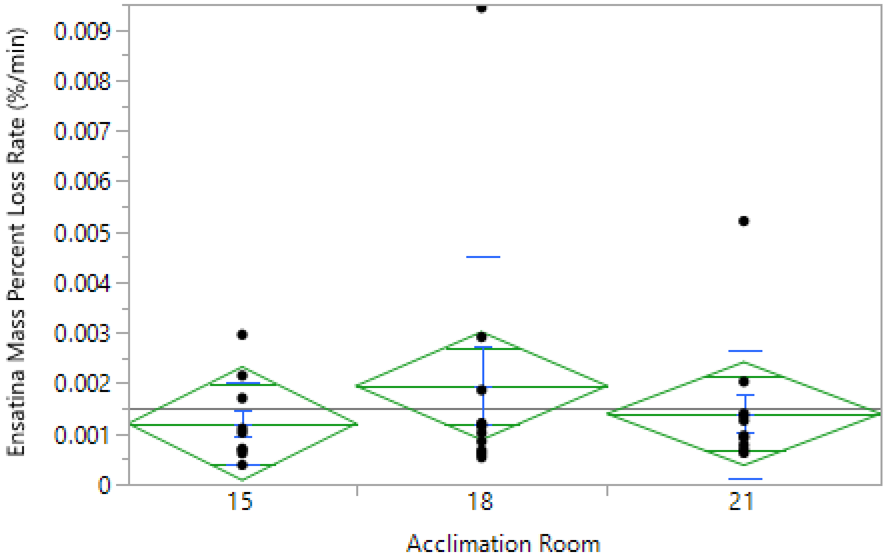


Fig. 19. A series of ANOVA tests comparing several measures of water los across all measured subspecies

3.15 Does skin resistance vary between acclimation treatments?

I ran additional ANOVA tests to find differences in rates of water loss between acclimation treatment groups. No significant difference was found for any of the rates (0.4216 ≤ p ≤ 0.5913).





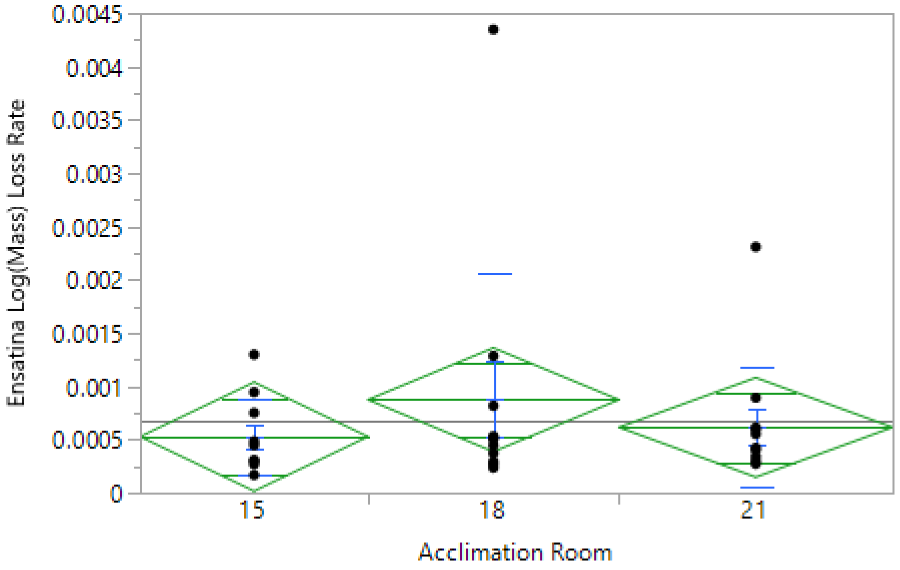
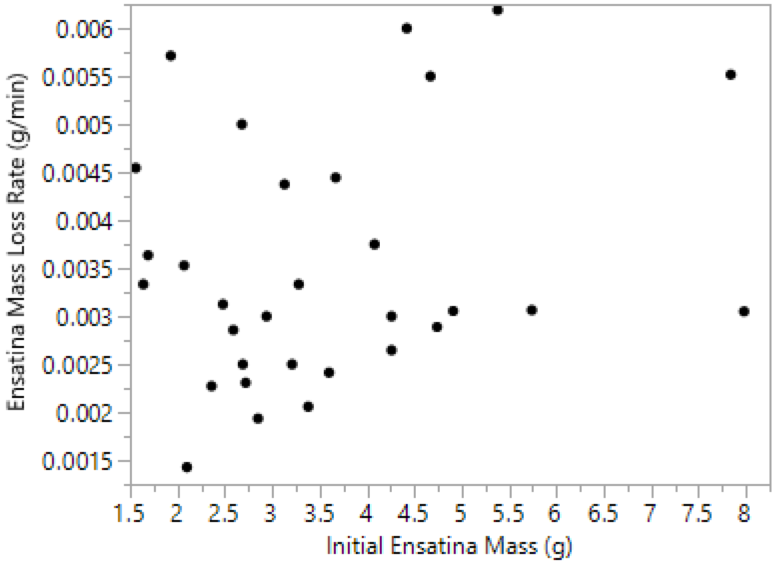


Fig. 20. A series of ANOVA tests comparing several measures of water los across all acclimation treatments.

3.16 How does skin resistance vary with mass?

I used several linear regressions to determine how water loss rates varied with body mass (both regular mass and logmass). Two individuals were excluded from this analysis, as they had far higher water loss rates than all others and were causing problems with data analysis.

There seemed to be no relationship between mass or logmass and mass loss rates (Fig. 21).



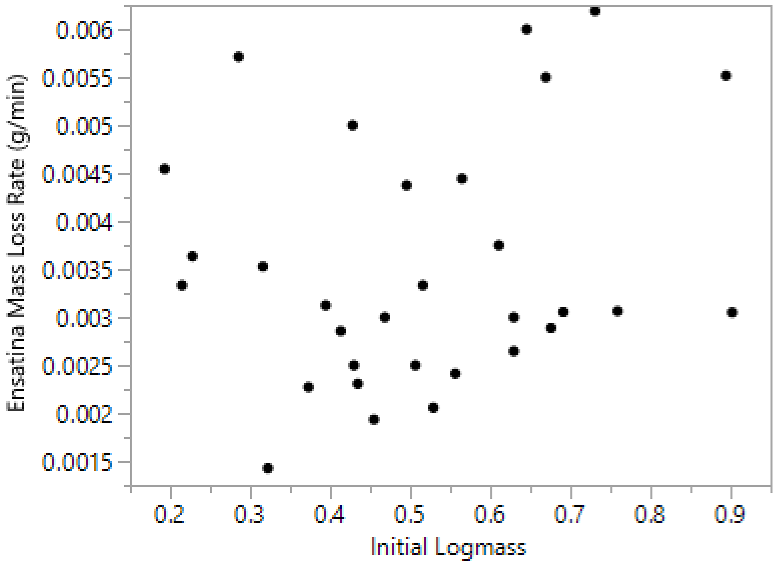
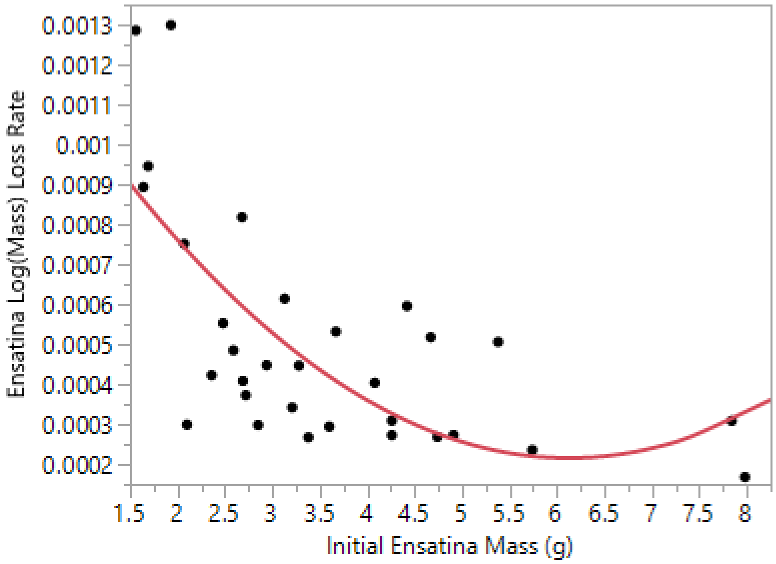


Fig. 21. Two dot plots comparing mass loss rates to initial mass and logmass of each animal

For Log(mass) loss rates, a quadratic model was a strong fit for both mass and Log(mass) ($p < 0.0001$).



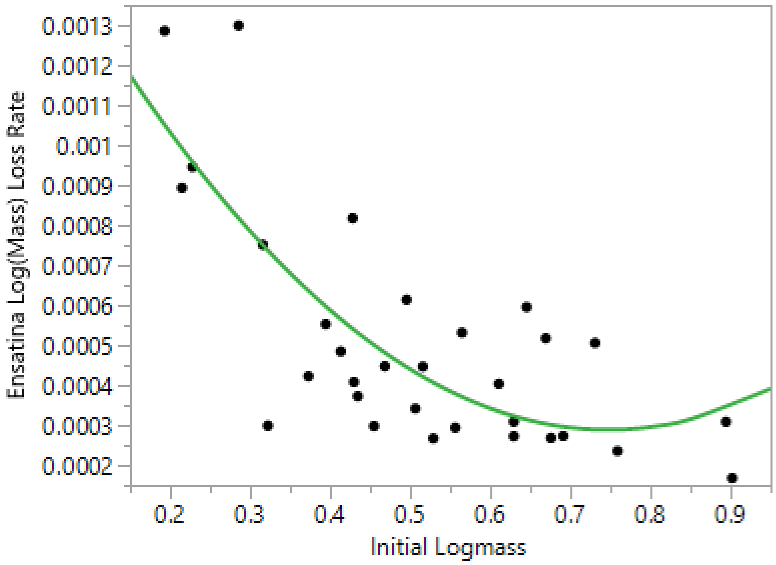
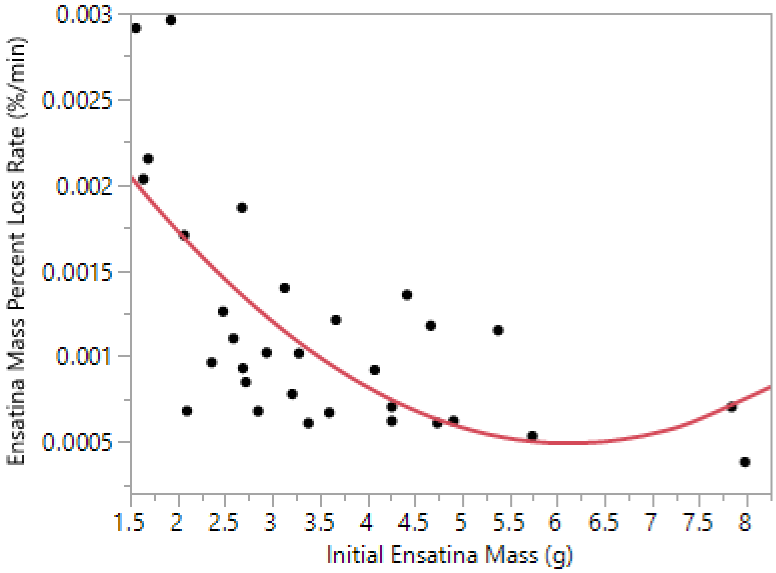


Fig. 22. Two quadratic regressions were a strong fit for log(mass) as a function of both mass and logmass ($p < 0.0001$)

The pattern was very similar for percent mass loss rates. A quadratic model was once again a strong fit for both mass and logmass (Fig. 23).



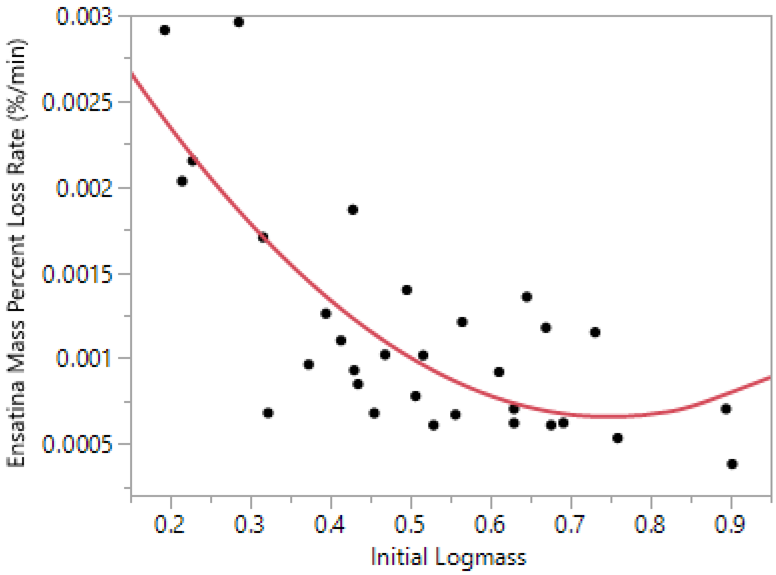


Fig. 23. Two quadratic regressions were a strong fit for percent mass loss as a function of both mass and logmass ($p < 0.0001$)

3.17 Which environmental factors are most important in predicting ensatina range?

Results from MaxEnt suggest several bioclimatic variables are useful predictors of Ensatina range (Fig. 24). Mean annual temperature is the most powerful predictor. Below it are mean temperature of the warmest quarter, precipitation seasonality, isothermality, and temperature seasonality as additional strong predictors. These results may be dubious, however. There was strong covariance between many of the bioclimatic variables tested, indicating that some variables may only be strong predictors because they contain the information of other, actually important variables. Also, models of this nature are somewhat limited in their ability to predict which factors determine a species' range. In reality, geographic ranges are partially determined by animal dispersal ability, the presence of geographic barriers, and some random chance (e.g. an organism's choice to migrate east instead of south may be based on climatic factors associated with the south, or the choice may be random) (Milanovich, Peterman, Nibbelink, & Maerz, 2010). That's not to say that the results from this model are useless, just that they should not be taken as gospel, especially without further study and model refinement.

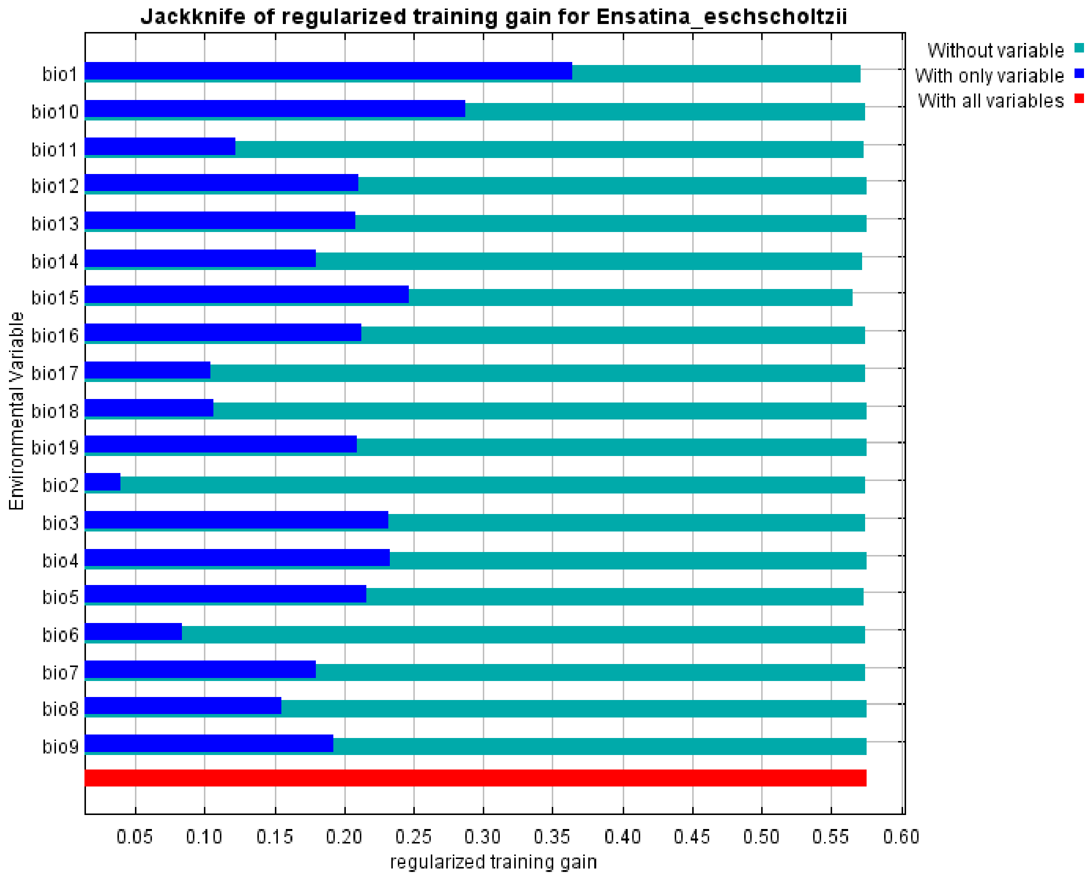


Fig. 24. Estimations of variable importance to predicting Ensatina range. Dark blue lines indicate the strength of a variable to predict Ensatina range on its own, or how much information it has on its own. Turquoise lines indicate how much information each variable has that other variables don’t. The similar length of all turquoise lines indicate significant covariance between variables.

BIO1 = Annual Mean Temperature
BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))
BIO3 = Isothermality (BIO2/BIO7) (* 100)
BIO4 = Temperature Seasonality (standard deviation *100)
BIO5 = Max Temperature of Warmest Month
BIO6 = Min Temperature of Coldest Month
BIO7 = Temperature Annual Range (BIO5-BIO6)
BIO8 = Mean Temperature of Wettest Quarter
BIO9 = Mean Temperature of Driest Quarter
BIO10 = Mean Temperature of Warmest Quarter
BIO11 = Mean Temperature of Coldest Quarter

BIO12 = Annual Precipitation
BIO13 = Precipitation of Wettest Month
BIO14 = Precipitation of Driest Month
BIO15 = Precipitation Seasonality (Coefficient of Variation)
BIO16 = Precipitation of Wettest Quarter
BIO17 = Precipitation of Driest Quarter
BIO18 = Precipitation of Warmest Quarter
BIO19 = Precipitation of Coldest Quarter

3.18 Where will ensatina go extinct under predicted climate change?

Data collected from this study has not yet been used to create an updated SDM model for predicting extinction. Nonetheless, I’ve included the results of the preliminary experiment here because they’re so pivotal to this experiment.

Average Tpref across all subspecies was input into the extinction risk model developed by Sinervo et al., and used to generate visual predictions of geographic extinction risk in 2050 and 2070 under two climate change scenarios (Moderate and high predicted severity) (Fig. 25; Fig. 26; Fig. 27; Fig. 28). Extinction risk was highest along the coast of central to southern California. In all scenarios, extinction risk of more northern populations (Northern california to Canada) was significantly lower than in other regions.

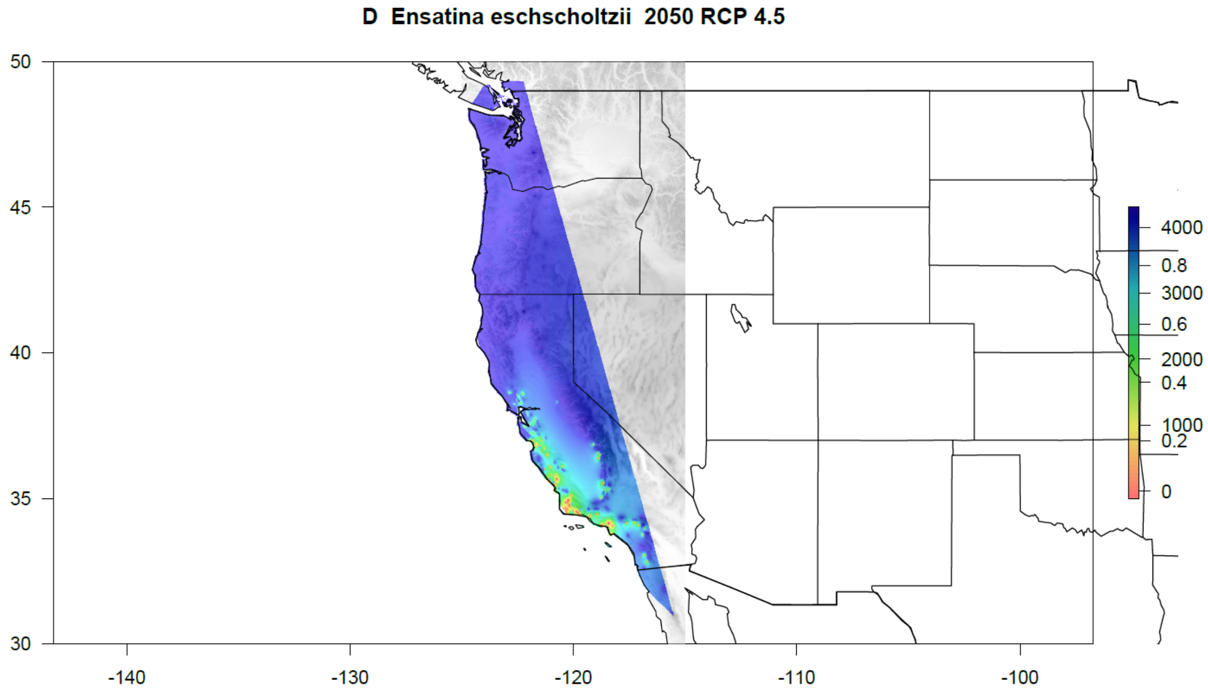


Fig. 25. Predicted extinction risk for Ensatina by 2050 under RCP 4.5, a moderate severity climate change scenario. Blue areas are low likelihood of extinction, red areas are high likelihood.

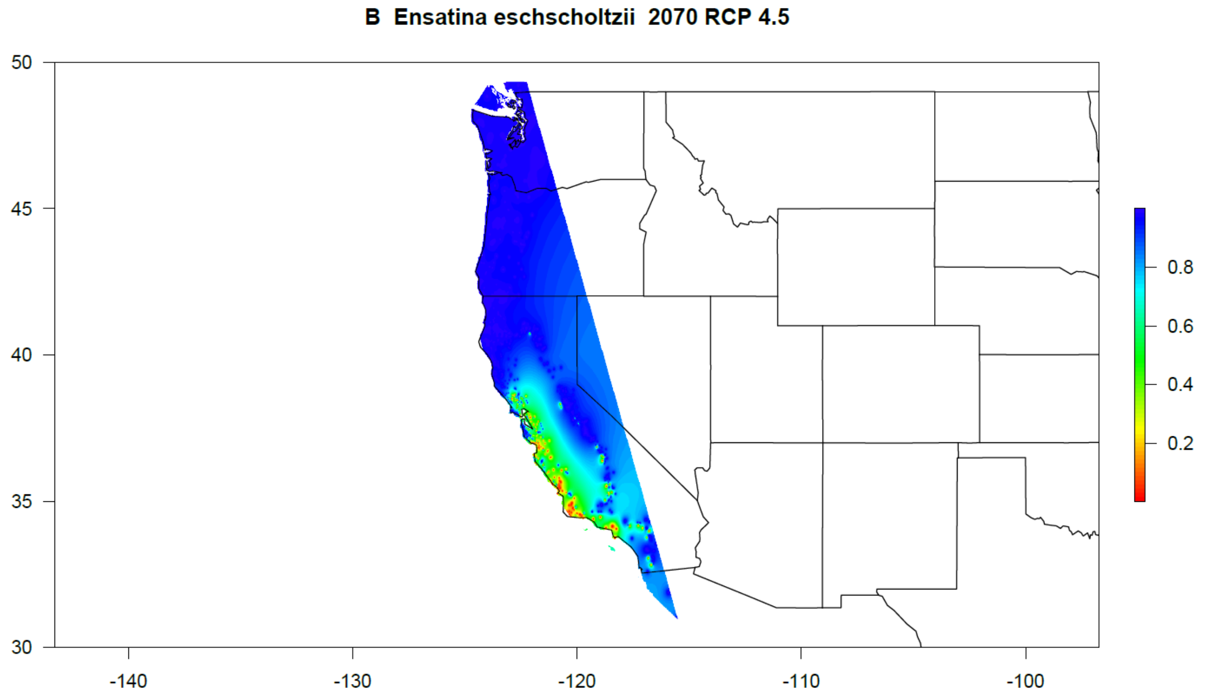


Fig. 26. Predicted extinction risk for Ensatina by 2070 under RCP 4.5, a moderate severity climate change scenario. Blue areas are low likelihood of extinction, red areas are high likelihood.

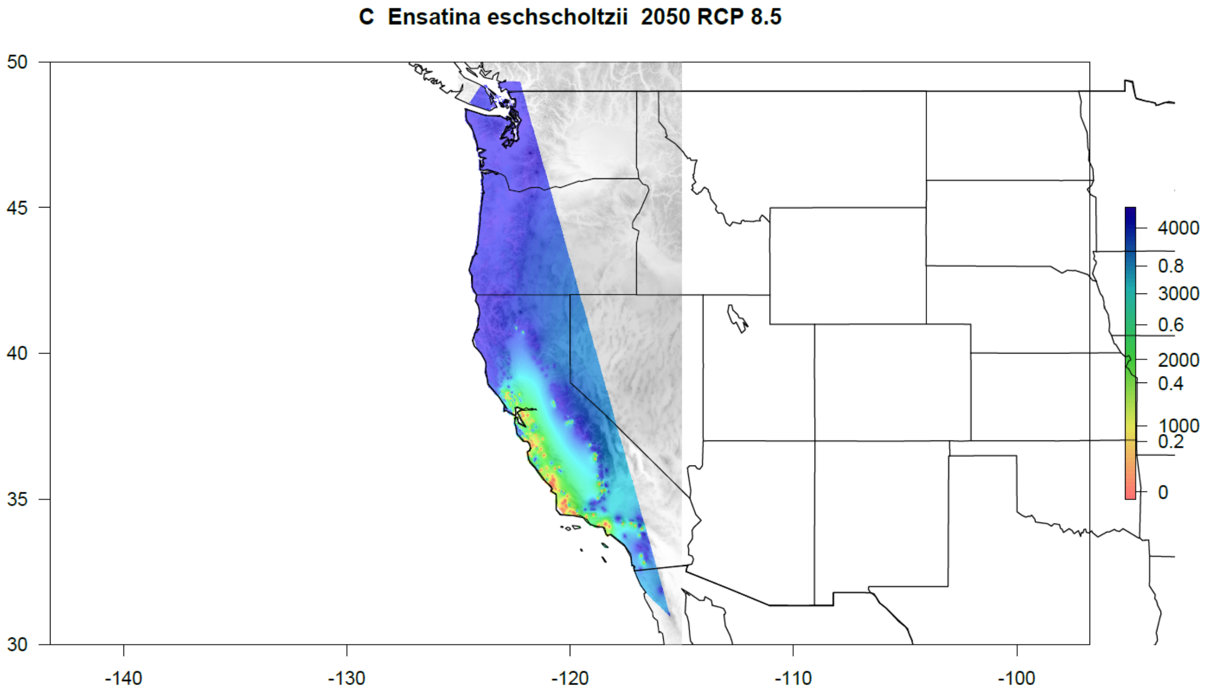


Fig. 27. Predicted extinction risk for Ensatina by 2050 under RCP 8.5, a high severity climate change scenario. Blue areas are low likelihood of extinction, red areas are high likelihood.

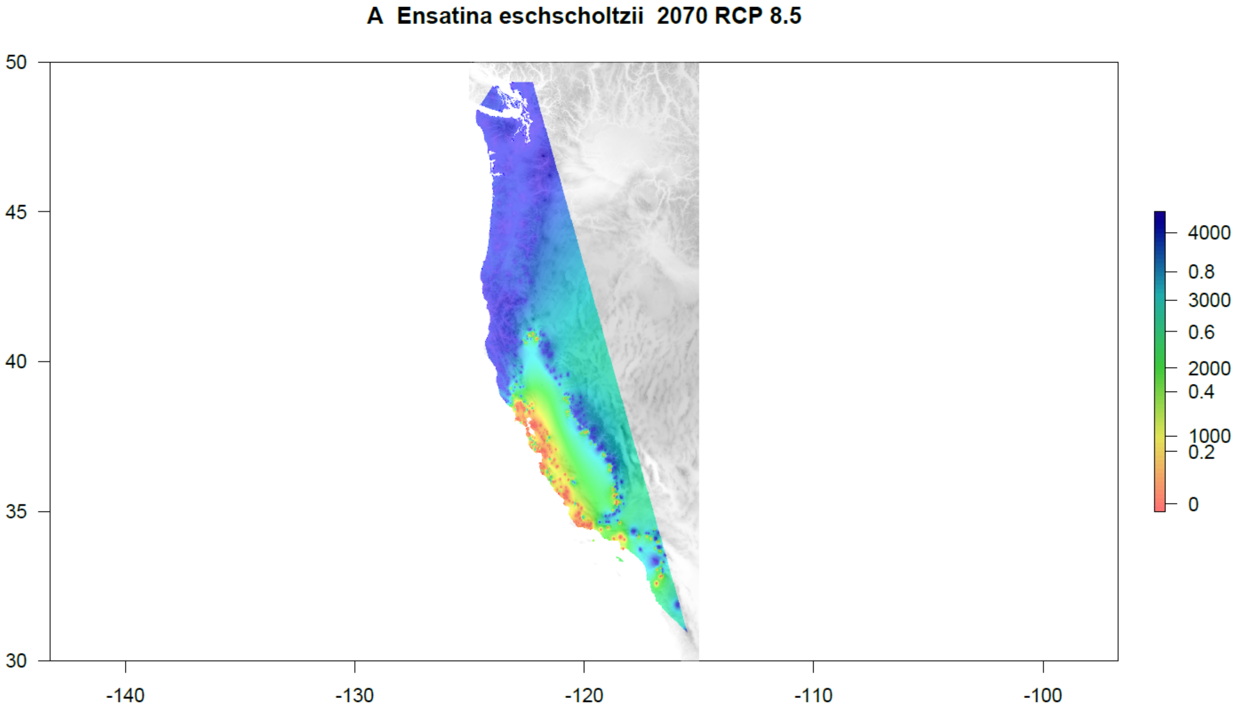


Fig. 28. Predicted extinction risk for Ensatina by 2070 under RCP 8.5, a high severity climate change scenario. Blue areas are low likelihood of extinction, red areas are high likelihood.

4. Discussion

4.1 Ensatina in the field

Our field data shows differences in the body temperatures of different subspecies. Average *e. picta* body temperature was significantly lower than *e. xanthoptica* and *e. oregonensis*. Given that *e. oregonensis* and *e. xanthoptica* were collected from locations no more than 30 miles apart, that may suggest that geographic location is a more important driver of thermal physiology in ensatina than subspecies.

A key assumption of this study is that ensatina will cease activity and burrow once temperatures exceed their preferred temperature. The boundary plot comparing air temperature to preference seems to support this, with 90% of individuals being found in air temperatures below their preference. When looking at ground temperature, the results are somewhat more muddled, with only about 69% being found in air temperatures below preference. Although this is still a majority, it seems to suggest that ensatina may sometimes remain active despite environmental temperatures exceeding their preference, and that there's a threshold other than tpref we should be looking for.

Substrate and body temperature follow an almost 1:1 line, strongly suggesting that ensatina body temperature is driven nearly completely by substrate temperature. This isn't a surprise given that they're ectotherms. What is surprising is that body temperature (and by extension substrate temperature) follow a quadratic trend in response to air temperature. This seems to imply that microhabitats stay at more moderate temperatures when the environment is especially cold or hot, but only to a degree. The matched pairs test found that substrates were often warmer than the environment when environmental temperatures were very cold. That and the diminished range of Tsub values vs. Tout values supports this hypothesis.

4.2 Thermal Preference

Pre-Acclimation thermal preference was similar across most species. Excluding *e. oregonensis*, it ranged from 14.93 to 18.37 degrees. The reasons for individual differences are unclear. *e. picta*'s mean thermal preference was about 6 degrees higher than its average field body temperature, while *e. oregonensis* was about 2 degrees lower. This seems to suggest that a single measure of field body temperature is a poor predictor of thermal preference. It's entirely possible that preference is more dependent on long-term climate patterns rather than seasonal field temperatures. Further analysis is required to confirm this.

It's very clear that thermal preference changed significantly after the salamanders were acclimated for several weeks, but the patterns are...strange. Assuming 15 degrees to be the baseline environmental temperature, *e. picta* seemed to demonstrate normal acclimation, with preference increasing the more environmental temperatures increase. *e. eschscholtzii* effectively did not acclimate. *e. xanthoptica* seemed to exhibit reverse acclimation, with preference decreasing as environmental temperatures increased. I'm not sure what happened with *e. oregonensis*. It had a maximum increase in the 18-degree treatment, and minimum in 15 degrees.

It's possible that *e. oregonensis* was exhibiting a pattern of normal acclimation, but the 21-degree room became too stressful and preference decreased in response. Many of our animals begin to exhibit stressed behavior in the 21-degree room after these trials were completed, so it's possible. For this as well, analysis of long-term climate differences between the regions of each subspecies may shed light on why each subspecies showed a different pattern.

4.3 Thermal Performance

The pre-acclimation thermal performance curve shows a clear pattern of performance increasing at a steady rate from 5 to 24 degrees. This is consistent with previous studies on other plethodontids, which have found maximum performance between 20 and 24 degrees Celsius (Young & Gifford, 2013; Clay & Gifford, 2017). This consistency suggests that ensatina thermal physiology may be indicative of the whole clade, or at least several other plethodontids. Although performance increases to 24 degrees, several ensatina began exhibiting stressed behavior and some even perished in the 21 degree treatment. This suggests a physiological tradeoff to high temperature exposure in ensatina, namely that they can't handle long-term exposure, even if short-term exposure could be beneficial.

Previous studies on lizards have found a trend of intimidation behaviors being more prevalent at low temperatures, while evasive temperatures are more prevalent at high temperatures (Hertz, Huey, & Nevo, 1982). This doesn't seem to be the case with ensatina, as both types of behaviors had generally increased performance as temperatures rose.

Although it's clear that thermal performance changed as a result of acclimation, the pattern by which it did is unclear. The only general trends I can see are increased performance at lower temperatures and decreased at higher temperatures, almost like a homogenization of performance, across all three acclimation treatments. It's unclear why this occurred, but it's possible that TPC was acclimated primarily in response to lab conditions rather than to the actual temperature treatments.

Leverage plots indicated that subspecies and trial temperature were both extremely important predictors of performance. This suggests that differences between subspecies likely played a significant role in TPC, and further analysis broken down by subspecies may help clarify what pattern the post-acclimation curves are following.

4.4 Skin Resistance

There's very little I can actually say about skin resistance, since it wasn't calculated in proper but rather crudely approximated. If the approximations are accurate, skin resistance seems to be uniform across different subspecies and acclimation treatments. This may imply that skin resistance in ensatina isn't very plastic, and has low acclimation potential. Body mass seemed to have the greatest influence in water loss rates, following a negative quadratic pattern. For implementing skin resistance into an SDM model for ensatina, it may be more important to estimate average ensatina size in different regions rather than breaking down differences by subspecies.

4.5 Which environmental factors are most important in predicting ensatina range?

MaxEnt predicted mean annual temperature to be the most significant driver of Ensatina range, suggesting that they are very temperature sensitive. It also predicted both precipitation seasonality and isothermality to be important drivers of Ensatina. This seems to support the idea that plethodontids are very poorly adapted for long-term climatic change. Since they typically aestivate during periods of high environmental stress, an extended period of high temperatures or frequent shifts between suitable and unsuitable conditions could decimate a population. Adding climatic stochasticity to the extinction risk model may improve projection accuracy. In general, performing more sensitivity analysis of what bioclimatic variables beyond temperature affect Ensatina and how they do so will allow us to create more accurate predictions of range and extinction risk.

4.7 Where will ensatina go extinct under predicted climate change?

Our preliminary SDM results suggest that extinction will be very likely along the California coast, possibly eradicating *e. xanthoptica* and *e. eschscholtzii*. This model did not take into account skin resistance or acclimation, and hinged on the key assumption that activity ceases below thermal preference.

Our results suggest that some subspecies acclimate their preference in response to increasing temperatures, while others inverse acclimate. This would make extinction less likely for *e. picta* and more likely for *e. xanthoptica* than what the preliminary SDM results suggest. A substantial amount of ensatina were found when ground temperatures were above their thermal preference, suggesting they may still remain active in some capacity. If this is true, it would likely reduce the risk of extinction across the board.

4.8 Future directions of research

For the sake of completeness, this study should be repeated with *e. croceator* and *e. klauberi*, as well as more individuals of *e. eschscholtzii* (since we only used 4). Once data from all subspecies has been accumulated, a more complete picture of extinction risk can be drawn, both in terms of genetic and geographic differences.

Desiccation trials should probably be redone. The apparent lack of stability in our set-up made it impossible for me to conventionally calculate skin resistances, relying instead on questionable approximations.

Analysis of how the differences in the long-term climate patterns of each subspecies, as well as habitat differences, could explain some of the more bizarre patterns in our results, and further breakdown of the effects of subspecies on TPC acclimation may also clear up some patterns.

An SDM model should be created with not only updated data from all the subspecies, but also acclimation potential, differences in acclimation between subspecies, skin resistance, and adaptability of skin resistance. Of course this would have to be done after properly measuring skin resistance and its adaptability.

Reacclimating these same salamanders multiple times would identify individual effects, and greatly increase the power of the data.

Running TPC trials at higher temperatures, up to 30 degrees perhaps, would help make comparisons with previous studies in the literature and create a more complete TPC curve for ensatina.

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