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THE FUTURE OF NORTH AMERICAN AMPHIBIANS: A STORY OF ECOPHYSIOLOGY, PLASTICITY AND CONSERVATION

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by

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Abstract

THE FUTURE OF NORTH AMERICAN AMPHIBIANS: A STORY OF ECOPHYSIOLOGY, PLASTICITY AND CONSERVATION

by

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As the climate changes at an unprecedented rate, rising temperatures will have complicated consequences for organisms. Ectotherms are particularly vulnerable because rely directly on environmental temperatures to regulate their body temperatures and perform necessary functions. While extinction risk for reptiles is well studied, amphibian present a more complex system due to their life histories and their fundamental tradeoff between maintaining activity at higher temperatures versus increased rate of water loss. To understand the climate change extinction risk of amphibians, I must first understand how they respond to current and predicted future environmental conditions. To accomplish this, I first investigate how canopy density and microclimates affect environmental conditions and those cascading effects on anuran thermal physiology in **Chapter 1.** I used agar frog models to estimate the thermal and hydric capacities of frogs and found that many environmental variables impact frog operative temperature and water loss rates. My results suggest that, with access to diversity of microhabitats, decreasing canopy coverage provides a larger range of thermal conditions without increasing the risk of water loss for frogs. In **Chapter 2**, to understand the long-term acclimation potential of these animals, I examine the within and inter-generation acclimation potential of maternal, incubation, and late rearing temperature on offspring thermal physiology. My results demonstrate

thermal inter- and within-generation plasticity in amphibians and show that larvae can quickly increase thermal preference and receive a major buffer to climate change. In **Chapter 3**, I test for outbreeding depression between two genetically distinct populations using an admixture propagation design. I found no evidence for outbreeding depression between pure line and genetic crosses. This suggests that admixture propagation is a safe method for genetic restoration and human facilitated gene flow could be used to break extinction vortexes. The results of this dissertation provide a picture of amphibians' response to the environment, within and between generations, and at the population level. I unite all my chapters to understand the large-scale physiological patterns of amphibians facing climate change, clarify the extinction risks for these animals, and show how I can use this information in conservation projects.

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Introduction

Broad Context

Climate change is a global threat to organisms across ecosystems and is already causing local population extinctions (Hof et al. 2011; Urban 2015; IPCC 2018). Ectotherms are especially at risk because they rely directly on environmental temperatures to regulate their own body temperatures and perform necessary functions (Deutsch et al. 2008; Kearney et al. 2009). However, ectotherms can respond to climate stressors in different ways that protect them from environmental conditions and lower their risk of extinction (Davis et al. 2005).

First, with sufficient time and ability to disperse, species' ranges and distributions may shift to more favorable environments (Wilson et al. 2005). There is, however, limited empirical evidence of latitudinal range shifts, suggesting that terrestrial vertebrates are limited in their dispersal capacity under climate change, with range expansion being limited largely to elevational changes (Sinervo et al. 2010). Second, ectotherms could modify their behavior to adjust their body temperature through thermoregulation in response to climate change (Huey 1982; Stevenson 1985). Evidence shows that ectotherms are able to modify body temperatures between daily and seasonal variation by altering basking posture and retreating from undesirable conditions (Heath 1975; Huey and Pianka 1977; Huey et al. 1989). But, behavioral thermoregulation may not buffer an organism from climate change over the long term (Huey et al. 2003; Kearney et al. 2009). Third, organisms may adjust to new environmental conditions through adaptive evolutionary responses

(Davis et al. 2005; Huey et al. 2012). With the climate changing quicker than previously expected (IPCC 2018), however, organisms may not be able to evolve quickly enough to prevent extinction. Finally, organisms that can thermally acclimate, either through within- or inter-generational plasticity, may be buffered against climate change, at least in short term. There is evidence of this in some invertebrates (Terblanche et al. 2005), but less is known about the acclimation potential of vertebrate ectotherms (Angilletta 2009). All these responses are limited by the amount that ectotherms can adjust physiologically, but in combination, these responses could guard ectotherms against the long-term threat of climate change.

If organisms do not respond to climate change in these ways, they face local or species level extinction. Scientists can predict their responses using extinction risk models that include physiology, demography, and climate predications (Deutsch et al. 2008; Williams et al. 2008; Chown 2012). Currently, models show the impact climate change will have on extinction probability, but most do not include biotic processes that might mitigate these effects, like local evolution, thermoregulation, or acclimation potential. And, while detailed models have been created for reptiles, less is known about amphibian extinction risk from climate change, even though amphibians are considered the most threatened vertebrate group (Wake and Vredenburg 2008; Raffel et al. 2013).

Amphibians present a complex system for understanding extinction risk due to their life histories and myriad of other threats – disease, pollution, and habitat loss (Rohr and Raffel 2010; Rohr et al. 2013). Many species interact with land and water,

with each habitat having their own microclimates that will be altered by climate change, and there is a fundamental tradeoff between maintaining activity at higher temperatures versus increased rate of water loss (Lertzman-Lepofsky et al. 2020a; Sinervo et al. In prep a). Additionally, the life history stages of amphibians—including eggs, larvae, metamorphs, and adults—may each experience a different environment (Kingsolver and Huey 1998), and have different thermal sensitivities and acclimation potentials (Davison 1969; Marais and Chown 2008; Kingsolver et al. 2011; Levy et al. 2015).

In this dissertation, I aim to understand amphibians' responses to climate change by studying the thermal physiology of three amphibian species. I used natural variation in environmental conditions, as well as experimentally manipulated temperatures, to test for effects on traits such as body temperatures, water loss rates, and thermal preferences, in both individuals and across populations. Specifically, I investigated: how environmental variables impact amphibian ecophysiology (**Chapter 1**), the potential for within- and inter-generational plasticity (**Chapter 2**) and if there is outbreeding depression in a local conservation project (**Chapter 3**). These topics are all critical to address because anthropogenic climate change continues to threaten species persistence (Trisos et al. 2020). With this approach of studying thermal physiology at multiple levels and life stages, I can get a more complete understanding of amphibian responses to climate change.

Dissertation Outline

Before scientist can predict how climate change affects amphibians, I must first understand the intricacies of how environmental variables effect the ecophysiology of amphibians in different habitats. In **Chapter 1**, "Canopy density, light, and moisture affect thermoregulatory trade-offs in an amphibian breeding habitat," I investigated the effect of canopy coverage and other environmental variables on the available thermoregulatory conditions of a breeding pond of the California red-legged frog, *Rana draytonii.* I used agar frog models to estimate the thermal and hydric capacities frogs would experience in locations with different canopy densities and microclimates. With this information, I can clarify how significant the fundamental trade-off between thermoregulating at higher conditions and the increased risk of dehydrations is, and I can understand how changing environmental conditions directly affect amphibians.

Once I understand how amphibians respond to thermal conditions immediately, I need to understand how acclimation responses alter their thermal physiology over time. In **Chapter 2**, "Intergenerational plasticity, as well as within generation plasticity, influences thermal preference of *A. mexicanum* at three time points", I investigated if amphibians could acclimate to thermal conditions through within- and inter-generational plasticity. To assess this capacity for plasticity, I tested how maternal, incubation, and late-rearing temperatures and their potential compounding interactions affect offspring thermal preference. This information

provides insights into how plastic thermal physiology is in amphibians and whether acclimation capacity is likely to provide a buffer against climate warming.

Finally, I must study if I can use this knowledge to actively conserve amphibians. I tested these methods in **Chapter 3**, "Using admixture propagation to create assisted gene flow, increase genetic diversity, and boost resiliency to climate change in an endangered, endemic amphibian." I used my knowledge of thermal physiology to test for outbreeding depression in a local conservation project on Santa Cruz Long-toed Salamanders (SCLTS), *Ambystoma macrodactylum croceum*. To increase genetic diversity, I proposed a genetic restoration plan that created artificial gene flow through an admixture captive breeding program. I crossed low genetic diversity populations with higher diversity populations and tested for any signs of outbreeding depression in physiological traits between the crosses compared to pure genetic lines. The results and conclusions presented in this chapter will help to determine if admixture propagation is a safe method for genetic restoration and if assisted gene flow can be used to break extinction vortexes in amphibians.

The results of this dissertation provide a picture of amphibians' response to the environment, within and between generations, and at the population level. I unite all my chapters to understand the large-scale physiological patterns of amphibians facing climate change, clarify the extinction risks for these animals, and show how I can use this information in conservation projects.

Chapter 1: Canopy coverage, light, and moisture affect thermoregulatory trade-offs in an amphibian breeding habitat

Abstract

When amphibians thermoregulate, they face a fundamental trade-off between the ability to maintain activity and an increased rate of dehydration at higher temperatures. Canopy coverage affects both the thermal and hydric conditions of the environment and can therefore influence amphibian thermoregulation. Frogs require proper conditions to thermoregulate to successfully grow, survive, and reproduce. But while we know how canopy and environmental variables typically affect operative temperature, less is known about effects on amphibian water loss rates. In this study, we measure the effect of canopy coverage on the conditions available for thermoregulation at a breeding pond of the California red-legged frog, *Rana draytonii.* We use agar frog models to estimate the thermal and hydric capacities frogs would experience in locations with different canopy coverage and microhabitats. At each site, we deployed models under four microhabitat treatments: wet/sun, wet/shade, dry/sun, and dry/shade. We modeled how environmental variables affected operative temperature and evaporative water loss from agar frogs. We found positive effects of air temperature, the sun treatment, and reduced canopy cover on operative temperature, and negative direct or indirect effects of these variables on evaporative water loss, consistent with the hypothesized trade-off between thermoregulatory behavior to increase temperature and the increased

desiccation risk due to higher water loss. Additionally, our results indicate that the availability of wet microhabitats can allow frogs to reduce water loss, potentially mitigating the risk of desiccation when thermoregulating to achieve higher operative temperatures. Our findings suggest, that with access to proper microhabitats, amphibians can mitigate the fundamental trade-off and receive benefits of thermoregulating at high temperatures.

Keywords: Canopy coverage, evaporative water loss, operative temperature, California red-legged frog, thermoregulation, canopy management, microhabitat

Introduction

Ectotherms facing extinction risk from climate change and other anthropogenic habitat alterations can respond to stressors with range shifts (Wilson et al. 2005), evolutionary adaptations (Visser 2008), and behavior (Stevenson 1985). But the dispersal capacity of many terrestrial vertebrates may be limited to elevational range shifts, instead of predicted latitudinal range shifts (Sinervo et al. 2010), and there may be inadequate time for species to evolve new adaptations (Chevin et al. 2010). Facultative behavioral change is the most rapid of these responses that could allow ectotherms to retain their physiological performance by staying within their thermal limits (Kearney et al. 2009). Ectotherms rely directly on environmental temperatures to regulate their body temperatures and perform necessary functions (Deutsch et al. 2008; Kearney et al. 2009). Ectotherms can also modify their thermoregulatory behavior, by altering positions or moving into favorable microhabitats, to maintain body temperatures for optimal performance (Huey 1982). However, thermoregulation adjustments are more complex for amphibians than for other ectothermic vertebrates, due to their highly permeable skin and thus increased risk of desiccation. We must study amphibian response to hydric limits, in addition to thermal limits, to understand their range of thermoregulatory abilities.

Terrestrial amphibians face a fundamental thermoregulatory trade-off between the benefits of higher thermal performance at warmer temperatures (e.g., increased foraging activity, Vickers et al. 2011 or immune responsiveness, Raffel et al. 2006; Maniero and Carey 1997) and higher rates of water loss (Lertzman-Lepofsky et al.

2020a). Amphibians have moist skin, which is required for cutaneous respiration but increases evaporative water loss (Boutilier et al. 1992). After spending time at high temperatures, amphibians may need to return to retreat sites to rehydrate and prevent desiccation. This need to maintain skin moisture limits amphibian mobility during breeding seasons, migrations, or adjustments to thermoregulatory behavior. Indeed, there is evidence that amphibians prioritize hydration over maintaining an optimum temperature (Moore and Gatten 1989; Anderson and Andrade 2017). Conversely, evaporative water loss can also be used as a physiological mechanism to help lower body temperatures (Rome et al. 1992). This initiates a cycle of bidirectional effects between temperature and water loss, where higher body temperatures increase water loss, and in turn, higher water loss rates help to lower body temperatures (Rome et al. 1992). These contrary demands underscore the need to consider the risk of water loss must be considered, in addition to temperature, when calculating amphibian survival risk within a given habitat. Nevertheless, predictions of species extinction risk rely primarily on temperature limits without accounting for water loss (Sinervo et al. 2010; Duarte et al. 2012), or the interactions between the two.

There are many habitat conditions that may affect this fundamental trade-off, such as environmental temperature, precipitation, vegetation, topography, and canopy cover. Canopy cover, which could have a major effect on amphibian body temperatures and evaporative water loss rates, is of particular interest because it can be altered by land managers. However, amphibians are facing multiple stressors, like climate change and habitat modification, that might result in conflicting effects on

how canopy coverage affects survival. In recent decades, many regions have experienced trends towards increased canopy coverage caused by climate change (Mantyka-Pringle et al. 2015; Song et al. 2018) and the spread of invasive flora (Crooks 2002; Watling et al. 2011). In a warming climate, increased shade from a denser canopy could help prevent amphibians from overheating or desiccating, but it might also result in the absence of warm microhabitats needed for proper thermoregulation, especially in temperate regions (Kearney et al. 2009). Before we can begin to consider canopy cover as a potential amphibian conservation tool for land managers, we need to understand the potentially complex ways that canopy cover may affect the balance of evaporative water loss and body temperature for amphibians in various types of microhabitats.

We know that dense canopy coverage can have negative consequences on amphibian population size. In a long-term survey of amphibian distribution, frogs, including Ranids, were absent from closed-canopy ponds and localized extirpations were positively associated with overgrowth (Skelly et al. 1999). There are other studies that also support that canopy overgrowth is linked with a higher risk of disease (Raffel et al. 2010; Becker et al. 2012) and probability of extinction (Skelly et al. 2002; Lambrinos and Kleier 2003). Similarly, sparse canopy coverage could lead to dehydration and force amphibians to take refuge in moist microhabitats (Rothermel and Semlitsch 2002; Rothermel and Luhring 2005), potentially leading to aggregation patterns that increase disease transmission and population declines (Burrowes et al. 2004). A proposed mechanism of these declines is that increased canopy coverage

reduces availability of warm and well-lit microhabitats, resulting in fewer thermoregulation opportunities to achieve warm body temperatures needed to help clear Bd infection (Raffel et al. 2010; Skelly et al. 2014). However, shade from increased canopy coverage may mitigate dehydration risk, but it is unclear how canopy coverage affects this complex relationship between the thermal environment and potential for water loss. Many studies have focused singularly on water loss or temperature to understand threats to amphibian populations (Sinervo et al. 2010; Duarte et al. 2012), but to predict how canopy cover affects trade-offs between thermoregulation and water loss we must study both factors simultaneously (Greenberg and Palen 2021). Previous studies conducted measurements of animals in their natural environments (Skelly et al. 2002), providing a snapshot of a moment in time but not capturing potential effects of extended exposure to different microhabitats.

At our study site, dead vegetation was historically cleared, and the canopy was thinned for aesthetic purposes and recreation (Brett Hall, personal communication). But when *Rana draytonii* was federally listed in 1995, habitat management ceased and the riparian overstory has grown an estimated 50-80% over the last 25 years (Brett Hall; Mark Allaback, personal communication). Subsequent surveys have shown a decreasing population size over the last three decades (Allaback personnel communication; Jones & Stokes 2002). While a temporally confounded correlation does not necessarily imply causality, multiple prior studies found increased Bd infection with increased canopy coverage (Raffel et al. 2010; Becker et al. 2012). We

hypothesized that increased canopy cover at this site negatively affected the frog population by reducing access to warm microhabitats, leading to reduced opportunities to achieve elevated body temperatures via behavioral thermoregulation. A central goal of this study was to evaluate a key prediction of this hypothesis, that increased canopy cover results in reduced availability of microhabitats where frogs can achieve elevated body temperatures. We also sought to explore potential trade-offs between body temperature and evaporative water loss, and the potential for bidirectional effects between these two key variables.

To test for potential effects of extended exposure to various combinations of canopy coverage, light, and water conditions, we placed agar frogs—models that mimic amphibian thermal and hydric responses—in different microhabitats within a known amphibian breeding habitat. We used these model agar frogs to measure how environmental conditions, including percent canopy coverage, affect the evaporative water loss rates (EWL) and operative body temperature (T_e) of a frog utilizing each microhabitat. Here, EWL is defined as the percent rate of evaporative water loss, and T_e is defined as the body temperature of an individual in thermal equilibrium with its environment. We used natural site-level variation in canopy coverage to evaluate effects of this variable on T_e and EWL, and we tested for effects of light and moisture at each site by intentionally placing agar frogs in different experimental conditions (sun versus shade; wet versus dry) within each site. To evaluate the effects of extended exposure to each condition, we measured variation in EWL and T_e throughout an entire season. We predicted that temporal variation in air temperature

would correlate positively with both EWL and T_e, that the wet treatment would decrease EWL (due to increased water availability) and T_e (due to increased evaporative cooling), and that the shade treatment and increased canopy coverage would both decrease EWL and T_e due to decreased solar radiation. We also predicted a bidirectional relationship between T_e and EWL, in which elevated T_e would increase EWL due to the direct effect of temperature on evaporation, while EWL would tend to decrease T_e due to evaporative cooling.

Materials and Methods

Study System

We studied the environmental conditions available to California Red-Legged Frogs (CRLF), (*Rana draytonii*) in a freshwater reservoir on the lower campus of the University of California, Santa Cruz Arboretum (36° 59' 01.09" N, 122° 03'44.33" W). *Rana draytonii* has a vulnerable status and is a medium-bodied frog that is a representative of typical California frog behavior and habitat usage. The pond is approximately 75 feet in diameter, the only known breeding location for this CRLF population, and characterized by dense emergent and woody vegetation (Jones & Stokes 2002).

Study Design

To measure the thermoregulatory conditions at the pond, we divided up the pond into 8 wedges, each with an area of approximately 51.28m^2. We deployed agar

frogs in each site's shoreline for 24-hr periods from March-September. We used natural differences in canopy coverage to test how percent canopy coverage affects EWL and T_e on agar frogs. For list of variables and their definitions, see Table 1.

To measure the full range of thermal and hydric conditions an amphibian could experience, agar frogs were placed in different microhabitats using a 2 x 2 factorial design with two levels of light treatment (sun vs shade) and two levels of water treatment (wet vs dry, Figure 1). We tracked T_e with internal dataloggers, EWL from mass changes in agar frogs, and other environmental conditions (Table 1, Shaffer 1989; Dzialowski 2005; Lertzman-Lepofsky et al. 2020a). With this design, we will first test how percent canopy coverage affects agar frogs' T_e and EWL individually. Then, we will test the interaction between their T_e and EWL using structural equation modeling. Finally, we test a real-life example of thinning excess vegetation and its effect on EWL and T_e.

Canopy Coverage

Holding a camera at 1 meter height and pointing the lens up vertically, we took photographs of the canopy overstory at two separate points approximately 5m apart within each site to calculate percent canopy coverage. We analyzed photos in Adobe Photoshop and converting pixels of sky to white and pixels of vegetation to black. Using Image J, we calculated the number of black vs white pixels and estimated percent canopy coverage by comparing the number of pixels of vegetation to total pixels. Average percent canopy coverage was calculated for each site.

Agar Creation and Validation

Agar frogs are regularly used to estimate the T_e and EWL an amphibian could potentially access in its environment (Nowakowski et al. 2017; Anderson et al. 2018). The free evaporation from agar captures the evaporative properties experienced by live frogs, including the cooling aspects caused by EWL, since we are unable to use live frogs due to their threatened status (Spotila and Berman 1976; Navas and Araujo 2000; Köhler et al. 2011). Agar EWL has been validated in comparison to live frogs (Lertzman-Lepofsky et al. 2020b), and agar models exhibit internal temperatures that match live amphibians (Bartelt and Peterson 2005; Anderson et al. 2018). While there has been recent debate about using agar models to compare to live thermoregulating amphibians (Christian et al. 2017; Riddell et al. 2017), we minimized identified limitations by using agar frogs that represent live frogs in size, shape, posture, and inactivity, and replaced models facing extreme dehydration to avoid inaccurate readings of EWL (Christian et al. 2017). We also acknowledge that agar frogs cannot replicate live frog behavior and movements, so we instead use agar frogs to study the available microhabitats with different thermal and hydric opportunities for frogs, as opposed to simulating what a live frog would experience.

We made agar frog models in latex molds, which were initially created with plaster casts from museum specimens spanning the size range of anurans in the Pacific Northwest: two large-bodied frogs, *Bufo boreas and Rana draytonii,* one small frog, *Pseudacris regilla,* and a small ellipse shape (3 cm diameter). We filled molds with agar (4.74 g in 100 ml) tinted green with acrylic paint and allowed them

to solidify around a thermocouple probe in the center. The agar formula we used was denser than previous studies to achieve a similar density to live amphibians and obtain more accurate T_e (Navas and Araujo 2000; Lertzman-Lepofsky et al. 2020b).

Agar Frog Deployment

We deployed the agar frogs for 24 hours every two weeks during the 2017 breeding season, March-September. Within each of the 8 wedges, one small (6–17 g) and one large (17–45 g) agar frog were deployed under four microhabitat treatments: wet/sun, wet/shade, dry/sun, and dry/shade (Figure 1, adapted with permission from Lertzman-Lepofsky et al. 2020a). Agar frogs in the sun light treatment were placed in the site under the location of lowest canopy coverage and shade light treatment frogs were placed under fallen branches to be fully shaded. Agar frogs in the dry water treatment were placed on dry substate near the pond bank, and wet water treatment frogs were placed on the water line, so half the agar was in the water and half was on saturated substrate. As the season progressed and water level changed, we adjusted the wet treatment location to keep agar frogs on the water line. Every agar frog was deployed with an internal thermocouple that measured internal model temperature at one-minute intervals to calculate T_e (Bakken 1992; Dzialowski 2005).

To determine EWL for each weigh period, agar frogs were weighed and deployed at 5:00 pm and weighed three times the following day (morning, noon, and evening). The weigh period was defined as the 4–14 hour period between model deployment and the time when weighing occurred (weigh point). The first 15 minutes

after each weigh point were removed from calculations to ensure agar frogs had achieved equilibrium again following handling. Any agar frogs that had lost 10% or more of their body weight were replaced with fresh agar frogs, to obtain consistent EWL. Cages, constructed from chicken wire, were placed over agar frogs to prevent damage from birds. A relative humidity datalogger was placed at the main site 2 meters above the ground in shade conditions to log ambient air temperature and relative humidity at one-minute intervals.

EWL and T_e Calculations

EWL was measured as percent water loss per hour, calculated as the change in agar mass over the deployment period. Operative temperature, T_e, was calculated from the internal thermocouple data. We calculated average, minimum, and maximum temperature for each weigh period as well as the standard deviation and full range difference of temperatures. The ambient data logger was used to calculate air temperature and relative humidity for each weight period. We calculated average, minimum, maximum, standard deviation, and range of both air temperature and relative humidity.

Effects of Vegetation Trimming

At one wedge site along the pond, site managers trimmed the canopy and removed dead vegetation to simulate historical management. We used this site in our study, but we additionally compared how this artificially trimmed canopy affected

EWL and T_e, compared to sites with naturally thin canopy coverage. We aimed to test if artificial thinning can return potential thermoregulatory benefits, such as increased T_e, that naturally thin canopy provides.

Statistical Analysis

Environmental Variable Effects on Average T_e and EWL

To examine how percent canopy coverage, water treatment, and light treatment impacted agar frogs, we fit a linear mixed model using the *lmer* package in R ver 3.6.1 (www.r-project.org). This modeling framework allowed us to examine the effect of many environmental variables (i.e., fixed effects) on multiple agar response variables and include two nested random effects of "session/period" and "site/microhabitat" (see Table 1 for list of variables used). We created two models to test how these environmental variables influenced two primary response variables: 1) EWL and 2) average T_e.

We started with a base model that includes the nested random effects above and the planned experimental variables as fixed effects: percent canopy coverage, water treatment, and light treatment. We choose these variables to start because percent canopy coverage is the predictor variable of interest, and the water and light treatments were manipulated in our experimental design. Next, we added additional predictor variables using a traditional forward selection procedure (Legendre and Legendre 1998; Blanchet et al. 2008) In each step, we added the fixed effect that contributed most to the model (lowest P-value) and continued to add predictors until

none significantly improved the model ($P>0.05$). Significance of the fixed effect was assessed by using an *F* test in the *anova* function in R, by sequentially adding the variable of interest and comparing with the previous model, which did not include the effect (Zuur et al. 2009). To reduce problems with multicollinearity, we only included the best single predictor variable in each of the following categories of highly correlated predictors: air temperature, agar frog temperature, and relative humidity (Table 1). After one predictor was added from each category, other variables from that category were removed from consideration as future predictors.

Interaction between T_e and EWL

To evaluate hypothesized causal pathways linking the EWL and average T_e of the agar frogs, including a potentially bidirectional relationship between T_e and EWL, we used piecewise structural equation modeling (SEM). This statistical technique estimates the strengths of the effects using the linear relationships between variables and can account for nested random effects (Shipley 2000). For discrete variables, such as light treatment and water treatment, we set them as binary values (0 and 1) to model as numeric variables. The hypothesized causal links between variables were determined by the LMMs created in the previous section for EWL and average T_e. To fit our models, we used the piecewiseSEM package in R (Shipley 2009).

Results

Environmental Variable Effects on Average T_e and EWL

Average air temperature and average relative humidity both had significant positive effects on agar frog average T_e (Figure 2d, 2f, Table 2). EWL had a positive significant effect on T_e, but water treatment did not (Table 2). In the final model, higher canopy coverage decreased T_e (Figure 2b, Figure 4b, Table 2) and the sun treatment significantly increased T_e (Figure 3a, Table 2). Day/night condition was not a significant predictor of T_e ($p > 0.05$).

Average air temperature and average relative humidity both had significant negative effects on agar frog EWL (Figure 2c,2e, Table 2). Average T_e of the agar frog had a positive effect on EWL, but light treatment and canopy coverage did not significantly affect EWL (Figure 4a, Table 2). Day/night condition significantly affected EWL, with measurements during the day having significantly higher EWL than those at night (Table 2). In the final model, the wet treatment significantly decreased EWL (Figure 3b, Table 2). For both models, agar frog mass, surface area, and shape were not significant predictors of the response variable tested (all $p > 0.05$).

Interactions between T_e and EWL

The SEM revealed the relative strengths of pathways linking percent canopy coverage, water treatment, light treatment, day condition (day vs. night), air temperature, and relative humidity to our two key response variables: agar frog EWL and T_e (Figure 5). It also included the bidirectional relationship between the two

response variables (Figure 5). Standardized coefficient estimates indicate the relative strength of each direct effect, while the associated p-value indicates whether this coefficient is significantly different from zero after controlling for the effects of other predictors.

The SEM revealed that average air temperature and average relative humidity both had significantly positive effects on T_e and negative effects on EWL, though the effects of air temperature were both stronger than effects of relative humidity (Figure 5). Day condition significantly affected EWL (higher EWL during day than night, Figure 5), but was not included as an effect on T_e because it was not significant in the linear mixed model. For the light treatment, sunny conditions significantly increased T_e but did not significantly affect the EWL (Figure 5). The water treatment had the opposite effect, with a wet condition having a significant negative effect on EWL but no significant effect on T_e (Figure 5). Canopy coverage had a significant negative effect on T_e and no significant effect on EWL (Figure 5). The SEM indicated that T_e and EWL both had significant positive effects on each other, with T_e more strongly affecting EWL than vice versa (Figure 5).

Effects of Vegetative Trimming

We found that that artificially thinned canopy resulted in similar T_e and EWL consistent with results observed with naturally thin canopy coverage (Figure 4). The artificially thinned site had a mean EWL similar to sites with similar canopy coverages and the site that had 10% more coverage (Figure 4). The site's mean T_e was

slightly lower than the similar naturally thin canopy coverage site, but was still warmer than most denser sites (Figure 4).

Discussion

Consistent with our primary hypotheses and recent studies (Sinervo et al. 2010; Sunday et al. 2014; Nowakowski et al. 2017), we found positive effects of air temperature, the sun treatment, and reduced canopy cover on T_e of agar frog models. However, these three variables had negative or nonsignificant direct effects on EWL, contrary to our prediction. These results seem to suggest that higher air temperatures and more sun may allow frogs to achieve elevated T_e without incurring the cost of increased desiccation risk. However, this interpretation ignores potential indirect effects of these variables on EWL, mediated by the very strong direct effect of T_e on EWL. For example, air temperature had either positive or negative effects on EWL depending on if we consider its direct effect (negative) or its indirect effect as mediated by T_e (positive). When we examined effect of air temperature on EWL as a single predictor, the overall effect was positive (coef = 0.045, $F_{1,58.1} = 11.5$, P = 0.001). The SEM results indicate that this positive effect was entirely mediated by the direct effect of air temperature on T_e. After accounting for this indirect effect, the direct effect of air temperature switched signs and became negative. A possible explanation for this pattern is that higher air temperatures correlated with higher relative humidity, which tended to reduce EWL. The only predictor variable with no significant direct effect on T_e (day vs. night) had a strong positive direct effect on

EWL (i.e., higher during daytime). Overall, the results show consistently positive effects of air temperature, the sun treatment, and reduced canopy cover on T_e, and negative direct or indirect effects of these variables on EWL, consistent with the hypothesized trade-off between thermoregulatory behavior to increase T_e and increased desiccation risk due to higher EWL (Lertzman-Lepofsky et al. 2020a).

The wet treatment reduced EWL, supporting our hypothesis that frogs are buffered from desiccation when aquatic microhabitats are available. However, the wet treatment had no significant direct effect on T_e, contrary to our prediction that wet conditions would result in increased evaporative cooling. A possible explanation for this result is that wet microhabitats might have higher relative humidity than dry microhabitats, resulting in less evaporation and thus less evaporative cooling. Indeed, higher relative humidity significantly increased T_e of agar frog models, consistent with decreased evaporative cooling under humid conditions. However, the SEM provided no clear evidence of an evaporative cooling effect when we explored the direct effect of EWL on T_e, which had a positive coefficient in the full model (opposite our prediction). Interpretation of this result is complicated by it being part of a bidirectional relationship between T_e and EWL, resulting in potential confoundment between effects going in either direction. It seems likely that some amount of evaporative cooling occurred but was too weak to overcome the much stronger positive effect of T_e on EWL. Overall, our results indicate that the availability of wet microhabitats can allow frogs to reduce EWL, potentially mitigating the risk of desiccation when thermoregulating to achieve higher T_e. This is

important, as both lab and field studies suggest that amphibians seek environments that maintain hydration levels at the cost of experiencing sub-optimal temperatures (Moore and Gatten 1989; Anderson and Andrade 2017).

Individuals often move between sunny and shaded microhabitat types to avoid physiological limits while accomplishing necessary activities like foraging, breeding, and fighting of disease (Huey 1982; Sunday et al. 2014). Such habitat selection has the potential to ameliorate exposure to harmful temperatures and high rates of water loss under extreme climatic conditions (Scheffers et al. 2014). Many studies show that access to different microhabitats can reduce risk of amphibians approaching thermal limits (Stevenson 1985; Scheffers et al. 2014; Nowakowski et al. 2017), but we additionally show that access to microhabitats can also reduce risk of reaching hydric limits. However, we artificially created these microhabitats and cannot assume that real amphibians always have access to the full range of microhabitat types observed in this study. Many real-word amphibian habitats may have a more restricted set of available and accessible microhabitats, especially in degraded habitats with strong anthropogenic impacts. Additionally, we assume that amphibians can move freely and quickly between microhabitats without cost. But, there are costs to frequent movement between habitats, such as higher risk of predation (Beever et al. 2017), increased energetic demands (Rohr and Palmer 2013), and possibly increased risk of water loss while traveling through undesirable conditions (Thorson and Svihla 1943). This potential for additional water loss could tip the scale of the fundamental trade-off with thermoregulating at higher temperatures. While moving between

microhabitats can provide the behavioral buffer amphibians need to thermoregulate, future work should focus on costs of this movement on the balance of the trade-off.

When behavioral adjustments are not enough to stay within hydric and thermal limits, animals face increased risk of death (Sinervo et al. 2010; Sunday et al. 2014) or increased opportunity costs due to the need to retreat to thermal refugia (e.g., burrows or deep-water habitats, Sinervo et al. 2010). As the climate changes, there may be added pressure on amphibians due to these other risks and costs, in addition to the fundamental trade-off of elevated T_e increasing EWL. As summer seasons lengthen and precipitation patterns change, more days will exceed physiological thresholds and aquatic habitats may become less available (Bartelt et al. 2010; Lertzman-Lepofsky et al. 2020a). This will likely force frogs to retreat to refugia more frequently and to restrict activities for more of the year, including during critical periods for migration and breeding (Walls et al. 2013). Furthermore, more frequent large-scale ecosystem disturbances, like increased wildfires and spread of infectious diseases, may magnify these struggles (Westerling et al. 2006; Raffel et al. 2013). In addition to decreasing amphibian habitat, wildfires can also alter habitat conditions (Hossack and Pilliod 2011), potentially affecting EWL and T_e. As chytrid continues to spread, availability of thermoregulatory conditions to fight off infections will be critical for population survival (Richards-Zawacki 2010), especially in temperate regions where shaded environments may be too cool for many species to fight off Bd infection (Kearney et al. 2009). For example, *Rana draytonii* at our study site are able to thermoregulate to warmer T_e to fight off chytrid, but very dense canopy cover

prevents them from achieving sufficiently high T_e (Becker et al. 2012). This finding led to a management decision to thin the overstory at our study site. With increased threats from climate change, it will be critical that ectotherms have access to habitats with proper hydric and thermal conditions to avoid restricting activity (Rozen-Rechels et al. 2019). While behavioral restrictions act at the level of the individual, these effects may have population level consequences, similar to documented effects of seasonal and interannual climate variation on population growth rates and local extinction probabilities (Kissel et al. 2019).

Our results suggest that decreasing canopy coverage increases the availability of microhabitats where frogs can achieve high T_e. This could be highly beneficial to some frog species, especially if there is access to wet microhabitats to help reduce the trade-off of increased desiccation risk due to the positive effect of T_e on EWL. We also found that artificial thinning is consistent with effects of naturally thin canopy coverage, suggesting that land managers can thin canopy to increase amphibians' access to higher T_e and receive other benefits of clearing (Skelly et al. 2014). While we used a specific breeding pond as a test case for estimating a pond's thermal and hydric conditions, these findings are likely to apply to amphibians more broadly. For example, we see similar trends of canopy and microhabitat treatments effects on T_e in other habitat types (Duarte et al. 2012) and in other amphibian species (Scheffers et al. 2014; Anderson et al. 2018). There are also studies that support evidence of a trade-off between T_e and EWL in other amphibians (Köhler et al. 2011; Lertzman-Lepofsky et al. 2020a; Greenberg and Palen 2021). Furthermore, we found similar

patterns of T_e and EWL responses regardless of the shape or size of the agar frog model, suggesting that different amphibian species will have similar responses to the observed conditions. Our analysis of the complex effects of canopy coverage and other environmental characteristics on EWL and T_e of agar frogs may also be of direct use to land managers trying to predict the effects of management actions on thermoregulatory conditions for frogs and other amphibians. For example, managers of the UC Santa Cruz Arboretum have used this information to alter their management plans to thin overstories, monitor vegetation growth, and remove invasive sun-covering species. A better understanding of how environmental characteristics affect the fundamental trade-off amphibians face between water loss and thermoregulation will be essential for predicting species responses to continued climate change and their extinction risk.

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Tables

Table 1.1 Variables measured in the experiment and used to build our linear mixed models.

Variable	Definition	Units	Spatial Scale
Evaporative Water Loss Rate (EWL)	Percent mass loss from an agar frog per unit time due to evaporation	% mass lost/hour	microhabitat
Operative body temperature (T_e)*	Internal 'body' temperature of agar frog, representing the temperature of a frog in thermal equilibrium with its environment	ºC	microhabitat
Canopy coverage	Percent of the sky that the canopy covers at each site	% coverage	site
Water treatment	Placement of agar frog in water's edge or on shoreline	wet or dry	site
Light treatment	Placement of agar frog in open sunlight or in shade	sun or shade	site
Relative humidity*	Density of water vapor relative to temperature at single location near the pond for every deployment period	%	pond
Air temperature*	Temperature at 1 m above the ground at a single location near the pond	ºC	pond
Day condition	Condition depending on if deployment period was during daytime or at night	day or night	microhabitat
Initial mass	Starting mass of agar frog before deployment	grams	microhabitat
Surface area	Surface area of agar frog before deployment (calculated from mass)	cm^2	microhabitat
Shape	Agar frogs were produced in four shapes	Ellipse, Hyla, Rana, Bufo	microhabitat
Session	Date each agar frog was deployed		pond
Site	Individual letter ID for each of 8 deployment sites in the pond		pond
Period	Deployment time period (4-14 hours) over which T_e and EWL were calculated	morning, afternoon, overnight	
Microhabitat	Within-site combination of treatments where an agar frog was deployed	sun/dry, sun/wet, shade/dry, shade/wet	

*Variable measured every minute with a temperature or humidity loggers. For analysis, we calculated the minimum, maximum, range (maximum—minimum), average, and standard deviation over each deployment period.

Table 1.2 Summary of the results for the linear mixed models testing the effect of environmental variables on the response variables of EWL and average T_e. Two nested random effects were fixed (session/period and site/microhabitat). Variables marked with (avg) represent that the average measurement of that variable was most significant in the final model (See Table 1.1). Shown are the fixed effects and p-values. Significant p-values are italicized.

Model	Variable	Fixed Effects Coefficients	F Statistics	Degrees of Freedom	p values
Average T_e	Water treatment	0.109	0.687	1	0.416
	Light treatment	0.886	46.439	1	*<.001*
	Canopy coverage	-0.035	9.007	1	*0.028*
	Air temperature (avg)	0.71	229.404	1	*<.001*
	EWL	0.353	123.177	1	*<.001*
	Relative humidity (avg)	0.041	5.115	1	*0.027*
Percent EWL Rate	Water treatment	-0.719	145.588	1	*<.001*
	Light treatment	0.029	0.216	1	0.646
	Canopy coverage	-0.01	4.283	1	0.108
	T_e (avg)	0.187	115.41	1	*<.001*
	Air temperature (avg)	-0.167	56.723	1	*<.001*
	Relative humidity (avg)	-0.027	21.415	1	*<.001*
	Day Condition	-0.511	12.942	1	*<.001*

Figure

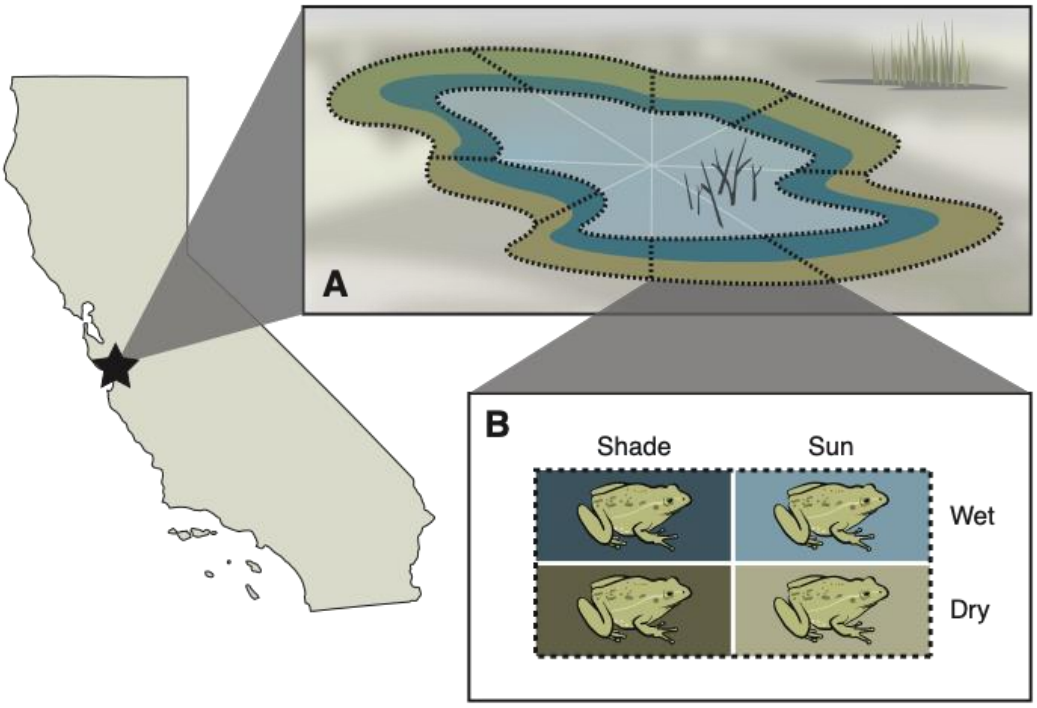

Figure 1.1 Panel A provides a representation of the study site, consisting of an amphibian breeding pond divided into 8 site location set around the shoreline. Panel B zooms in on one site location where one small and one large agar frog were deployed under four microhabitat treatments: wet/sun, wet/shade, dry/sun, and dry/shade. Adapted with permission from author Lertzman-Lepofsky et al. 2020a.

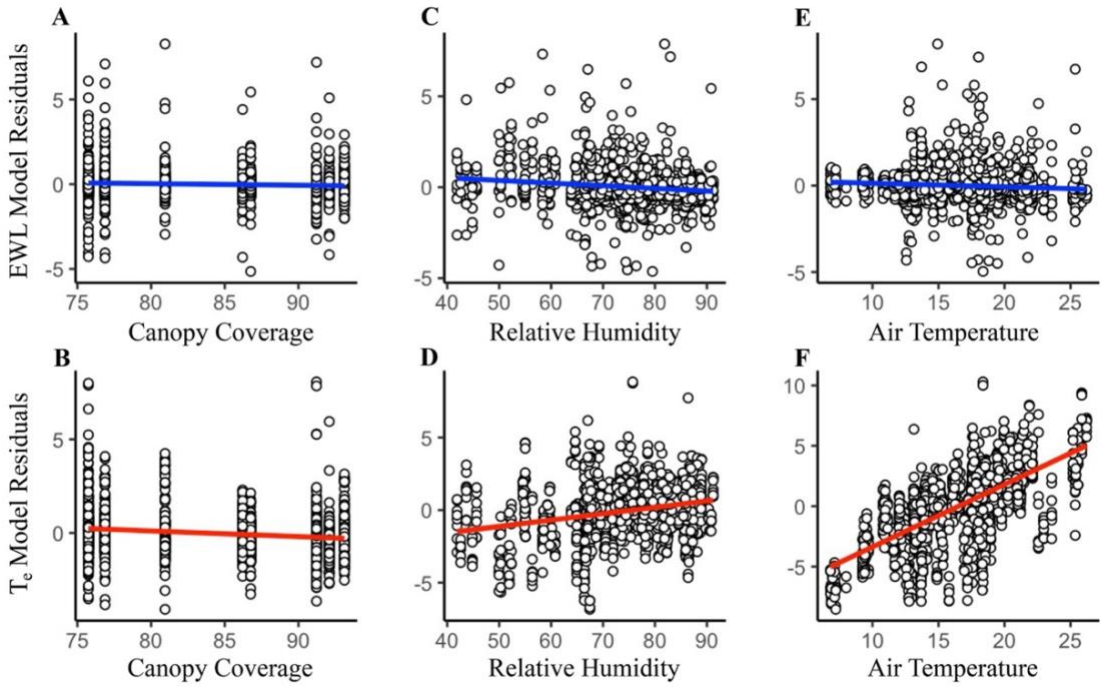

Figure 1.2 Three environmental variables— percent canopy coverage, average relative humidity, and average air temperature —affected evaporative water loss (EWL) and average operative temperature (T_e) of agar frogs. *A, B* – Canopy coverage. As canopy coverage increased, there was a significant decrease in T_e and no significant effect on EWL. *C, D* – Humidity. As humidity increased, there was a small but significant decrease in EWL and a significant increase in T_e. *E, F* – Air temperature. As air temperature increased, there was a small but significant decrease in EWL and a significant increase in T_e. Each residual plot was generated by removing the predictor of interest and associated random effects from the full model and plotting the residual errors from this model against the predictor of interest. Each line represents a simple linear regression fit to the residuals.

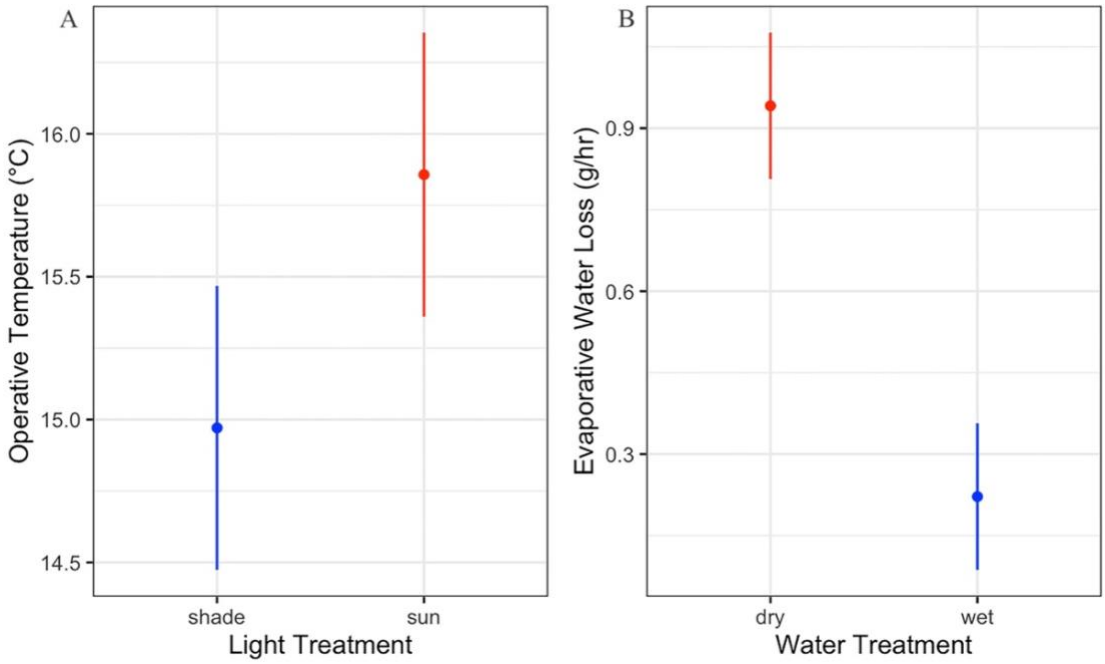

Figure 1.3 Least square means of light treatment on T_e and water treatment on EWL. A) Sun treatment has a significantly higher T_e then shade treatments. B) Wet treatments have a significantly lower EWL then dry treatments. Points represent mean values and bars represent standard error.

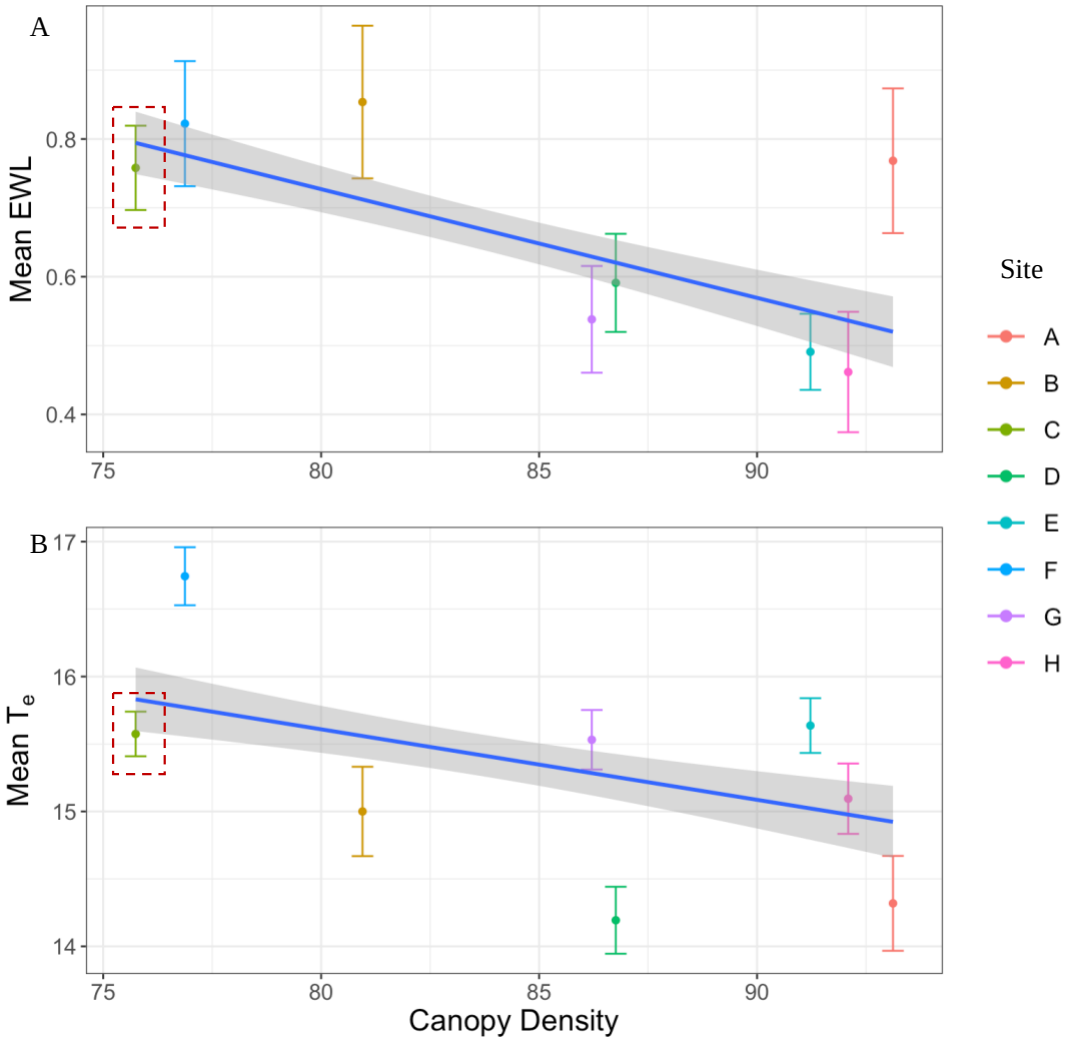

Figure 1.4 Canopy coverage by site effects on A) EWL and B) T_e. Points represent mean values with standard error bars. Color represents site and the red dashed box represents the artificially trimmed site. The blue line represents the model predictions and confidence intervals.

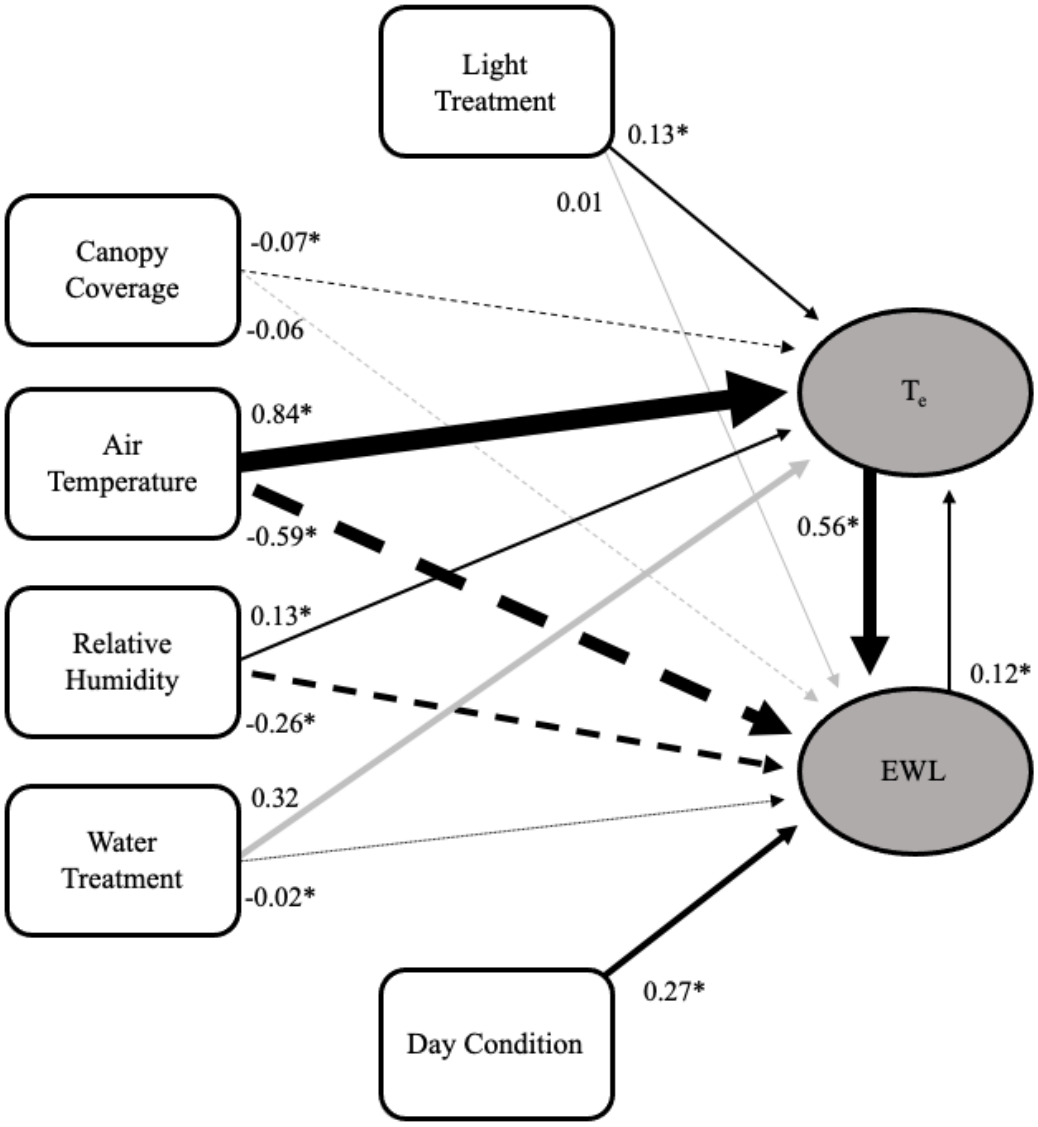

Figure 1.5 Visual relationship of the structural equation model. Standardized coefficient estimates indicate the relative strengths of all direct effects of one variable on another, with grey arrows representing insignificant effects, while black arrows and asterisks show significant p-values, controlling for the effects of the other predictors. Dashed arrows represent negative effects, while solid arrows show positive effects controlling for the effects of the other predictors. The weight of the arrows represents the strength of the effect. Response variables are shown in grey circle, and predictor variables white squares.

Chapter 2: Intergenerational plasticity, as well as within generation plasticity, influences thermal preference of *A. mexicanum* at three time points

Abstract

Genetic change via evolution can take many generations, but intergenerational plasticity (IGP) can allow parents to produce offspring with phenotypes that match current environmental demands in a single generation. Parental effects on offspring in response to environmental cues are well documented in general. However, whether parental thermal environments can prime offspring to adapt to thermal conditions is largely unknown. To assess this capacity for thermal intergenerational plasticity, we tested how maternal, incubation, and late-rearing acclimation temperatures and their potential cumulative interactions affect offspring thermal preferences. We found that egg incubation temperature was positively correlated with offspring thermal preference immediately after hatching, consistent with a developmental acclimation effect. In older larvae (33 days), both late-rearing temperature and the three-way interaction among maternal, incubation and rearing temperature positively affected offspring thermal preference. Larvae with a consistent thermal history at 18°C had the lowest thermal preferences, almost 2°C lower than their counterpart larvae raised consistently at 21°C, which had the highest thermal preference. Our results demonstrate thermal IGP and within-generation plasticity in amphibians. With the positive cumulative interaction between maternal, incubation, and rearing temperature, larvae can quickly increase thermal preference in response to rising

temperatures, providing a potential physiological buffer to climate change. We demonstrate that thermal IGP is a real possibility, which is critical because growing evidence suggests that IGP might be an essential adaptation available to organisms needing to escape climate change. This information will be useful to more accurately predict future population level extinction probabilities, alter management decision to focus on species lacking IGP rescue, and continue to broaden our understanding of the importance and complexity of IGP. We must expand our studies on IGP to understand the interplay of IGP and within GP, understand the long-term effects of IGP over generations, and the interplay of IGP and evolution.

Keywords: maternal effects, acclimation, thermal preference, axolotl, *Ambystoma mexicanum*, intergenerational plasticity

Introduction

As the climate changes at an unprecedented rate (Diffenbaugh and Field 2013; Smith et al. 2015), organisms can respond immediately with physiological responses or avoid stressors with range shifts (Wilson et al. 2005) and behavioral changes (Stevenson 1985). But, when organisms reach the limits of migration or behavioral thermoregulation, they lose their buffer against climate change, and their survival will depend on evolutionary change or plasticity (Davis et al. 2005; Gunderson and Stillman 2015; Seebacher et al. 2015). Acclimation, a form of phenotypic plasticity within a generation that reflects the influence of the environment on an individual's phenotype, either morphologically or physiologically, could provide a cushion against ecological change (Chevin et al. 2010; Forsman 2015; Rohr et al. 2018). Organisms can acclimate to many environmental conditions including salinity (Bianchini et al. 2008) and nutrient levels (Grossman 2000), but acclimation to temperature conditions may be particularly important for conservation efforts given predicted levels of climate warming (Huey et al. 2012).

Thermal acclimation (defined as the capacity by which an organism alters a thermal trait in response to experienced environmental temperatures, Rohr et al. 2018) affects an ectotherm's ability to respond and cope with temperature variability and increasing temperatures, both features of climate change (Somero 2010; Huey et al. 2012). Extended exposure to higher temperature can cause physiological changes (Sinclair et al. 2016) in metabolism (Terblanche et al. 2005), behavior (Lara-Reséndiz et al. 2015), and immunity (Raffel et al. 2013). We can measure the potential for

thermal acclimation by assessing whether physiological traits shift as a function of a temperature experienced in the recent past (Huey et al. 2012). One such trait, thermal preference—the temperature an organism chooses when allowed to freely thermoregulate—can measure thermal sensitivity (Sinervo et al. 2010; Huey et al. 2012). Thermal preference is a proxy for whether an animal will persist over climate change (Sinervo et al. 2010), and positive acclimation of thermal preference could ameliorate extinction risk. If ectotherms can increase their temperature preference through acclimation, and maintain normal functions and behavior, they could survive exposure to the elevated temperatures expected under climate warming (Gvoždík 2011). But this only remains true if organisms can acclimate quickly enough before reaching the limits of behavioral buffering; otherwise ectotherms face climate change related decline and disruption of ecological interactions between organisms with different thermal acclimation capacities (Rohr et al. 2013, 2018). Ectotherms could also acclimate more quickly to increasing temperature if acclimation occurring within an ectotherm's life time is magnified by plasticity from between generations (Mousseau and Fox 1998; Räsänen and Kruuk 2007; Ho and Burggren 2010).

While less studied than within-generation plasticity (WGP), thermal intergenerational plasticity (IGP; also referred to as environmental parental effects or transgenerational plasticity) could also allow organisms to respond adaptively to changing thermal environments (Baugh and Day 2020). Specifically, the thermal environment of a parent might alter the phenotype of future generations (Via 1993; Jablonka and Raz 2009). Genetic change via evolution can take many generations, but

IGP could allow parents to respond to their current thermal stressors and produce offspring with thermal physiology that matches environmental demands in a single generation. One potential mechanism of IGP is maternal effects, changes in offspring phenotype determined by the phenotype or environmental conditions experienced by its mother (Falconer and Mackay 1996), through non-Mendelian inheritance mechanisms like epigenetics (Kirkpatrick and Lande 1992). We see many examples of maternal effects in the literature (Bernardo 1996): maternal condition can affect offspring size (Pick et al. 2016), predator-exposed parents can affect risk-prone behavior in offspring (Hellmann et al. 2020), and parent immune experience can affect offspring immunity (Reid et al. 2006). If thermal intergenerational plasticity can occur, it could allow ectotherms to cope with rapid environmental variation and provide a potential mechanism to buffer organisms from climate warming (Donelson et al. 2012). For example, in an ecophysiological model with a maternal effect acclimation capacity, lizards that could acclimate their thermal preference between generations had 10% lower extinction risk by 2070, enough to buffer many species from extinction, in the case of positive acclimation (Sinervo et al. 2018), or exacerbate extinction in the case of inverse acclimation (Bestion et al. Submitted). Over longer time periods, maternal effects can produce qualitatively different evolutionary outcomes than Mendelian inheritance and over generations, maternal effects can even change the rate and direction of evolution (Kirkpatrick and Lande 1992; Wolf et al. 1998). Intergenerational plasticity can also potentially interact with within-generational plasticity, but very few studies have addressed this directly. We

see studies providing indirect support by addressing IGP and WGP separately, like how maternal temperature can affect egg size (Bownds et al. 2010; Collin and Salazar 2010), whereas offspring rearing temperature can also affect egg size (Radmacher and Strohm 2010). Therefore, we must study both WGP and IGP simultaneously to understand if WGP can enhance, override, or counteract parental effects.

While thermal IGP on offspring thermal traits has not been previously documented, there are many examples of non-genetic intergenerational inheritance, spanning from plants to invertebrates to humans (Jablonka and Raz 2009). Through intergenerational plasticity, parents can provide offspring with increased tolerance of environmental perturbations such as contaminants (Marshall et al. 2008), food shortages (Bashey 2006), desiccation (Yoder et al. 2006), and shading (Galloway and Etterson 2007). Intergenerational plasticity, specifically maternal effects, has already been documented in several traits in amphibians: egg size and number (Kaplan 1987), morphology, growth, and locomotor performance (Kaplan and Phillips 2006). While there is growing evidence of parental effects in response to environmental stressors, the ways in which parental thermal environment prime offspring for thermal conditions is largely unknown. Studies in reptiles, have shown that viviparous snakes maintained at hotter temperatures have offspring that prefer warmer temperatures (Blouin-Demers et al. 2000), and lizards' thermoregulatory behavior can alter progeny's thermal preference by 1°C (Paranjpe et al. 2013). But, little is known about how amphibian mothers influence their offspring's thermal traits, even though amphibians have great potential thermal plasticity (Refsnider et al. 2019;

Bodensteiner et al. 2021).

Amphibians are likely to have thermal plasticity because of their life history strategies and developmental plasticity (Salinas and Munch 2014; Thorson et al. 2017), and are great candidates for studying thermal intergenerational plasticity. While there are many examples of parental thermal conditions influencing offspring non-thermal traits, very few studies have ever measured parental temperature effects on offspring thermal traits. We see a few examples, like parental temperature effects on temperature dependence of growth (Salinas and Munch 2012) or on gene expression (McCairns et al. 2016), but effects of parental temperature on offspring thermal preference has never been measured in vertebrates (Clusella-Trullas and Chown 2014). In this study, we ask how maternal temperature, as well as incubation and rearing temperature, affect offspring thermal preference. Additionally, many papers studying thermal intergenerational plasticity focus on raising parents in two climatically extreme temperatures to differentiate trends, but this is not a fair representation of what animals will experience in the wild (Walsh et al. 2014; Betini et al. 2020). We used a realistic increase in maternal temperature, mirroring projected climate increases, to measure effects on offspring thermal physiology to provide a more realistic understanding of how fast IGP could occur. Lastly, while many studies address IGP or within-generation plasticity separately, our experimental design focused on the interactive effect of parent and offspring temperature simultaneously to test for potential conflicting or cumulative effects on thermal preference.

To assess this capacity for thermal intergenerational plasticity, we tested how parental and offspring temperature affect offspring thermal preference. We genetically crossed *Ambystoma mexicanum* (the Mexican Axolotl*)* raised in two different temperatures ("maternal temperature") and then randomly assigned eggs from these crosses to two new temperatures ("incubation temperature") to measure the effect of maternal and incubation temperature on offspring thermal preference (Figure 2.1). Later in larval development, these offspring were randomly assigned to two new temperature treatments ("late-rearing temperature"), resulting in a fully crossed randomized block design (maternal temperature × incubation temperature × late-rearing temperature). We used an aquatic thermal gradient to test how maternal temperature, incubation temperature, late-rearing temperature, and potential interactions between these treatments affected offspring thermal preferences at three time points.

Materials and Methods

Study System

Ambystoma mexicanum, the Axolotl, is native to central Mexico and is an obligate paedomorph, retaining its aquatic larval form for its entire life. With no ability to migrate, access to climate refuges and reduced genetic diversity from historic bottlenecks (Parra-Olea et al. 2012), plasticity could be one of the species only means to persist in the face of climate change. *A. mexicanum* is also likely to have plastic traits, based on its regenerative abilities, and we see some evidence of

thermal plasticity in metabolism and critical thermal maximum (Orille et al. 2020; McKeon 2022). *A. mexicanum* is also easy to breed and we have a detailed understanding of its physiology, because this species is also a model organism historically used to understand amphibian biology (Voss et al. 2009).

To test for interactions between inter- and within- generation plasticity on thermal preference in *A. mexicanum*, we performed a randomized block experiment with two temperature treatments over three different exposure stages: maternal (IGP), incubation (WGP), and late-rearing (WGP). With this design, we can test how each temperature in an animal's thermal history individually affects offspring thermal physiology. We predicted that warmer maternal, incubation, and late-rearing temperatures would each lead to higher offspring thermal preference. This represents a positive plastic response to an experienced temperature; however, we could see an inverse acclimation if a warmer experienced temperature leads to cooler thermal preference. Our design also lets us test the interactive effect of WGP and IGP by comparing the interaction term of maternal, incubation, and late-rearing temperatures. This effect could be cummulative, with WGP enhancing IGP, WGP could override IGP effects, or WGP could counteract IGP. We hypothesize that incubation and late-rearing temperature will have an cumulative effect with maternal temperature, and the offspring experiencing the warm treatment for all three temperatures will have the highest thermal preference.

Breeding

Three male and six female adults, that had been raised their entire lives at 18°C, were acclimated to lab conditions for one year. All males and three females remained at 18°C, while the other three females were moved to 21°C for a minimum of three months to simulate the stress of increased temperatures from climate change.

Males were randomly paired with females from 18 °C and then 21°C treatments in large breeding tanks, creating a half-sibling design to test maternal temperature effects (Figure 2.1a, Falconer and Mackay 1996; Roff 1998). Once paired, males released spermatophore packets within 24 hours and females picked these packets up and laid their eggs within 1-3 days. After a female completed laying eggs, the eggs were divided equally among 18 and 21°C to test for incubation temperature effects. Males were given a minimum of 7 weeks between breeding events to allow for sperm regeneration (Duhon n.d.).

Hatchling Thermal Preference

Eggs hatched after a period of 2-4 weeks, depending on incubation temperature. Five days after hatching, larvae were run through temperature preference trials to measure effects of maternal and incubation temperature at birth (N = 80/mom per temperature, N =960 larvae total). Full details on these thermal preference trials are given below. We could not conduct trials with larvae immediately after hatching because they do not eat external food for 2-3 days when they are still digesting their yolk. Animals do not properly thermoregulate or choose not to thermoregulate until

they are regularly digesting food (Sinervo personal observation), so we waited until the animal were eating regularly at five days after hatching to run thermal preference trials.

We conducted all thermal preference trials between 09:00 and 14:00, the normal active period of *A. mexicanum.* We designed an aquatic thermal gradient with an inner metal track where the animal could freely swim, which was temperature controlled by being placed in an external track that was filled with water and cooled with ice on one end and heated with an aquarium heater on the other (Lillywhite 1971; Hill et al. 1975; Hutchison and Hill 1976). This created a consistent thermal gradient of 5 to 30°C and we created a total of 24 tracks. We cooled the larvae for fifteen minutes prior to the thermal preference trial at 5-8°C to encourage thermoregulation, and then placed the larvae in the thermal preference track for a period of one hour.

Because of their small body size (2-3cm), larvae have little thermal resistivity and are typically the same temperature as their substrate (Brattstrom 1963; Spotila 1972; Spranger personal observation), so we assumed the water temperature near the abdomen of the larvae was essentially the same as their body temperature. We measured the temperature near the abdomen of the larvae in the thermal preference track every five minutes and calculated thermal preference by averaging these values, after removing the first two measurements of every trial to account for time for larvae to explore the track and start thermoregulation.

After these thermal preference trials, we continued to raise larvae at their

original incubation temperature (either 18 or 21°C) for two more weeks and then measured their thermal preference a second time at 19 days old. Following this second set of thermal preference trials, larvae from each clutch were randomly assigned to one of two new temperatures to test for the effect of late-rearing temperature on thermal preference (Figure 2.1a). This created a treatment design where larvae from each clutch had one of eight potential thermal histories (Figure 2.1b). After two weeks at the late-rearing temperature, we measured thermal preference a final time at 33 days old.

Statistical Analysis

We used a linear mixed effects model to test how the thermal history from maternal, incubation, and rearing temperature affects the response variable of thermal preference at 5, 19, and 33 days. Linear mixed models are useful for studies with related individuals because the model has combined effects of independent explanatory variables (i.e., fixed effects) while also accounting for within-clutch and among-parent variability (i.e., random effects, Zuur et al. 2009). We used single fixed effects to test how maternal, incubation and late-rearing temperature individually impact offspring thermal preference. We used two-way and three-way interaction terms between maternal, incubation, and late-rearing temperature to test for effects of WGP either enhancing, overriding, or counteracting IGP.

We first used a linear mixed effects model (r package lme4; Bates et al. 2015) to analyze differences in thermal preference at five days. We included fixed effects of

maternal temperature, incubation temperature, and their interaction, with random effects of clutch ID nested within maternal and paternal identity (separately), trial start time, and track number. All predictor variables were treated as categorical variables. The same linear mixed effects model was run with thermal preference at 19 days as the response variable. For thermal preference at 33 days, we used the same linear mixed effects model with the additional fixed effects of late-rearing temperature and the 3-way interaction among maternal temperature, incubation temperature, and late-rearing temperature.

To distinguish if thermal preferences were different between the 8 treatment groups, we used a one-way ANOVA with the fixed effect of treatment group on thermal preference with random effects of clutch ID nested within maternal and paternal identity (separately), trial start time, and track number. We then performed a post-hoc Tukey test to compare between treatment groups. We also tested for pairwise interactions and obtained estimated marginal means (r package emmeans) for our 33-day linear mixed models to contrast mean thermal preference within a treatment group between other temperature histories.

Any animal that spent more than 4 measurements below 8.5°C or above 26°C was considered stuck and was removed from the data set because are no longer able to thermoregulate properly. We removed 32 extremely low and 22 extremely high outliers where animals got stuck in a thermal extreme and were unable to continue thermoregulation.

To select the most appropriate model, we first estimated a full model

including all fixed effects and compared it with an intercept-only model to test for overall model significance (Zuur et al. 2009). If the full model was significantly different from the intercept-only model, model selection was initiated. We used backward, stepwise model selection based on a series of *F* tests to determine the order of testing for fixed effects (Zuur et al. 2009). Significance of the fixed effect was assessed by a Likelihood Ratio Test using the *anova* function in R, by sequentially removing the variable of interest and comparing with the previous model, which included the effect (Zuur et al. 2009). Non-significant effects were sequentially removed and significant effects were retained in the final model (Zuur et al. 2009).

Animal Welfare

All protocols involving live animals were approved by the University of California, Santa Cruz Institutional Animal Care and Use Committee (IACUC, Office Code: Sineb2108).

Results

Thermal Preference at 5 days

Larvae had a range of thermal preferences from 11.67 to 25.11°C at 5 days old. Incubation temperature was positively associated with offspring thermal preference (Table 2.1, Figure 2.2, with animals incubated at 18 having an average thermal preference of 18.1°C and those hatched in 21°C an 18.9°C thermal preference). Maternal temperature did not have a statistically significant effect on

offspring thermal preference at 5 days old, and the interaction term between maternal and incubation temperature also was not significant (Table 2.1).

Thermal Preference at 19 days

We found no evidence that maternal temperature, incubation temperature, or their interaction explains variation in offspring thermal preference at 19 days: The full linear mixed model, which included maternal temperature, incubation temperature, and their interaction on offspring thermal preference at 19 days was not significantly different from the intercept-only model, so we did not initiate model selection (χ^2 =6.7064, df =3, p = .08187).

Thermal Preference at 33 days

At 33 days, axolotl larvae thermal preferences ranged from 12.13 to 24.17 °C. Late-rearing temperature was positively associated with offspring thermal preference (Table 2.2, Figure 2.3); animals experiencing a rearing temperature of 18°C had a significantly cooler thermal preference than larvae reared at 21°C. Maternal temperature and incubation temperature, individually, were not statistically significant main effects of variation in offspring thermal preference at 33 days old, and neither were any of the two-way interaction terms between maternal and incubation temperature, maternal and rearing temperature, or incubation and rearing temperature (Table 2.2). However, the three-way interaction among maternal, incubation and rearing temperature was significant (Table 2.1, 2.2), meaning that the

thermal preference of larvae at 33 days was positively and significantly affected by the combined effect of these three temperature experiences. This suggests evidence of intergenerational plasticity, because maternal temperature interacts with the within-generational plasticity of incubation temperature and late-rearing temperature.

While there were four treatment groups at the 19-day measurement, our experiment involved division of these four groups between two late-rearing temperatures to test for WGP, which created a total of eight treatment groups during this final phase of the experiment (Figure 2.1b, Figure 2.4). Animals that were moved to a late-rearing temperature of 21°C (no matter their previous thermal history) had significantly higher thermal preferences, and animals moved to a late-rearing temperature of 18°C had significantly lower thermal preferences (Figure 2.4). The greatest change in thermal preference happened from larvae who were already living in 21°C and then secondary rearing temperature was also 21°C, suggesting either a cumulative effect or that age of measurement collection matters. On the same note, the largest decrease in thermal preference came from animals whose previous thermal history was all at 18°C and were kept at 18°C, dropping almost an entire degree.

The observed three-way interaction between maternal, incubation, and new rearing temperature suggests the potential for cumulative additive effects of temperature: Larvae experiencing the same temperature for all exposure periods (e.g. maternal, incubation and late-rearing) have the most distinct thermal preference, with larvae having incubation and rearing temperature opposite of their maternal temperature being more intermediate, and offspring with other temperature

combination having a median thermal preference (Figure 2.5). Larvae raised completely at 18°C (maternal, incubation, and rearing temperatures) had the lowest average thermal preferences and larvae raised completely at 21°C had the highest thermal preference (Figure 2.5). Animals from a full 18°C history had an average thermal preference of 17.4°C, almost 2°C lower than their 21°C counterparts with a thermal preference average of 19.3°C (Figure 2.6). In our one-way ANOVA followed by Tukey tests, we found 3 sets of treatment groups that were statistically different (lines, Figure 2.5): the larvae with full 18 °C histories to full 21°C histories, one with maternal and incubation temperature at 18°C then late-rearing at 18°C compared to 21°C, and one with maternal and incubation temperature at 21°C then late-rearing at 18°C compared to 21°C.

It was also useful to explore these cumulative effects of temperature, by considering the predicted two-way interaction between exposure periods on larval thermal preferences (Table 2.3). We saw a similar trend of late-rearing temperature having the highest effect on offspring thermal preference, specifically in groups where maternal and incubation temperature were 18°C, maternal and incubation temperature were 21°C, and maternal temperature was 18°C with incubation temperature 21°C. We can visualize these patterns with the pairwise interaction of estimated marginal means: A warmer late-rearing temperature always raised thermal preference and the most dramatic increase was for larvae that previously only experienced 18°C (Figure 2.7). We also see the least difference in offspring's' final

thermal preference between treatments when incubation temperature was different from maternal and late-rearing temperature.

Discussion

Rising temperatures will have unprecedented and complicated consequences, particularly for ectotherms. There are limited studies on IGP in general, let alone IGP for vertebrate thermal traits. Here, we present the first study to experimentally manipulate temperature to measure intergenerational effects on offspring thermal preference. In addition, we simultaneously examined within and between-generation temperature effects and their potential interactions. We show that incubation temperature had a significant effect on larval thermal preference 5 days after hatching, but that late-rearing temperature had a greater effect on thermal preferences of later-stage larvae. We also showed a positive cumulative interaction between maternal, incubation, and rearing temperature, representing a combination of IGP and WGP. Our results suggest evidence of intergenerational plasticity because maternal effects, through maternal temperature, interacts with the within-generational plasticity of incubation temperature and late-rearing temperature. There is growing evidence that IGP might be an essential response organisms have to escape climate change, short of evolutionary change in thermal traits (Davis et al. 2005). Here we demonstrate that IGP and WGP can have cumulative effects that could help organisms respond to increasing temperatures when they can no longer migrate or alter behavior. With the potential mechanism of maternal effects, we show

acclimation can occur in short time periods with significant changes on offspring physiology. Our results support that amphibians do have the potential for thermal IGP, as well as within-generation plasticity, and their combination is a realistic coping mechanism to thermal stress.

Thermal plasticity has the potential to ameliorate the impact of climate change if a species exhibits positive acclimation in response to warming (Sinervo et al. 2018), or exacerbate the impacts of climate change if a species exhibits inverse acclimation (Tsuji 1988; Wang et al. 2013; Bestion et al. Submitted). We chose to measure thermal preference because it is a critical component of ecophysiology (Gvoždík 2011; Chown 2012), but it is also the best signal for how an animal will persist over climate change. We can compare an organisms' thermal preference to the environmental temperature it experiences to predict extinction risk (Sinervo et al. 2010; Huey et al. 2012). Currently, most extinction risk models consider the thermal physiology of a species as static, even if there is potential for acclimation. Few models include hypothetical or review-based parameters for plasticity and adaptive potential (Sinervo et al. 2010), and fewer still include empirical acclimation data, let alone IGP (Reed et al. 2011; Rohr et al. 2018). However, a study modeling a hypothetical maternal effect increasing offspring thermal preference by +1°C shows that this can rescue a species and ameliorate extinction risk for 10 out of 11 species (Sinervo et al. 2018). We provide empirical evidence, that in a little over a month, a combination of thermal experiences can change an individual's thermal preference by 2°C. This could provide the buffer they need for the next 50 years, as we predict

climate will rise on average 1-3°C by 2070 (IPCC 2007; Pielke et al. 2022), and potentially eliminate most of the thermal extinction risk of axolotls for the near future. With such a large buffer provided, we must highly consider IGP as a potential mechanism of climate rescue and focus future research on other responses to thermal IGP, test effects of multiple generation, and search for other temperature interactions.

Not all organisms have the same capacity for acclimation, which is likely related to selection arising from climate variability (Rohr et al. 2018). The range of temperatures an organism experiences may determine the capacity for coping to increasing temperatures (Tewksbury et al. 2008) and organisms that live in homogenous environments (Huey et al. 2012), have low heat tolerance (Huey et al. 2009), or already live in areas close to operative temperature (Deutsch et al. 2008; Amarasekare and Savage 2012) will also have lower acclimation potential. Additionally, it is theorized that ectotherms in places of higher climate variability have greater acclimation abilities then those in low variability climates (Johnson and Kelsch 1998; Gabriel et al. 2005; Angilletta 2009). Axolotls, animals that historically live in buffered, more homogenous aquatic systems, should in theory have a low plastic response for acclimation. However, our results show that between changes of maternal, incubation, and rearing temperature larvae can have a plastic change in thermal preference by almost 2°C. Other amphibians, especially those with developmental plasticity and metamorphosis and who experience more variable thermal environments, like migrating from natal ponds to other habitat types, could have even greater plasticity of thermal traits and be even more buffered against a

warming climate. However, up to this point, we have assumed thermal plasticity has a positive effect on organismal adaptation, but we must consider the possibility of negative plasticity, or inverse acclimation. For example, lizards exposed to extremely high temperatures start to decrease metabolism (Tsuji 1988) and could prefer cooler temperatures, and we see other cases of inverse acclimation in reptiles (Feder and Pough 1975; Gvoždík et al. 2007). If animals have negative plastic responses to temperature, or the buffer of positive plasticity is maxed out by climate increases, then these organisms must revert to other methods of rescue like retreating, behavioral adjustments, and migrations, or face death. We must continue to work to understand how much of a buffer IGP, within GP, and their combination, can realistically provide organisms to understand their future extinction risk.

While there is limited thermal IGP research focusing on offspring thermal traits, research that measures thermal IGP on other non-thermal offspring traits has typically focused on extreme differences between temperatures (Walsh et al. 2014; Betini et al. 2020; Lee et al. 2020). For example, a study in minnows measured effects on offspring growth by parental thermal treatment of 24 and 34°C parental thermal treatment (Lee et al. 2020), and we see a common trend of 10-20°C differences in parental treatments. While using these extreme parental temperatures allows us to clearly see underlying trends, it does not represent realistic change. Most animals experiencing climate warming will not experience such an immediate, significant increase, but a more gradual rise in mean temperature. We show that with the exposure difference of just 3°C, mimicking the predicted climate warming in the

next 50 years, organisms can still have positive thermal acclimation on thermal physiology. Very few studies also directly address the interactive effects of parent, incubation and offspring temperature simultaneously (Salinas and Munch 2012), and instead address them individually. While, we see some individual effects of maternal or offspring temperature on physiology (Huey et al. 2012; Rohr et al. 2018), some of the studies suggesting negative or no results might be underrepresenting the potential for IGP because they are missing cumulative effects or measuring offspring traits at inappropriate time intervals. We show that focusing on a single treatment temperature or taking offspring measurements at certain ages or life stages may not show the full effect of plasticity, and it is necessary to consider multiple thermal influences and check for cumulative effects over multiple generations.

As climate warms, plasticity may provide a buffer, but organisms will need to adapt to more than temperature effects on their physiology. Rising temperature will have other critical consequences like shifts in phenology (Kissel et al. 2019), precipitation variation and hydrological changes in aquatic habitats (Bartelt et al. 2010), and consequences for population dynamics (Sæther et al. 2000). Animals will need to simultaneously adapt to these consequences of climate warming in addition to thermally acclimating. And while plasticity can benefit an organism's survival and reduce costs like lost foraging or mating opportunities (Sinervo et al. 2010), it can also have fitness costs (DeWitt et al. 1998). We must consider these costs, such as metabolic change (Dillon et al. 2010), increased disease transmission (Rohr and Raffel 2010), and decreased reproductive success from reduced sperm production or

ovarian growth (Schleicherová et al. 2014), while considering how much benefit IGP actually provides an animal. Additionally, as environmental conditions are rapidly changing, IGP could potentially dampen selection resulting from a change in environment, creating a dilemma for future generations (Donelson et al. 2016). But, if IGP could mitigate population decline by alleviating a phenotype-environment mismatch, it could ultimately buy time for evolutionary rescue (DeWitt et al., 1998; Marshall et al., 2010; Harmon and Pfennig, 2021).

Our results suggest that, with exposure to different temperatures both inter- and within-generations, larval salamanders can acclimate their thermal preference. We demonstrate that a single temperature or a single point in time may not have significant effects on physiology, but there is a complex interactive effect between maternal, incubation, and rearing temperature. We show evidence of thermal IGP, through the mechanism of maternal effects, and we can use this information to more accurately predict future population level extinctions, alter management decisions to focus on species lacking IGP rescue, and continue to broaden our understanding of the importance and complexity of IGP. We must expand our studies on IGP to understand the relationship of IGP and within generation plasticity, understand the long-term effects of IGP over generations, and the interplay of IGP and evolution.

Acknowledgements

We acknowledge the support of Alexandra Baldacci for assistance running trials and Fausto Méndez-de la Cruz for advice and input on this experimental design.

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Tables

Table 2.1 Results of building the linear mixed effects analyses showing thermal effects on offspring thermal preference at 5 and 33 days (r package lme4; Bates et al. 2015). The full model at 19 days was not significantly different from the intercept-only model, so model selection was not initiated. The table lists the fixed effects considered in the starting models. Non-significant fixed effects were removed sequentially in order from top to bottom through backward, stepwise model selection. Significance determined by a Likelihood Ratio Test. Fixed effects retained in the final models are in bold and indicated by an asterisk.

Response	Fixed Effect	χ^2	df	P value
Thermal preference, 5 days				
	Maternal x Incubation temperature	1.787	1	0.181
	Maternal temperature	0.792	1	0.374
	Incubation temperature	**11.265**	**1**	**< 0.001***
Thermal preference, 33 days				
	Maternal x Incubation x Late-rearing temperature	**4.461**	**1**	**0.035***
	All other two-way interactions and single terms kept in model			

Table 2.2 Final results for the linear mixed effects analyses showing thermal effects on offspring thermal preference at 5 and 33 days (r package lme4; Bates et al. 2015). The full model at 19 days was not significantly different from the intercept-only model, so model selection was not initiated. The table lists all fixed effects in the final model. Significance determined by a Likelihood Ratio Test. Fixed effects that were significant are in bold and indicated by an asterisk.

Response	Fixed Effect	χ^2	df	P value
Thermal preference, 5 days				
	Incubation temperature	**13.281**	**1**	**< 0.001***
Thermal preference, 33 days				
	Maternal x Incubation x Late-rearing temperature	**6.328**	**1**	**0.012***
	Maternal x Incubation temperature	0.236	1	0.627
	Maternal x Late-rearing temperature	2.505	1	0.113
	Late-rearing x Incubation temperature	0.002	1	0.964
	Maternal temperature	1.258	1	0.262
	Incubation temperature	2.192	1	0.139
	Late-rearing temperature	**35.375**	**1**	**<0.001***

Table 2.3 Model estimated marginal means for the three-way interaction of mother, incubation, and rearing temperature at 33 days (r package emmeans). We contrast mean thermal preference within a treatment group between other temperature histories. Treatments that are significantly different in models are in bold.

Simple Contrasts	Contrast Estimate	SE	df	T ratio	p value
Maternal temperature					
Incubation 18 Late-rearing 18	-0.74	0.513	8.6	-1.442	0.185
Incubation 21 Late-rearing 18	-0.476	0.498	8.96	-0.955	0.364
Incubation 18 Late-rearing 21	0.275	0.501	8.27	0.548	0.598
Incubation 21 Late-rearing 21	-0.66	0.494	8.63	-1.336	0.216
Incubation temperature					
Maternal 18 Late-rearing 18	-0.578	0.489	25.2	-1.181	0.249
Maternal 21 Late-rearing 18	-0.314	0.471	25	-0.666	0.512
Maternal 18 Late-rearing 21	0.016	0.49	25.1	0.033	0.974
Maternal 21 Late-rearing 21	-0.919	0.469	24.7	-1.957	0.062
Late-rearing temperature					
Maternal 18 Incubation 18	-1.246	0.247	599	-5.041	**<.001**
Maternal 21 Incubation 18	-0.231	0.246	417	-0.939	0.348
Maternal 18 Incubation 21	-0.652	0.249	817	-2.623	**0.009**
Maternal 21 Incubation 21	-0.836	0.231	512	-3.615	**<0.001**

Figures

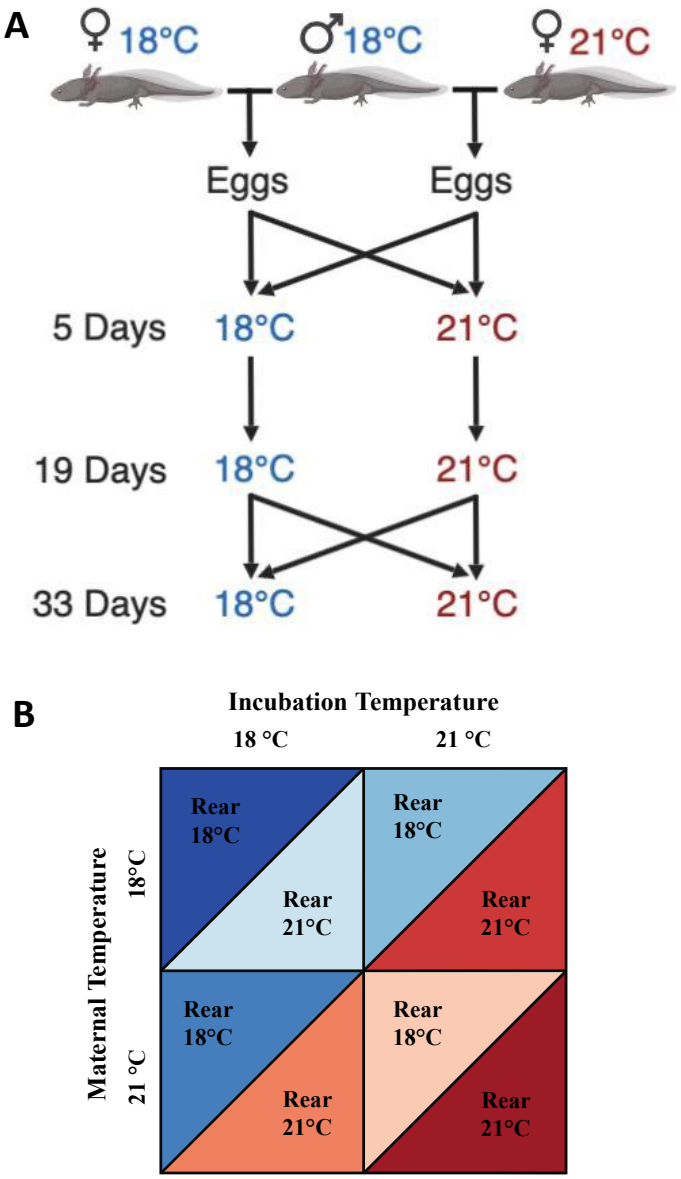

Figure 2.1 A) In our experimental design, males from 18°C were randomly paired with females from 18°C and then paired with females from 21°C treatments in large breeding tanks. After a female completed laying eggs, the eggs were divided between two incubation temperature treatments (18°C and 21°C). We then measured larval thermal preference at 5 days and 19 days old. Next, these offspring were split between two late-rearing temperature treatments (18°C and 21°C). Finally, after two more weeks, we measured thermal preference at 33 days old. B) Experimental diagram of larval treatments. Mothers were exposed to 18°C or 21°C, eggs were incubated at 18°C or 21°C, and rearing temperature was switched at 19 days to 18°C or 21°C to create a full-factorial, split-clutch design. This creates a total of 8 final treatment groups.

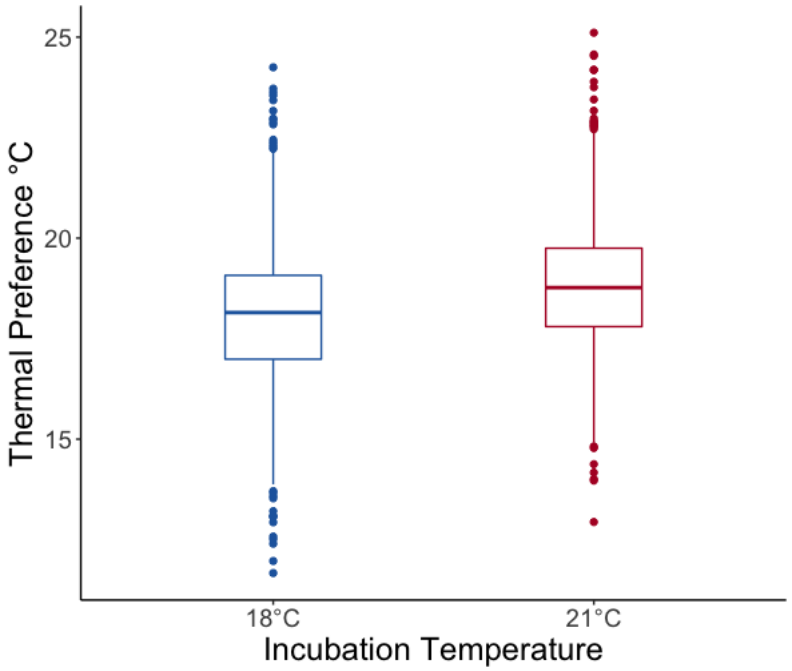

Figure 2.2 Egg incubation temperature significantly affects offspring thermal preference at 5 days old (χ^2 =13.281, df =1, p= 0.0002681). Offspring incubated at 18°C (blue plot) had a significantly lower mean thermal preference than offspring incubated at 21°C (red plot). For each box plot, the line represents the mean thermal preference, the upper and lower edges of the box represent the lower and upper quartile, the lines above and below the box represent the maximum and minimum values, and the individual points represent outliers.

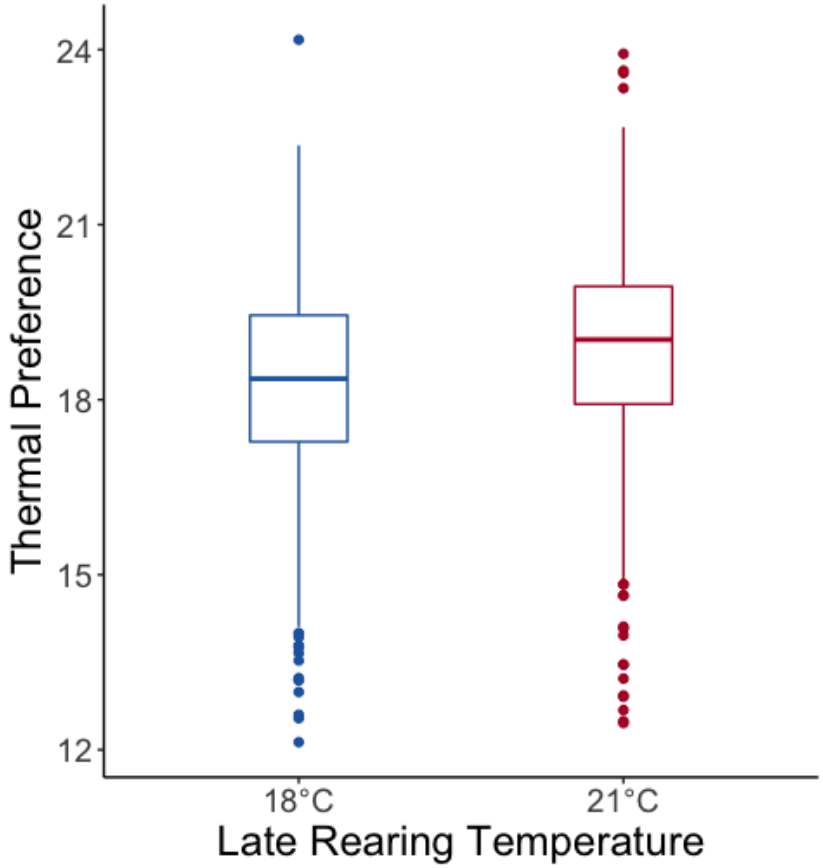

Figure 2.3 Late-rearing temperature significantly affects offspring thermal preference at 33 days old (χ^2 =35.3754, df =1, p=2.719e-09). Offspring recently reared at 18°C (blue plot) had a significantly lower thermal preference than offspring recently reared at 21°C (red plot). For each box plot, the line represents the mean thermal preference, the upper and lower edges of the box represent the lower and upper quartile, the lines above and below the box represent the maximum and minimum values, and the individual points represent outliers.

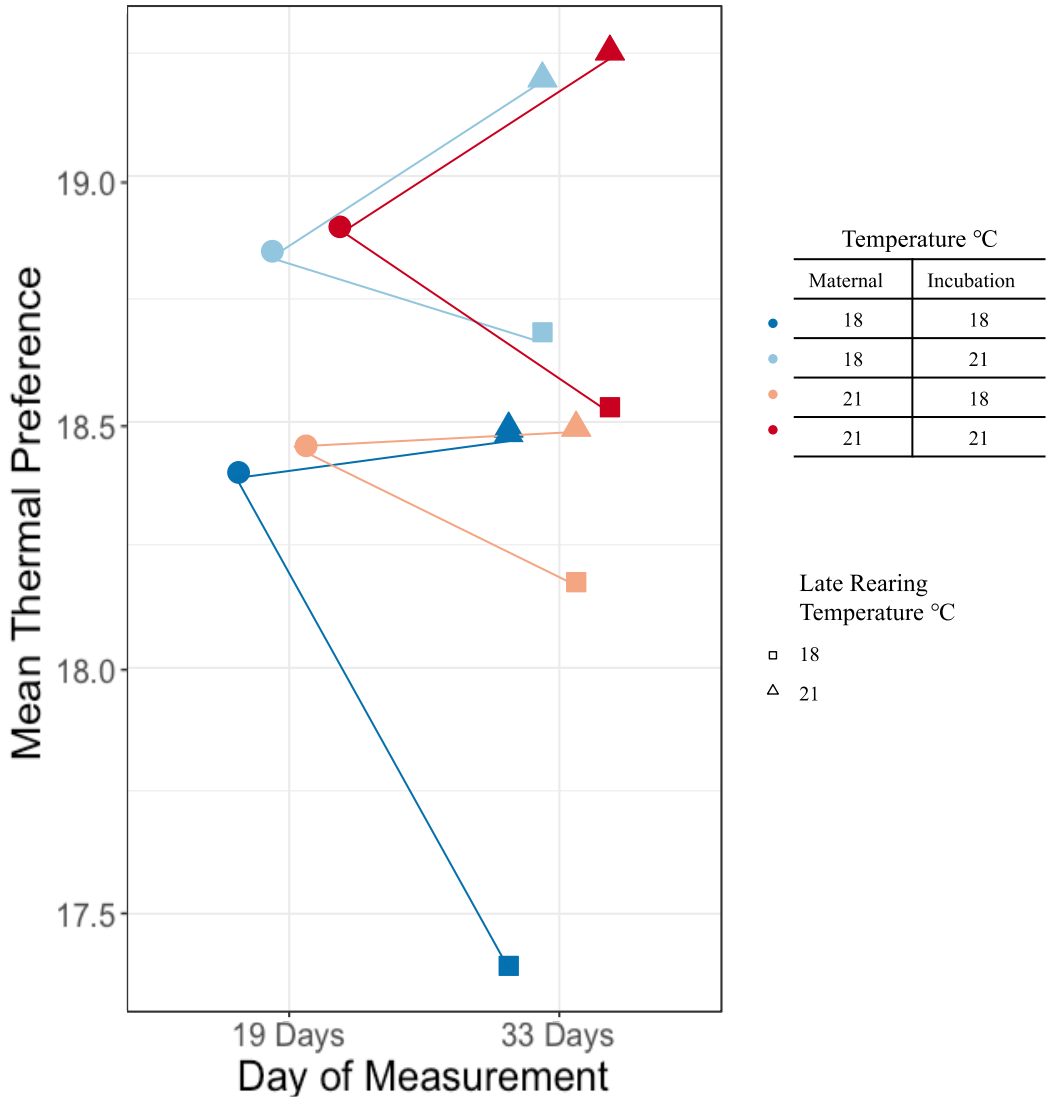

Figure 2.4 Mean thermal preference changes from 19 to 33 days, after animals are moved to late-rearing temperatures. Animals that were moved to a late-rearing temperature of 21°C (no matter their previous thermal history) tended to have higher thermal preferences than animals moved to a late-rearing temperature of 18°C. The greatest increase in thermal preference from 19 to 33 days happened for larvae whose incubation and late-rearing temperatures were both 21°C. The largest decrease in thermal preference from 19 to 33 days happened for animals whose incubation and late-rearing temperatures were both 18°C. Color represents the thermal history of the animal at 19 days (maternal and incubation temperature), and shape represents the late-rearing temperature that the animals were transferred into (triangle = 21°C, square =18°C).

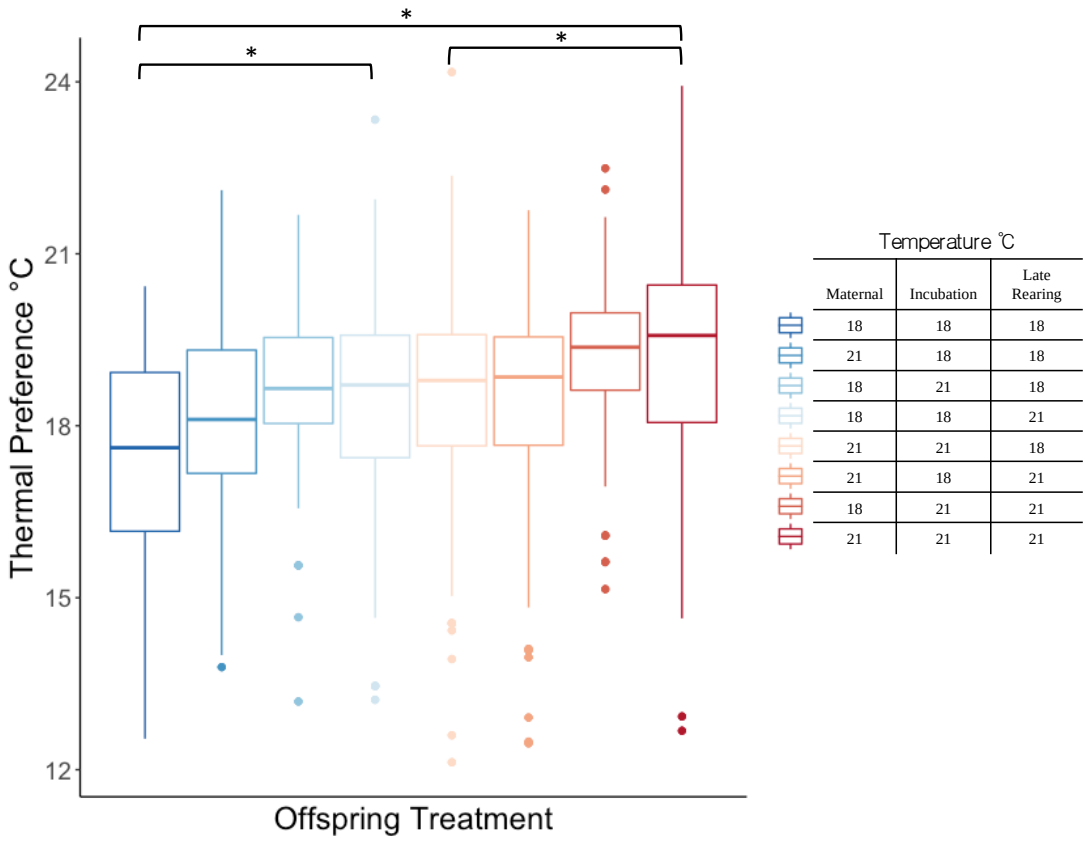

Figure 2.5 This figure shows the thermal preference for the eight possible thermal histories that larvae could have experienced at 33 days, representing the three-way interaction of maternal temperature, incubation temperature, and late-rearing temperature. Color represents an increasing thermal preference (darker blue=colder, and deeper red =warmer). Brackets represent treatment groups that have significantly different thermal preferences. Larvae raised completely at 18°C (maternal, incubation, and rearing temperatures) had the lowest thermal preferences (dark blue), and larvae raised completely at 21°C had the highest thermal preference (dark red). For each box plot, the line represents the mean thermal preference, the upper and lower edges of the box represent the lower and upper quartile, the lines above and below the box represent the maximum and minimum values, and the individual points represent outliers.

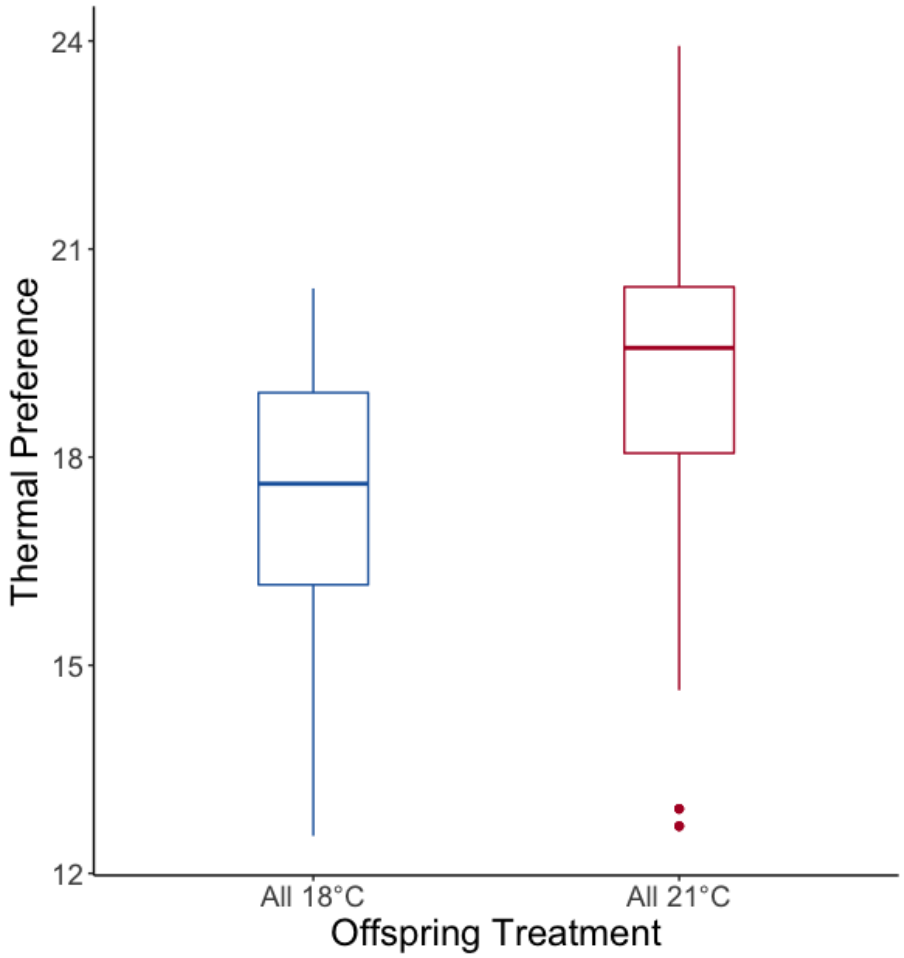

Figure 2.6 The two extreme combinations of rearing histories differ significantly in thermal preference at 33 days. Larvae raised from 18°C mothers, had 18°C incubation temperatures, and were reared their whole lives at 18°C (blue plot) have a significantly lower thermal preference then offspring with 21°C maternal, incubation, and rearing temperatures (red plot). Animals from 18°C have an average thermal preference of 17.4°C, almost 2°C lower than their 21°C counterparts with a thermal preference of 19.3°C. For each box plot, the line represents the mean thermal preference, the upper and lower edges of the box represent the lower and upper quartile, the lines above and below the box represent the maximum and minimum values, and the individual points represent outliers.

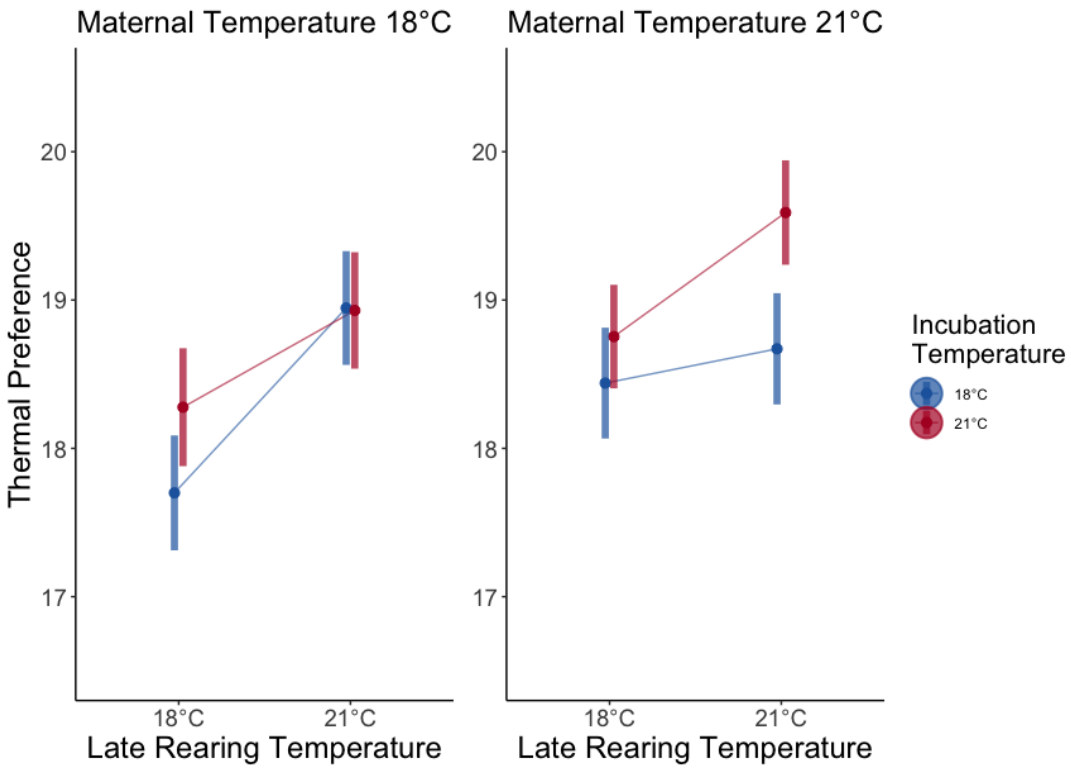

Figure 2.7 This graph represents model predictions of marginal means for final thermal preference (33 days post-hatching) estimated at 18 °C and 21 °C maternal temperatures and the pairwise interactions between incubation and late-rearing temperature (r package emmeans). A warmer late-rearing temperature always raised offspring thermal preference and the most dramatic increase was for larvae that previously only experienced 18°C maternal and incubation temperatures. We also see the least difference in final thermal preference between treatments when incubation temperature was different from maternal and late-rearing temperature. Each point represents the mean thermal preference from the model predictions and the line above and below represent the standard error.

Chapter 3: No evidence of outbreeding depression in admixture propagation before assisted gene flow in an endangered, endemic amphibian

Abstract

Small populations, like endemic species or isolated metapopulations, have a high risk of losing genetic diversity and are more susceptible to demographic and environmental stochasticity. This combination can interact and reinforce each other in a downward spiral, causing an extinction vortex. One current species facing such a vortex is *Ambystoma macrodactylum croceum* (Santa Cruz long-toed salamander; hereafter SCLTS), an endangered, highly endemic subspecies. Populations are continuing to decline, and low genetic diversity and inbreeding depression have been identified as major threats to species persistence. We created a human assisted gene flow plan to make SCLTS more resilient to climate change by increasing genetic diversity and adaptive potential, but first we needed to test for outbreeding depression between populations. To do this, we proposed an admixture captive breeding program between genetically distinct populations. We crossed low genetic diversity populations with higher diversity populations and tested for differences between the crosses compared to pure genetic lines. We test for outbreeding depression in survival and physiology traits that are proxies for organism fitness. We found no difference in numbers of failed eggs, infertile eggs, or larval death, which provides no evidence for developmental differences between pure populations and crosses. Additionally, we found no difference in growth rates or thermal preference. No significant differences

between pure lines and genetic crosses in any traits suggests there was no outbreeding depression between populations. Given these results, we moved forward with the human assisted gene flow plan and all larvae were released into struggling populations to increase genetic diversity and hopefully therefore boost resiliency to climate change. This novel approach will become a proactive genetic restoration method for SCLTS, and potentially other isolated, endemic species. This will potentially stop the extinction vortex and contribute to population viability, both immediately and under future climate changes.

Keywords: *Ambystoma macrodactylum croceum*, genetic rescue, translocations, admixture breeding, outbreeding depression

Introduction

Species are currently facing extinction rates exceeding any pre-anthropogenic estimates (Barnosky et al. 2011). While the crisis is seen across taxa and habitats (Sinervo et al. 2010; Brauer and Beheregaray 2020; Nic Lughadha et al. 2020; Munstermann et al. 2022), small, isolated species are particularly vulnerable (Lande 1988; Frankham et al. 2002). In addition to being more susceptible to demographic and environmental stochasticity, they also face more genetic threats. This combination can interact and reinforce each other in a downward spiral, called an extinction vortex (Gilpin and Soule, ME 1986; Fagan and Holmes 2006). Extinction vortexes occur when small populations have low genetic diversity, which in turn prevents populations from recovering from disturbance, further shrinking the population size, and ultimately cycling downwards into extinction (Blomqvist et al. 2010; Palomares et al. 2012).

These small populations, like endemic species or isolated metapopulations, typically have low genetic diversity initially caused by fragmentation of natural habitats (Templeton et al. 1990) or population bottlenecks from stochastic events (Bouzat 2010). These genetic consequences are further exacerbated when populations remain separated. After populations split or shrink, there are restricted mating opportunities that can lead to inbreeding depression—the reduction of offspring fitness caused by matings between related individuals and an increase in recessive deleterious alleles (Charlesworth and Charlesworth 1987). Second, populations that remain isolated over many generations continue to lose genetic variation, due to the

random fixation or loss of alleles through genetic drift (Ezard and Travis 2006). Yet, this genetic variation is necessary to respond evolutionarily to environmental stressors (Frankham et al. 2002; Willi et al. 2006). Lastly, unfavorable mutations are more easily accumulated because selection operates less efficiently in smaller populations (Frankham et al. 2002). With continued shrinking genetic diversity, fitness traits decline, there is a decreased chance of evolutionary rescue, and an increased chance of extinction risk (Spielman et al. 2004). This situation is further threatened by climate change. Populations facing climate change stressors, such as rising temperatures and shortened hydroperiods, will need to have the genetic variation required to adapt to volatile and rapidly changing conditions (Pauls et al. 2013).

In populations with low genetic diversity, we see less variation in phenotypes, complete loss of some alleles or genotypes, and increased deleterious alleles (Hughes et al. 2008). While all traits can be affected, we can most immediately see effects on survival traits and changes in physiology. Specifically, we see negative effects on individuals' performance that reduces their survival, reproduction, and resistance to environmental stress (Keller 2002; Reed and Frankham 2003). For example, a loss of genetic diversity can negatively affect individual fitness through decreased sperm quality (Hinkson and Poo 2020), reduced litter size (Hedrick and Fredrickson 2010), increased juvenile mortality (Ralls et al. 1988; Pini et al. 2011), and increased susceptibility to disease and parasites (Coltman et al. 1999). These consequences can magnify and have population level consequences, such as lower effective population sizes (Newman and Pilson 1997), altered mating systems (Olsson and Madsen 2001;

Blomqvist et al. 2002), and decreased resistance to and quicker transmission of disease (Anderson et al. 1986; King and Lively 2012). Decreasing genetic diversity can also lead to larger ecological consequences (Hughes et al. 2008); there can be effects on species interactions (Turkington and Harper 1979), alteration of community dynamics (Birch 1960; Pimentel 1968), and even the stability and function of the ecosystem (May 2001; Hooper et al. 2005). With these compounding issues, we must find ways to conserve genetic diversity in threatened populations before it is irreversible and they vortex into extinction.

Scientists have developed many methods to conserve species facing extreme declines. First, we typically work to stabilize populations; for example, by protecting critical habitat, removing pollutants and invasive plants, and creating legislature or listing to safeguard species. While this is an important step, once a population loses genetic variation, even if the population size rebounds, genetic variation will not recover quickly (Westemeier et al. 1998). Genetic variation will only be restored slowly though the accumulation of random mutations over generation (Gregory 1965), leaving endangered species at risk for extinction long after population size has recovered. Instead, we must directly address genetic restoration in our conservation plan, actively conserving and increasing genetic diversity (Hedrick 2004; Tallmon et al. 2004). While this is still uncommon, plans that do address low genetic diversity usually have methods to increase natural gene flow between isolated populations—land bridges, tunnels under roads, or purchasing land to create habitat conductivity (Teixeira et al. 2013; Soanes et al. 2018). These methods could increase genetic

diversity, but it assumes species are able to migrate long distances and that population numbers are large enough for self-rescue. Many species already in an extinction vortex do not meet these criteria and need additional support.

We can directly increase genetic diversity with human assisted gene flow, such as translocations of animals from healthy populations to struggling populations, which simultaneously boosts population size and brings new genes into the population (Aitken and Whitlock 2013). We have seen this method strengthen populations (Pimm et al. 2006), remove detrimental variation from inbreeding (Westemeier et al. 1998; Madsen et al. 1999; Hogg et al. 2006), and restore genetic diversity to historical levels (Bouzat et al. 2009). Even with promising results, many criticize this method over concerns of unknown consequences, like spreading disease or genetic swamping of locally adapted populations, eliminating genetic distinctiveness (Clewell 2000). Specifically, there could be a potential for outbreeding depression (Hufford and Mazer 2003; Edmands 2007), which occurs when breeding between genetically distant groups results in a reduction of fitness (Edmands 2007). For example, we see lower survival and disruption of local adaptation in plants (Waser et al. 2000; Montalvo and Ellstrand 2001) and reduced reproduction in nematodes (Gimond et al. 2013) when genetically distant populations breed. With direct translocation, we are unable to test for outbreeding effects until after we relocate animals, leaving the potential for irreversible genetic damage to a population. Not only is there immediate harm to the population, but this also speeds up the extinction vortex. Even though overall evidence for outbreeding depression is still

rare (Frankham et al. 2011; Whitlock et al. 2013) and the benefits of outbreeding can outweigh the risks for populations at the brink of extinction with no adaptive potential to face climate change, we can reduce these potential threats. Before we use human assisted gene flow in the wild, we can use admixture captive breeding to test if there is outbreeding depression between populations.

Amybstoma macrodactylum croceum, or the Santa Cruz Long-toed Salamander (SCLTS), is a model system to test this strategy in because it is highly endemic with isolated populations. USFWS and collaborators also monitor the entire species which allows us to track and alter genetic variation in every single breeding population, something impossible for many other species. SCLTS meets the criteria of being in an extinction vortex: small populations, loss of genetic diversity, and no ability for self-rescue. Habitat fragmentation has led to 6 genetically isolated metapopulations in SCLTS, some healthy and robust, and some that are facing population extirpations. In declining metapopulations, low genetic diversity and inbreeding depression were identified as the major threats to persistence (U.S. Fish and Wildlife Service 2009, 2019). It is critical that existing, declining populations are bolstered with genetically diverse individuals. This not only increases population numbers, but also to increases genetic representation and resiliency to withstand stochastic events such as droughts and wildfires, which are becoming more common with climate change. To accomplish this, we proposed a genetic restoration plan that creates human assisted gene flow between populations with low and high genetic diversity. Before we enact this plan and start translocations, we must first test for

outbreeding depression between populations through an admixture captive breeding program. Therefore, we crossed low genetic diversity Monterey County populations with higher diversity Santa Cruz County populations and tested for outbreeding depression between the crosses compared to pure genetic lines. We tested for outbreeding depression in survival traits that are proxies for organism fitness: number of failed eggs, infertile eggs, and larval death, as well as growth rate and thermal physiology. If there is outbreeding depression, we predict that crosses would have inferior survival traits, growth rates, and thermal preference compared to pure genetic lines. If there is no evidence of outbreeding depression, we predict that populations would have no significance difference between traits, or potentially crosses would have increased variation in traits compared to pure lines due to increased genetic diversity and reduced inbreeding depression. No evidence of outbreeding depression allows us to move forward with our genetic restoration plan to translocate larvae to struggling populations to artificially boost genetic diversity and increase resiliency to climate change.

Materials and Methods

Study Species History

Ambystoma macrodactylum croceum (Santa Cruz long-toed salamander; hereafter SCLTS) is an endemic salamander from coastal California, limited to southern Santa Cruz and northern Monterey counties, with a range that is roughly 16 miles long by 5 miles wide. Approximately 30 confirmed breeding ponds were

identified when the species was first detected in 1954, but only 16 of which have known or assumed breeding as of 2019 (Figure 3.1; U.S. Fish and Wildlife Service 2019). With one of the smallest ranges of any ESA listed species, this subspecies is currently federally endangered, and state fully protected (U.S. Fish and Wildlife Service 1999). Destruction of habitat, from freeway, housing, and agriculture development, has resulted in the highly fragmented nature of suitable lands within the species range. This fragmentation has led to 6 metapopulations that no longer interbreed (Figure 3.1).

With this fragmentation SCLTS populations have recently undergone significant bottlenecks (U.S. Fish and Wildlife Service 2009) and are at extreme risk of inbreeding depression, low genetic diversity, and future bottlenecks. The little genetic research that has been conducted on the SCLTS suggests that restricted gene flow has resulted in the genetic isolation of each metapopulation, with some metapopulations experiencing greater isolation than others (Savage 2008, 2009). High genetic distances, indicating a lack of gene flow, is prominent between metapopulations that are bisected by Highway 1 and between the Santa Cruz and Monterey County sub-populations. Over the past two decades, several recovery activities have been implemented in Santa Cruz County, resulting in the stabilization and bolstering of Santa Cruz County metapopulations (U.S. Fish and Wildlife Service 2009, 2019). Scientists have monitored breeding adults, improved ponds and upland habitat, created management plans, acquired land, built new ponds, surveyed potential habitat, cleared invasive plants, developed conservation agreements with homeowners

and farmers, built tunnels, tracked egg and larval development, created salamander protection zones, measured pond characteristics, and much more. However, over the same timeframe, SCLTS populations in Monterey County have steadily decreased and local extirpations continue to occur. Monterey populations are at the southern end of the range and have been experiencing a recent history of extreme weather, including a three-year drought and head waves that led to very low reproduction in the wild. Currently, there are only five known functional breeding sites within Monterey County, and, based on annual aquatic surveys at all previously occupied sites over the last decade, it is believed that very low numbers of breeding adults remain throughout Monterey County.

As Monterey County populations continue to decline, low genetic diversity and inbreeding depression are the major threats to SCLTS persistence (U.S. Fish and Wildlife Service 2019; Ventura Fish and Wildlife Office and California Department of Fish and Wildlife 2019). These populations are less capable of enduring stochastic events, are extremely vulnerable to extirpation (Allentoft and O'Brien 2010), and will not recover without additional gene flow from translocations. This is because likelihood of natural recolonization of sites is extremely low due to habitat fragmentation and, as a result these, populations will, and some even have, gone extinct. With the risk of extinction being much greater than any risk of outbreeding depression, USFWS and collaborators agreed to artificially increase genetic diversity through captive admixture propagation to make populations more robust to climate change. This desperate need for artificial gene flow, in combination with a small

range and intensive regulation that allows us to monitor every population and track all translocations, makes SCLTS an ideal model system to implement genetic restoration.

Adult Collection

During the rainy season in late 2020-early 2021, USFWS and environmental consultants set up pitfall traps at three SCLTS breeding ponds. When overnight rain was predicted with over 40% chance of rain, pitfall traps were opened in the evening and checked the following morning. Any reproductive animals (males with swollen vents and gravid females) were collected and transported to UC Santa Cruz (Figure 3.2).

Adults were collected from Calabassas Pond, Upper Cattail Pond, and McClusky Slough (Figure 3.1). We chose Calabassas Pond as the source population for Santa Cruz County, as it is one of the largest, healthiest breeding populations (U.S. Fish and Wildlife Service 2009) and genetic studies indicate that it is in the sole remaining viable breeding complex in the Northern metapopulations (Savage personal observation 2014). We chose Upper Cattail and McClusky ponds, found in two different metapopulations, as the source populations for Monterey County genes. McClusky is a highly understudied breeding population and the only viable population in its metapopulation. As USFWS is losing land access to it, we chose McClusky adults to ‘save’ their unique genetic line and breed it back into other populations. We chose Upper Cattail because it was a historically successful

population with ideal adult upland habitat; however, it now faces extreme decline with only a few adults observed the last few years.

Breeding Set-up

Breeding tanks were situated outdoors, adjacent to the Coastal Science Campus at UCSC which has weather like the source populations (e.g., coastal fog once spring ends). We monitored tank temperatures with aquatic data loggers to ensure temperatures were similar to conditions found at natural ponds with data from loggers that were placed in Calabassas Pond in 2019 and supplemented with shade cloth as needed.

We created breeding tanks from 300-gallon cattle ponds and built wood-framed lids with construction mesh to allow for natural air and insect flow (Figure 3.3a). We filled tanks with 175 gallons of water, let water dechlorinate by off-gassing for 5 days and tested with chlorine strips. After, water was treated with a 5% Holtfreter's solution (suitable for *A. m. sigillatum* larvae, Spranger personal observation). Tanks were filled with a layer of mud, grassy plants, and sticks from Calabassas ponds to seed the pond with native invertebrates and provide natural material for females to lay eggs on (Figure 3.3b). We placed artificial stones to create a platform for adults to rest, if needed, and they also had a lip around the edge of the tank to rest. All seeded tanks rested for a minimum of 4 weeks to allow zooplankton colonies to establish before adults were introduced. Throughout the experiment, we measured water conditions, oxygen levels, and temperature twice weekly to ensure

appropriate conditions. Toward the end of spring, as evaporation increased and rain frequency decreased, we added an additional 15-20 gallons of water to each tank a week.

Breeding Design

We created pure Monterey lines, pure Santa Cruz lines, and admixture crosses of Monterey by Santa Cruz by keeping adults in mixed sex groups and allowing them to mate freely. With this design, we could test if there are negative outbreeding effects of crossing the different populations by comparing admixture crosses to pure lines. With limited rain received that year, we brought in 29 females and 35 males: 5 adults from Upper Cattail, 15 from McClusky, and 44 from Calabassas. We had a final pairing design of 2 pure Santa Cruz (Calabassas x Calabassas), 2 pure Monterey (McClusky x McClusky), and 6 crosses (4 Calabassas x McClusky and 2 Calabassas x Upper Cattail). We were unable to create a pure Upper Cattail line because only 1 female was caught at the population.

Breeding in the lab is most productive in other Ambystomatid salamanders with 2-3 females and 6-8 males per laboratory pond (Shaffer personal observation), because female choice is necessary for successful breeding (Spranger personal observation). We followed this design, but with our limited number of adults, and housed 1-4 females with 2-5 males and allowed them to breed freely.

Eggs and Survival Traits

Once eggs were laid, adult animals were removed and released to original capture locations and eggs were left undisturbed until hatching (usually 3-4 weeks). We were unable to count total eggs laid per tank because females laid in clumps and within plants, which made visual counting inaccurate. We did not separate the egg clumps and plants because we did not want to disturb the eggs during development. Approximately 2 weeks post lay date and after eggs started to develop, we visually inspected the embryos and counted numbers of infertile and failed eggs. Infertile eggs were defined as eggs that never started any developmental stages. Failed eggs were eggs that were fertilized and started to develop but were aborted. After larvae hatched, we recounted numbers of infertile and failed eggs, as we were then able to disturb hatched egg clumps and plants.

Larval Rearing and Growth Measurements

When larvae hatched, they fed off the seeded organisms (see above) in the tanks for approximately 4 weeks. As zooplankton colonies were depleted, we supplemented feedings with frozen bloodworms each day. We also noticed that some tubs had a higher density of larvae than others (from more mothers successfully laying in those tubs, Table 3.1). To adjust for this, we started to supplement extra food in April, and we split larvae within the densest tanks into two tanks in April-early May.

We checked for dead larvae 5 days a week every week to track number of larval deaths. We also visually inspected larvae for developmental abnormalities in morphology, such as malformations in head shape, limbs, spine, etc. After hatching, we measured the length of 15 larvae per tub each week to calculate growth rate. Larvae were collected and placed in a shallow bucket with a ruler. We placed the camera 0.5 meters above the bucket and took a photo. We analyzed the photo in Image J and used the ruler to calibrate length. We measured the total body length of each larva.

Thermal Preference

We chose to measure thermal preference —the temperature an organism chooses when allowed to freely thermoregulate—because it is a critical component of ecophysiology (Gvoždík 2011; Chown 2012). Thermal preference can also be used to accurately predict if an organism will persist under climate change by comparing how close thermal preference temperature is to its experienced environmental temperature (Sinervo et al. 2010; Huey et al. 2012).

We conducted all thermal preference trials between 09:00 and 14:00, the normal activity period of SCLTS (N=18 larvae/tub). We designed an aquatic thermal gradient with a 2-meter inner steel track where the animal could freely swim. This was temperature controlled by being placed in an external plastic or wood track that was filled with water and cooled with ice on one end and heated with an aquarium heater on the other (Lillywhite 1971; Hill et al. 1975; Hutchison and Hill 1976). This

created a consistent thermal gradient of 5 to 30°C (+/- 2 °C) and we created a total of 18 tracks. We cooled the larvae in individual cups for sixty minutes prior to the thermal preference trial at 5-8°C to encourage thermoregulation, and then we placed the larvae in the thermal preference track for a period of two hours. During the two-hour period, we measured the larvae's chosen temperature with an omega thermocouple every ten minutes. Because of their small body size, larvae have little thermal resistivity and are typically the same temperature as their substrate (Brattstrom 1963; Spotila 1972; Spranger personal observation), so we assumed that the water temperature near the center of a larva's abdomen was a reliable proxy measurement for larval body temperature. We calculated thermal preference by averaging these values, after removing the first two measurements of every trial to account for the larvae initially exploring the track before starting thermoregulation.

Statistics

Survival Traits

We used one-way Analyses of Variance (ANOVA) to analyze differences in the number of infertile eggs, failed egg, and larval deaths between the populations. Because there were a different number of females that bred per tank, there would be natural differences in numbers of infertile eggs, failed eggs, and larval deaths. To account for this, we divided these counts by the number of mothers in each tank and used that as the response variable. We included the fixed effects of cross type and conducted a Tukey post-hoc test to compare differences between the populations.

Growth

We averaged total length of larvae per tub each week. We then calculated average growth rate per tub per week (cm/week) by calculated the difference from the first to last week's measurements and dividing by total weeks. With the replicate at the tank level, we used a one-way ANOVA to analyze differences in growth rate between the populations. We included the fixed effects of cross type and conducted a Tukey post-hoc test to compare differences between the populations.

Thermal Preference

We used a linear mixed effects model (r package lme4; Bates et al. 2015) to analyze differences in thermal preference between the populations. We included fixed effects of cross type with random effects of trial start time and animal length. Significance of the fixed effect was assessed by a Likelihood Ratio Test using the *anova* function in R. We conducted a Tukey post-hoc test to compare differences between the populations.

Any animal that spent more than 4 measurements below 12.1°C or above 27°C was considered stuck and was removed from the data set because they are no longer able to thermoregulate properly. We removed 4 extremely low and 2 extremely high outliers where animals got stuck in a thermal extreme and were unable to continue thermoregulation.

Animal Welfare

All protocols involving live animals were approved by the University of California, Santa Cruz Institutional Animal Care and Use Committee (IACUC, Office Code: Sineb2108) and permitted by U.S. Fish and Wildlife Service (FWSVFWO-25, Section 20h) and California Fish and Wildlife Service (SC-003574 and MOU).

Results

Survival Traits

We observed both infertile and failed eggs in most breeding tanks (Table 3.1). However, there was no significant difference between number of infertile eggs (df =3, F value=0.353, p=0.789) or failed eggs by cross type (df=3, F value=0.348, p=0.792). We saw one outlier in a Calabassas x McClusky cross that had large number of infertile and failed eggs. While three females bred in this tank, we believe all eggs came from one female because these eggs were clumped together.

A total of 19 dead larvae were observed during monitoring of the larval rearing tanks (Table 3.1). The majority of these animals were very small, with two being deformed during egg development, and all died shortly after hatching. There were no significant differences in number of larval deaths by cross type (df = 3, F value=1.550, p = 0.296). Throughout the season, we did not observe any other visual developmental abnormalities in the larvae. While we only found 19 dead larvae, cannibalism was occurring in the tanks. Based on the size of larvae, their cryptic nature, and size of the breeding tanks, it was extremely difficult to track how many

larvae were lost to cannibalism. Suspected cannibals (larger individuals) were moved to separate, individual tanks in the greenhouse.

Given that we found no significant difference between survival traits in eggs and larvae, it is likely that any difference we see in growth or thermal preference is due to difference among populations and not due to in-situ selection during the experiment.

Growth

Larvae hatched at approximately 1.3 cm and grew on average 0.251 cm a week. We found no significant difference between pure genetic lines and genetic crosses in average weekly growth rate (Figure 3.4, df =3, F value =0.2076, p =0.888). While not significant, we did see more variation in growth rate in the Calabassas x McClusky cross (Figure 3.4). Larvae were released at 3.5-6 cm. While there was no significant difference in final growth rates, we did notice that there was initially a growth rate difference in week 5-6 (Figure 3.5). Tubs with a high density of larvae grew slower than tubs with a lower larval density. After we supplemental food and split dense tanks (see methods above), growth rate sped up for the smaller individuals and then we saw a more even size distribution across all tanks (Figure 3.5).

Thermal Preference

Larvae had a thermal preference range of 15.95 to 25.56°C. We found no significant difference between pure genetic lines and genetic crosses in larval average

thermal preference (Figure 3.6, χ^2 =5.069, df=2, p=0.167). While not significant, larvae with both parents from the Calabassas population had the highest average thermal preference of 22.1°C compared to Calabassas x Cattail with the lowest at 21.0°C.

Larval Release

With no detectable defects and similar survival traits between crosses, all larvae were deemed suitable for release by U.S. Fish and Wildlife Service and California Department of Fish and Wildlife. When animals were close to size of metamorphoses, we released over 2,000 larvae all into struggling Monterey County populations (Figure 3.1). Some larvae were released into small current breeding ponds to increase resiliency and genetic representation, and some were released into newly created ponds to increase redundancy. Before release, a small tissue sample from the tail tip was collected for future genetic work.

Discussion

We found no evidence of outbreeding depression between Santa Cruz and Monterey populations. Therefore, admixture propagation is a viable method for genetic restoration and stopping the extinction vortex. Consistent with no outbreeding depression, we found no difference in traits between pure lines and genetic crosses. We found no difference in numbers of failed eggs, infertile eggs, or larval death, which provides no evidence for developmental differences between pure populations

and crosses. Additionally, we found no differences in growth rates and thermal preference. With no difference detected between any traits, we concluded that there was no initial risk of outbreeding depression. These results allowed us to move forward with the human assisted gene flow plan and all larvae were released into struggling Monterey populations (Figure 3.1).

Future captive propagation will continue to bolster other suffering SCLTS populations, and we must test for outbreeding depression between other metapopulations. But we must also contemplate what our results mean for the species past genetic composition. Similar thermal preferences regardless of cross type could imply that there is no local population-level adaptation to temperatures. However, Calabassas and Upper Cattail ponds have different thermal conditions and McClusky is assumed to be quite cooler than other ponds, so it is unlikely there is no local adaptation to temperature. Instead, similar thermal preferences implies that larvae have highly plastic thermal traits (Huey et al. 2012; Rohr et al. 2018) and adjusted to the thermal conditions they were experiencing in our tanks. This implies that we can translocate young larvae between ponds without risk of phenotype-environment mismatch and that animals will quickly adjust to new pond thermal conditions. Plasticity of thermal traits could also ameliorate extinction risk: if SCLTS can increase their thermal preference through acclimation, and maintain normal functions and behavior, they could survive exposure to the elevated temperatures expected under climate warming (Sinervo et al. 2010; Gvoždík 2011). Plasticity in thermal traits, in addition to increased genetic diversity to stabilize against stochasticity,

further breaks the extinction vortex pattern. We also found that while growth rates are similar through populations, density of larvae does dramatically effect growth. We need to consider this as we decide how many larvae to release in each pond, and the consequence that would have on larvae naturally born in those locations. In situations where larvae are used to recolonize previously used ponds, we must survey whether other present protected amphibians, like California Tiger Salamanders and Red-legged frogs, or invasive species will outcompete larvae. For new man-made ponds, we also need to test that appropriate zooplankton species have colonized for released larvae to grow naturally. Chemical mosquito abatement is used erratically in breeding ponds and, while the chemical currently has known no direct consequences on vertebrates, the consequences on zooplankton and phytoplankton is uncertain. Implications of density on growth and plasticity of thermal preference are critical for planning future translocations, but we also must understand if there are any long-term consequences of releasing admixture crosses.

We saw no immediate outbreeding effects between crosses, but we also did not see any evidence of reduced inbreeding depression. This could be because inbreeding depression effects occur when it is the most difficult to detect (Blomqvist et al. 2010). For example, if deleterious genes are expressed very early in development, we may only see less inbred offspring to measure. We could face the same sampling bias for outbreeding depression, but we avoided this possibility by counting eggs that failed during development. Although, cannibalism in our tanks probably did remove the weakest larvae and could be a potential mechanism that

removed overly inbred or outbred animals. We saw no evidence of outbreeding depression in survival and physiology, but it is possible we can see different effects over many generations (Tallmon et al. 2004; Edmands 2007). Future individual fitness benefits from increased diversity can be amplified over generations (Hogg et al. 2006), but there are also possibilities of outbreeding depression not being detectable until the second or third generation because of deleterious interactions between homozygous loci (Lacy et al. 1993; Fenster and Galloway 2000; Marr et al. 2002). We will need to monitor our release sites to conclude if there is any outbreeding depression in future generations.

While we found that local adaptation to thermal conditions can be seemingly overridden by plasticity to thermal conditions, we must be careful about genetic swamping of other local adaptations. For example, asymmetric gene flow from larger core population to small, isolated population can spread alleles adapted to the core location (Fedorka et al. 2012) or create phenotype-environment mismatch (Paul et al. 2011). However, recent studies show that local adaptations can be maintained despite high gene flow (Tigano and Friesen 2016), and we see that gene flow doesn't swamp local adaptations in other *Ambystoma* species (Micheletti and Storfer 2020). If we accept there are no or low negative consequences of our admixture propagation, we can continue with the assisted gene flow and releasing larvae in struggling populations. SCLTS will benefit from bolstering populations numbers in both existing ponds and act as founders at newly built breeding sites, but also benefit from increased genetic diversity. In addition to individual fitness benefits, increased

genetic diversity allows populations to have higher tolerance for environmental stochastic events (Lande and Shannon 1996; Reed and Frankham 2003; Hughes et al. 2008). Populations should be able to better tolerate climatic events such as increasing droughts (Lopes et al. 2015), increasing temperatures (Sankar et al. 2014), and decreased hydroperiods by altering time to metamorphosis, all which will be more common in the SCLTS range. If there is no outbreeding in future generations, no genetic swamping, and an added buffer against stochastic events, then we could potentially stop the extinction vortex.

History supports that low genetic diversity is a major threat to SCLTS, especially in Monterey County populations and this threat will only be exacerbated under climate change pressures, creating an extinction vortex. Using admixture propagation, we found no evidence of outbreeding depression in any survival traits and that we can continue with genetic restoration plans. It is clear moving forward, that SCLTS conservation requires a new approach: human facilitated breeding and assisted gene flow. This supports self-sustaining populations, ensures existing ponds remain functional breeding sites, and provides the adaptive potential needed to endure against stochastic events. This novel approach will become a proactive genetic restoration method for SCLTS, and potentially other isolated, endemic species, and contribute to population viability, both immediately and under future climate changes. However, we must consider more than just immediate survival traits and physiology. True genetic rescue occurs when population fitness increases, but increased population growth must be sustained over many generations (Tallmon et al. 2004).

We must next investigate how translocations change the genetic composition of future generations and test if there are effects of outbreeding depression on reproductive success. Moving forward, we plan to gather and analyze comprehensive genetic data that would allow us to interpret reproductive success of translocated larvae, test new genetic composition at release sites, and guide future management translocation decisions.

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Tables

Table 3.1 We had ten breeding groups, with between 1-3 females and 4-6 males in each tank. This table represents what crosses occurred and the counts of failed eggs, infertile eggs, and larval deaths.

Tank ID	Cross	Cross Type	Lay Date	Hatch Date	Initial Density	Failed Eggs	Infertile Eggs	Larval Deaths
39	McClusky x Calabassas	Cross	2/2	2/26	low	75	75	1
52	McClusky x Calabassas	Cross	2/2	2/25	medium	1	0	0
26	Calabassas x Calabassas	Pure Santa Cruz	2/2	2/24	medium	7	1	0
25	Cattail x Calabassas	Cross	2/2	2/25	high	2	2	18
51	McClusky x Calabassas	Cross	2/2	2/25	low	1	0	0
38	Calabassas x Calabassas	Pure Santa Cruz	2/2	2/24	high	3	6	0
12	McClusky x McClusky	Pure Monterey	2/2	2/26	medium	0	0	0
13	Cattail x Calabassas	Cross	2/13	3/10	medium	1	0	0
11	McClusky x Calabassas	Cross	2/13	3/10	medium	0	0	0
50	McClusky x McClusky	Pure Monterey	2/13	3/8	medium	1	0	0

Figures

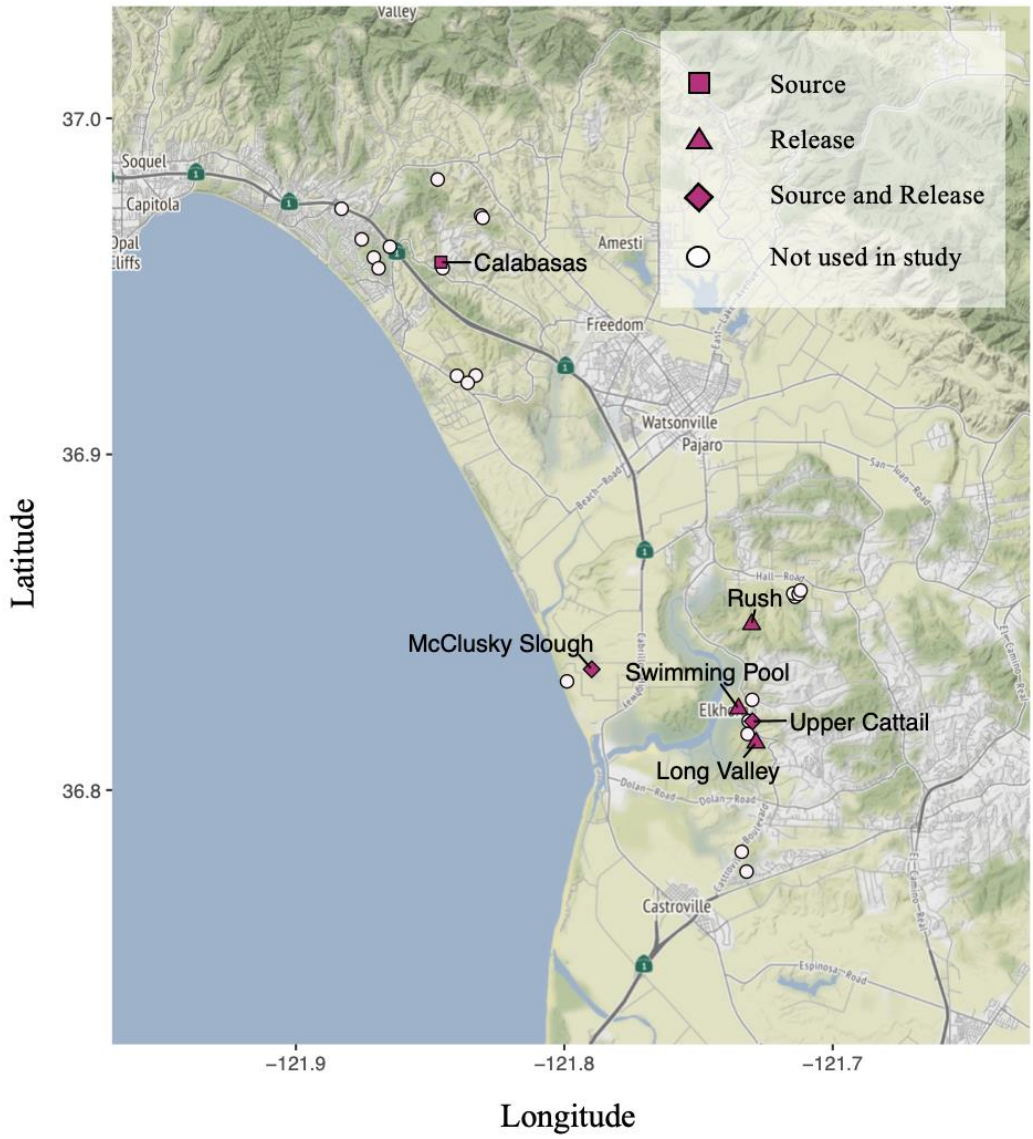

Figure 3.1 The range of SCLTS consists of southern Santa Cruz County and northern Monterey County with 28 current and historical breeding sites. Pink sites are ponds used in our study, and white sites represent ponds not used. Shapes represent how ponds were used in the study: triangles are larval release sites, squares are adult source sites, diamonds are both larval release and adult source sites, and circles represent sites not used in the breeding design.

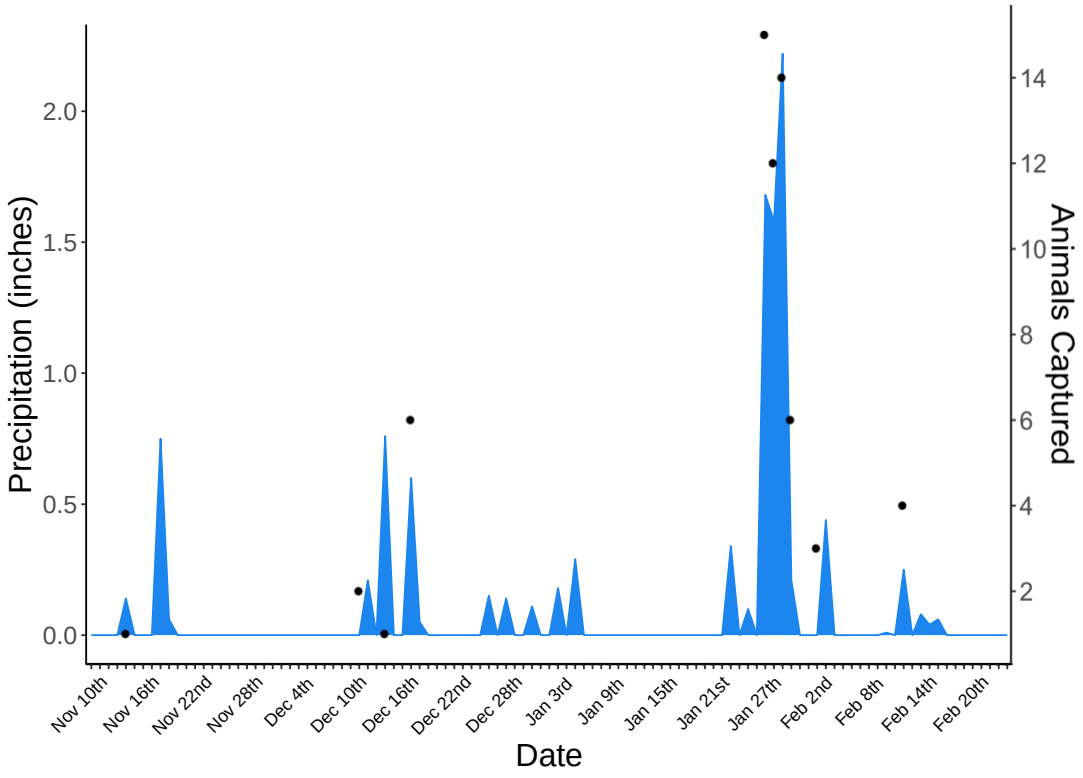

Figure 3.2 Number of adults collected in the 2020-2021 migration season with regards to precipitation levels. Blue chart represents rain fall in inches per day and black dots represent number of individuals collected each day.

Figure 3.3 Breeding tanks were 300-gallon cattle ponds with lids that allow natural air and insect flow and water treated with a 5% Holtfreter's solution. Tanks were filled with a layer of mud, grassy plants, and sticks from Calabassas ponds, to seed the pond with native invertebrates and provide natural material for females to lay eggs on. There are artificial stones to create a platform for adults to rest, if needed, and they also have a lip around the edge of the tank to rest.

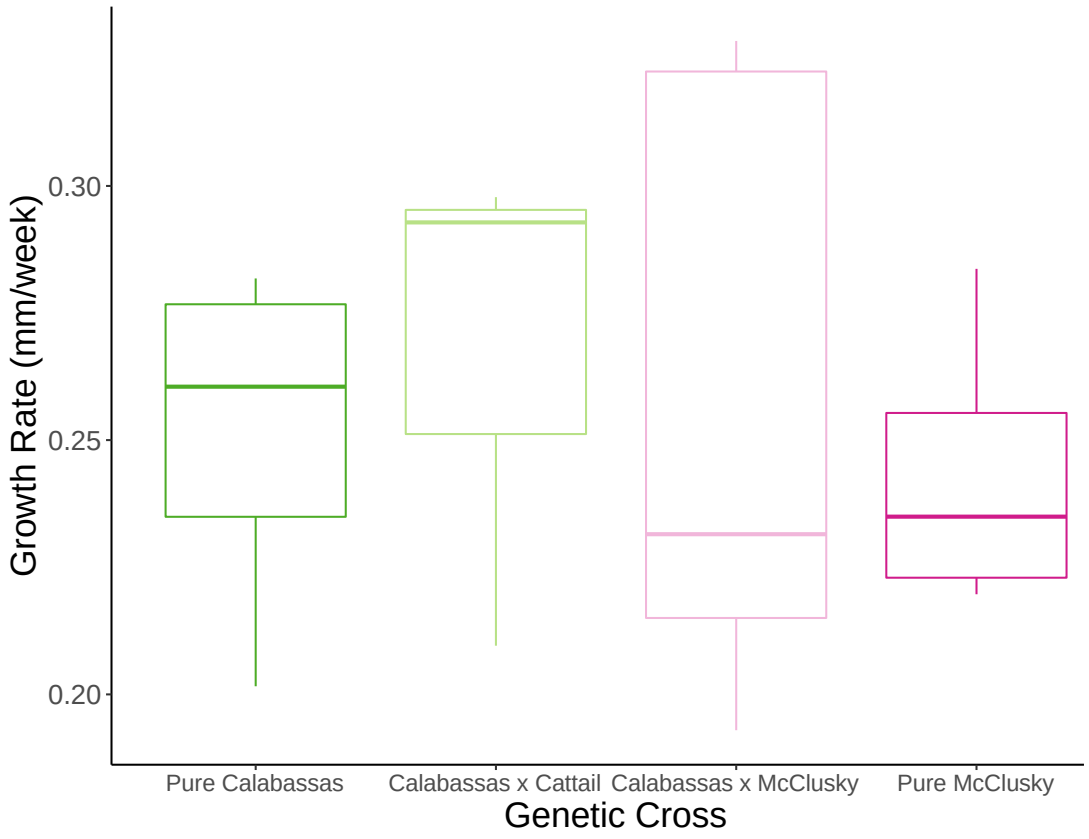

Figure 3.4 We found no significant difference between pure genetic lines and genetic crosses on averaged weekly growth rate (mm/week). For each box plot, the line represents the mean thermal preference, the upper and lower edges of the box represent the lower and upper quartile, and the lines above and below the box represent the maximum and minimum values.

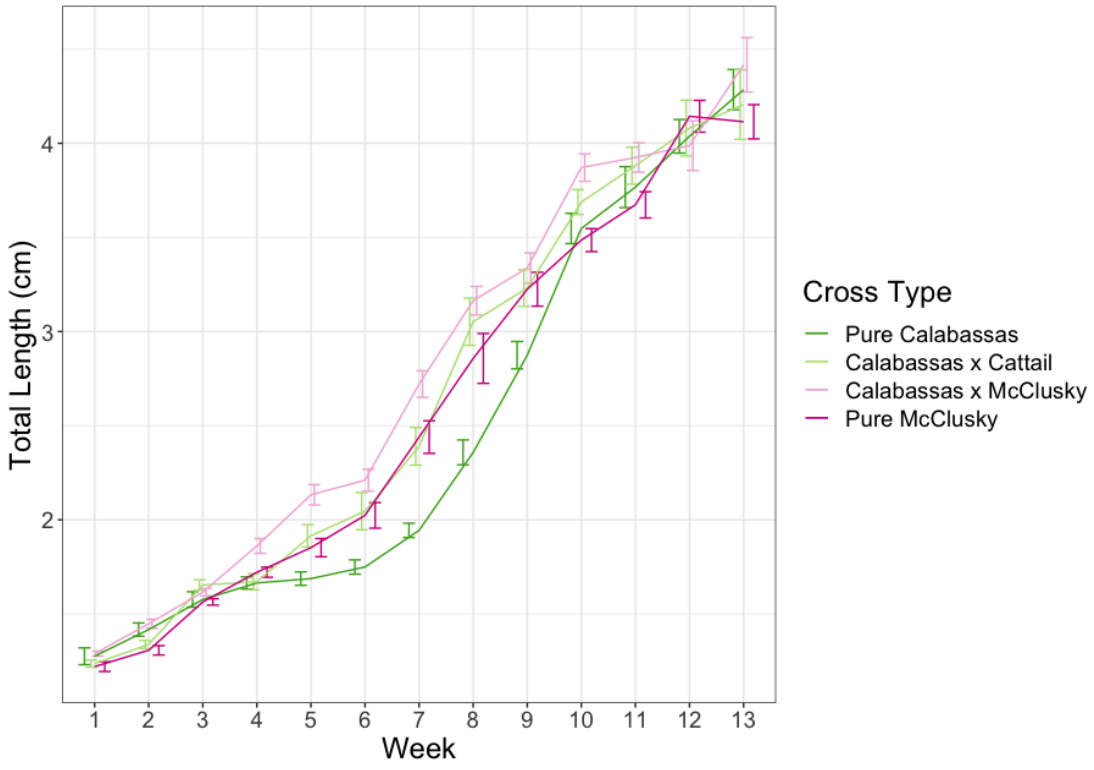

Figure 3.5 This represents average total length (cm) each week per genetic cross type. Animals were first measured in early March and last measured in late May. For high density tanks, growth rate was originally smaller, but after supplementing food and splitting tanks, growth rate sped up and equalized. Each point represents the mean total length and the line above and below represent the standard error.

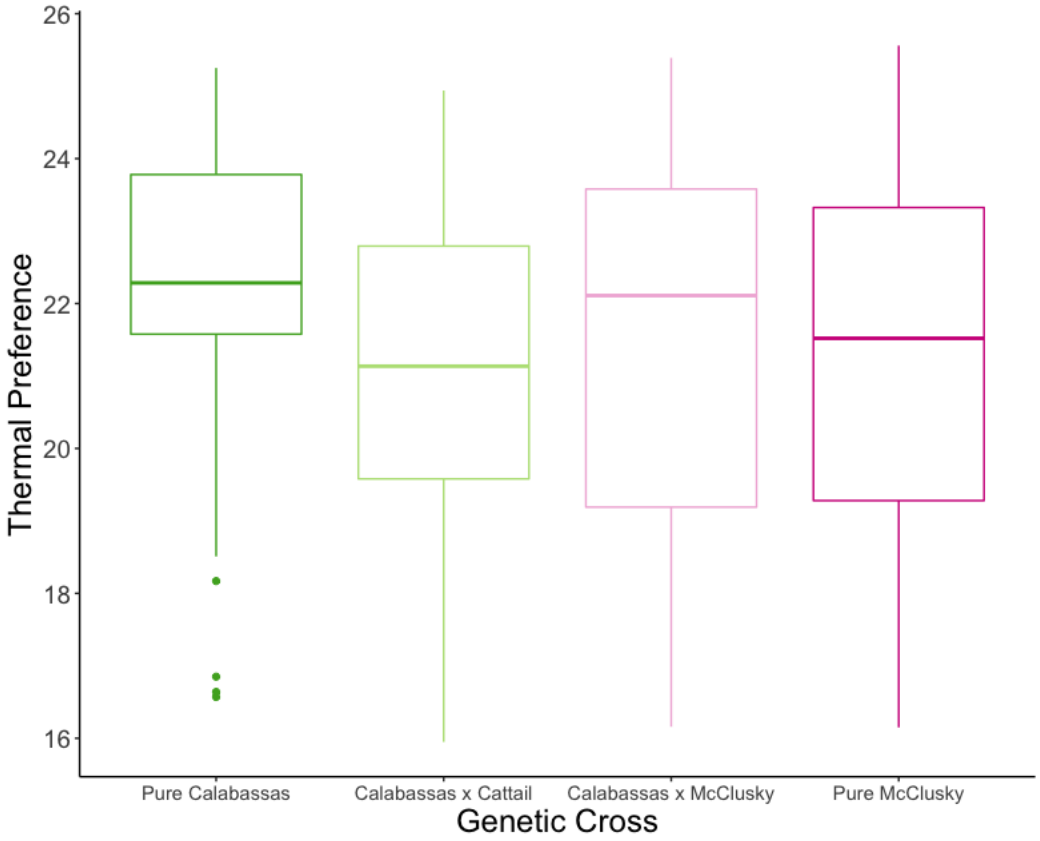

Figure 3.6 There is no significant difference in thermal preference between pure genetic lines and genetic crosses. For each box plot, the line represents the mean thermal preference (°C), the upper and lower edges of the box represent the lower and upper quartile, the lines above and below the box represent the maximum and minimum values, and the individual points represent outliers.

Synthesis

This dissertation has focused on the immediate and potential future response amphibians will have to environmental conditions. In **Chapter 1**, I investigated the effect of canopy coverage, and other environmental variables, on the available thermoregulation conditions at a breeding pond. I found positive effects of air temperature, the sun treatment, and reduced canopy cover on operative temperature, and negative direct or indirect effects of these variables on evaporative water loss, consistent with the hypothesized tradeoff between thermoregulatory behavior to increase temperature and the increased desiccation risk due to higher water loss. Additionally, my results indicate that the availability of wet microhabitats can allow frogs to reduce water loss, potentially mitigating the risk of desiccation when thermoregulating to achieve higher operative temperatures. My findings suggest access to sufficient microhabitat variation, especially wet microhabitats, may allow amphibians to mitigate the fundamental trade-off between higher temperature and the risk of desiccation. Furthermore, access to microhabitats may allow amphibians to use behavioral thermoregulation to ameliorate negative effects of anthropogenic climate change.

In **Chapter 2**, I investigated if amphibians could acclimate to thermal conditions through within- and inter-generational plasticity. Right after hatching, incubation temperature was positively correlated with offspring thermal preference. However, in older larvae, both late-rearing temperature and a three-way interaction among maternal, incubation, and rearing temperature increased offspring thermal

preference. These results demonstrate thermal inter- and within-generation plasticity in amphibians, which suggests that this combination is a possible coping mechanism to thermal stress. With the positive compounding interaction between maternal, incubation, and rearing temperature, larvae exposed to increasing temperatures can quickly increase thermal preference and receive a major buffer to climate change.

In **Chapter 3**, I tested for outbreeding depression in a local endangered salamander. I found that survival traits, growth rate, and thermal physiology were not significantly different between pure line and genetic crosses, suggesting no pattern of outbreeding depression. This research suggests that admixture propagation is a safe method for genetic restoration in SCLTS and I can continue my plan of human facilitated gene flow. All larvae were released into struggling breeding ponds to bolster population size, create gene flow between counties, and increase genetic diversity.

These three chapters show that amphibians are at lower risk of desiccation, have thermal plasticity that can protect them from future climate change, and I can use assisted gene flow to increase genetic diversity and bolster populations. This all seems like amphibians may persist better under climate change than previously predicted (Raffel et al. 2006; Wake and Vredenburg 2008; Kissel et al. 2019), but we must consider other factors when determining their extinction risk. Rising temperature will cause other negative consequences like shifts in phenology (Kissel et al. 2019), precipitation variation and hydrological changes in aquatic habitats (Bartelt et al. 2010), and changes in population dynamics (Sæther et al. 2000). Furthermore,

more frequent large-scale ecosystem disturbances, like increased wildfires, increased temperature variability, and spread of infectious diseases such as chytrid fungus, may magnify negative effects of climate change (Westerling et al. 2006; Raffel et al. 2013). Animals will need to simultaneously adapt to these consequences of climate change in addition to adapting to warmer mean temperatures.

In particular, altered hydroperiods are likely to be major problems for the persistence of many amphibian species. With climate change there will be fewer aquatic habitats and increasing drought in many regions, as well as faster drying of wetlands as the summer season lengthens (Bartelt et al. 2010; Lertzman-Lepofsky et al. 2020a). If breeding ponds dry too quickly, or never form, larvae will die before they reach metamorphosis (Semlitsch and Wilbur 1988; Griffiths 1997). Then, populations will quickly go extinct with no reproductive success and individuals will not live long enough to get any of the benefits I discovered in these research chapters. Future research must focus on plasticity in growth rates and other adaptations to changes in hydroperiods to understand amphibians' true extinction risk.

When taken together, these chapters highlight amphibians' complex and nuanced response to environmental changes. Changing environmental conditions will affect amphibians at multiple levels: ecophysiology at the individual level, plasticity at individual and generational levels, and genetic impacts at the population level. With this broader understanding, I can inform conservation methods to alter land management plans, improve extinction risk modeling to include plasticity, and help populations become more genetically resilient to climate change. My dissertation

adds to our understanding of how climate change affects ectotherms and their physiological responses, but we must expand research on how additional environmental conditions and stochastic events affect species persistence. We must continue work to have a full understanding of amphibian physiology to protect ectotherms from the increasing risk of extinction.

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