IMPACT OF INTROGRESSION ON THE DISTRIBUTION OF TRAITS ACROSS AN APPALACHIAN SALAMANDER HYBRID ZONE

by

CYNTHIA M. CARTER

(Under the Direction of John C. Maerz)

ABSTRACT

Hybrid zones provide a unique opportunity to untangle evolutionary mechanisms and challenge the way we think about biological and genetic diversity. Studies of hybrid zones can provide valuable insight into the dynamics and interactions that shape a variety of systems. Moreover, hybrid zones and hybrid lineages may themselves play an important role in the preservation and enhancement of adaptive potential in populations and communities under changing conditions. In this dissertation, I identify and characterize patterns in the distribution of traits across a *Plethodon* hybrid zone. In Chapter 2, I explore how climate has influenced the distribution of genomic and phenotypic traits of a *P. shermani* and *P. teyahalee* hybrid zone across a spatially complex hydroclimate gradient in the Coweeta basin. Chapter 3 examines the extent to which *P. teyahalee*, *P. shermani*, and hybrids in the Coweeta basin exhibit behavioral differences regarding their allocations of time to activities on the surface versus those occurring below-ground. I explore how patterns in behavior relate to hybrid index and weather across this system. In Chapter 4 I compare the physiological traits among *P. teyahalee*, *P. shermani*, and hybrid and explore the factors that influence rates of cutaneous water loss. Finally, in Chapter 5 I compare patterns in body size linked to demography and water loss and identify climatic and topographic variables that may be influencing patterns in morphology across this hybrid zone. Insights from this research will enable us to better understand the ecological and evolutionary processes that structure this system and improve our ability to make predictions about how lineage dynamics may shift in the future.

INDEX WORDS: Climate change, Evaporative water loss, Genomic clines, Hybrid index, Hybridization, Hybrid zone, Introgression, Multi-state model, Ordinal regression, *Plethodon shermani*, *Plethodon teyahalee*, Salamander behavior, Size at maturity, Surface activity, Zero one inflated beta regression

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DEDICATION

For MEBC, SLC, CCP III, KRLN, Oliver, and Winnie. I love you.

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A2016 04-009-Y3-A0, and A2020 06-028-Y3-A6. The collection of animals for use in this research was permitted by the North Carolina Wildlife Resources Commission under NC Wildlife Collection Licenses 14- SC00896, 15-SC00896, 16-SC00896, 17-SC00896, 18-SC00896, 19-SC00896, 20-SC00896, 21-SC 01421, 22-SC00896. Weather data was provided by the Coweeta LTER and the USDA Forest Service. Many thanks to Chris Oishi and Jason Love for logistical support and permission to use Forest Service and Coweeta LTER data.

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CHAPTER 1:

INTRODUCTION AND LITERATURE REVIEW

Conservation and management efforts may focus narrowly on populations or broadly on entire communities; but, in both cases, those units are typically viewed as groups or subsets of distinct species. Inherent in this perspective is the idea that a species is a fixed entity—a flowing network of genes contained within a boundary. However, there are many examples of lineages that lack clear genetic boundaries and do not fit neatly within this definition. The relationships between such lineages can be quite complex and may include varying degrees of hybridization (the interbreeding of individuals from genetically distinct populations) and introgression (gene flow between populations whose individuals hybridize) (Rhymer and Simberloff 1996).

Hybrid zones were once viewed as rare and unstable occurrences—primarily occurring at the range boundaries of species that have yet to sort out their evolutionary trajectories (Dobzhansky 1940; Wilson 1965; Wagner, 1969). We now understand that hybridization of lineages varies dramatically across systems. In some cases, hybridization can be limited to contemporary introgression resulting in the production of only early generation hybrids (i.e., F1 and F2) with no backcrossing into parental populations. In these systems, introgression may be constrained by hybrid inferiority, resulting in reduced survival or reproduction of hybrid individuals (Barton 1979). In other systems, hybrid zones or lineages can form because of historical introgression, which can range from a single event (e.g., the migration of a few individuals of one parent species into the range of another) to repeated or ongoing introgression (Nolte and Tautz 2010). In such cases, hybrid zones may contain only later generation hybrids, or, if the parent lineages remain in contact, a combination of early and late generation hybrids.

Furthermore, the extent of introgression can also vary across systems, ranging from broadly distributed swarms to narrowly restricted zones with limited introgression (Arnold 1997; Harrison and Larson 2016). This broad variation in conditions among hybrid zones makes the classification of individuals with hybrid ancestry a particularly challenging task (Arnold 1997; Mallet 2001; Hörandl and Stuessy 2010; Chaturvedi et al. 2020). In the case of populations with extensive historical and ongoing introgression, it may be appropriate to consider hybrids as distinct lineages. These lineages can be both evolutionarily active and dynamic and have the potential to exhibit a wide range of ecological and evolutionary outcomes distinct from those of either parent lineage.

Hybrid zones provide a unique opportunity to untangle evolutionary mechanisms and challenge the way we think about biological and genetic diversity. Ecologists have long understood the importance of maintaining genetic diversity of populations, particularly for imperiled species. Conservation efforts often aim to preserve the continuum of environments throughout a species' range in order to conserve local adaptation and promote gene flow (Bradburd and Ralph 2019). The ultimate goal of these efforts is to preserve genetic diversity and increase the likelihood that a population or species will be able to adapt to changing conditions and persist in the landscape. In some cases, the variation required to respond to these changes may not exist within a species. In others, the selective pressures created by changing conditions may simply outpace the ability for natural mutations to arise or existing variation to proliferate through a population (Gonzalez et al. 2013). However, in cases where distinct lineages can exchange genes, hybridization may serve as an additional pathway to alter the adaptive capacity of a system. Rather than relying on variation that exists within a species or population, introgressing lineages can exchange traits that have evolved under distinct sets of conditions. Introgression and hybridization also have the potential to yield novel combinations of traits by altering the relationships between co-linked traits and breaking phenotypic syndromes that exist within each parent lineage. When the introgression of genetic material confers a selective advantage to the recipient population, this process is referred to as "adaptive introgression."

While there are numerous well supported examples of adaptive introgression in plants, this phenomenon is not as well studied in animals. There are, however, several notable examples, including the beak shape in Darwin's finches, wing patterns in *Heliconius* butterflies, and pesticide resistance in western European mice (Song et al. 2011; Pardo-Diaz et al. 2012; Lamichhaney et al. 2015). A recent study on color polymorphisms in snowshoe hares (*Lepus americanus*) shows compelling evidence that polymorphism in adaptive winter camouflage is the result of hybridization with black-tailed jackrabbits (*L. californicus*) and the subsequent introgression of alleles related to coat pigmentation (Jones et al. 2018).

Though discussions of hybridization are often focused on the ways modern interactions might impact parent populations, historical introgression can also serve as an important source of adaptive potential and effectuate evolutionary novelty. Within some taxonomic groups, many contemporary "species" are in fact descendent hybrid lineages that persisted through historic periods of substantial climate change (Becker et al. 2013). In the fish family Cichlidae, hybridization appears to have played a crucial role in promoting all major adaptive radiations of this family across East Africa (Meier et al. 2017; Irisarri et al. 2018; Svardal et al. 2020). Similar patterns have also been observed in the mass-spawning coral genus *Acropora* (Willis et al. 2006). This illustrates the importance of hybrid lineages and dynamics to the persistence of populations in the face of future climate change (Willis et al. 2006; Janes and Hamilton 2017). Furthermore, hybrid lineages may require the development of unique conservation policies and management strategies in order to preserve their unique ecological contributions and evolutionary potential in the face of current global change.

Hybridization poses an interesting challenge to conservation and management decisions. While the field of ecology's understanding of these systems has evolved over time, current conservation and management policies rarely reflect these changes. Such is the case with the Endangered Species Act (ESA) of 1974—a landmark piece of legislation in the United States that is widely regarded as one of the world's strongest wildlife protection laws. As its name suggests, the ESA was designed to preserve imperiled plant and animal species and facilitate their recovery such that legal protections are no longer needed to sustain them. The law was written using the prevailing definition of what constitutes a species, Ernst Mayr's biological species concept (Mayr 1942; Mayr 1963). Mayr conceptualized species as "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr 1942). From 1977 to 1990, one of the agencies primarily responsible for administering the ESA—the U.S. Fish and Wildlife Service (FWS)—operated under a *de facto* policy that denied legal protection to hybrid individuals. Hybrids were considered a threat to "pure" parent populations because of their potential to cause outbreeding depression and genetic assimilation (Haig and Allendorf 2006). The FWS policy faced criticism from the scientific community, with Mayr himself arguing that failure to provide protection to imperiled species with documented instances of hybridization posed a greater threat to conservation than those hybrids themselves (O'Brien and Mayr 1991). Ultimately, the *de facto* policy was dropped, and while additional guidelines have since been proposed, no formal policies for the management of hybrids under the ESA have been approved.

Some of the primary obstacles to addressing the uncertainty surrounding the conservation and management of hybrid populations are the complex and widely variable range of circumstances under which hybridization can occur. Differing levels of conservation concern can add an additional layer of complexity to these systems, as seen in the hybrid zone formed by the

Balearic shearwater (*Puffinus mauretanicus*) and the Yelkouan shearwater (*P. yelkouan*). Classified as "critically endangered," *P. mauretanicus* is considered the rarest European seabird and is predicted to face global extinction within a matter of decades (Oro et al. 2004; IUCN 2023). In contrast, *P. yelkouan* has been assigned the somewhat more auspicious classification of "vulnerable" (IUCN 2023). These seabirds have largely allopatric distributions and are only known to co-occur and hybridize on a single island in the Balearic archipelago (Genovart et al. 2005). While this hybrid zone appears to be naturally occurring, concerns remain over the potential consequences of introgression on dwindling populations of *P. mauretanicus*, with possible threats ranging from outbreeding depression to genetic assimilation. However, there is currently no evidence to suggest that this hybrid zone is progressing toward either of these potential outcomes.

The increasing prevalence of hybrid zones encouraged by anthropogenic influences, such as habitat destruction or the human movement of species, has generated additional questions and concerns over the conservation and management of hybrids. For example, human-mediated introductions of the non-native barred tiger salamander (*Ambystoma tigrinum mavortium*) into the native range of the of the endangered California tiger salamander (*A. californiense*) has resulted in the production of morphologically, physiologically, and ecologically distinct hybrid offspring (Riley et al. 2003; Cooper and Shaffer 2021). These hybrids do not appear to fill the same ecological niche as the native *A. californiense* and seem to have a competitive advantage over both parent lineages in ponds where all three groups co-occur (Fitzpatrick and Shaffer 2007; Ryan et al. 2009). Over time, it is expected that introgression between these lineages will continue, and, eventually, genetically "pure" populations of *A. californiense* may no longer exist in the wild (Cooper and Shaffer 2021). While this would result in the loss of some alleles unique to those native salamanders, the apparently enhanced survival of hybrids could also increase the likelihood that some portion of *A. californiense*'s genome will persist and that some type of tiger salamander exists in those habitats. The broad impact this transition will have on the overall structure and function of these communities is currently unknown.

The shearwater and tiger salamander systems highlight key issues in the broader debates over how to manage hybrid zones. Despite broad agreement that species are not fixed entities and an increasing awareness of the ubiquity of hybrids, many regulatory frameworks still fail to accommodate these highly ecologically influential organisms, leaving them with no legal designation to guide their protection and management. Furthermore, the mere fact of a hybrid zone's existence is insufficient grounds for making decisions on how it should be managed. The unique and variable conditions of individual systems mean that overly-generalized management policies have limited utility. A policy that promotes genetic diversity and preserves ecological functions in one community may have detrimental impacts when applied to another.

Shifting global environmental conditions are likely to impose selective pressures that strongly impact a variety of organisms. There is a pressing need to develop conservation and management strategies that preserve the evolutionary processes that are likely to influence the persistence of particular groups of organisms, and associated ecosystems processes, into the future. Achieving these goals will require a thorough understanding of the underlying factors that drive these processes. Studies of hybrid zones can provide valuable insight into the dynamics and interactions that may be operating to shape a variety of systems. Moreover, hybrid zones and hybrid lineages may themselves play an important role in the preservation and enhancement of adaptive potential in populations and communities that may struggle to adapt under changing conditions.

In this dissertation, I aim to identify and characterize patterns in the distribution of phenotypic, genomic, physiological, and behavioral traits across a hybrid zone between two salamander species in the genus *Plethodon* (family Plethodontidae). These insights will enable us to better understand the ecological and evolutionary processes that structure this system and improve our ability to make predictions about how lineage and population dynamics may shift in the future.

SUMMARY OF STUDY SYSTEM & RESEARCH OBJECTIVES

Plethodontidae is the most diverse family of salamanders in the world. Members of this family lack lungs and are entirely reliant on cutaneous gas exchange. Their reliance on thin, highly vascularized skin makes their ecology highly dependent on the availability of moisture in their environment (Feder 1983). Despite this restriction, they have adapted to fill a broad suite of ecological niches, and their primary distribution ranges from temperate forests in North America to neotropical rainforests in Central and South America. The highest concentration of plethodontid diversity occurs in the southern Appalachian Mountains. Much of this diversity is contained within the genus *Plethodon*, a group of terrestrial direct-developing salamanders. Many North American salamanders have a biphasic life-history, which requires some proximity to aquatic habitats to accommodate larval development. Because *Plethodon* lack an aquatic larval stage, they are better equipped to exploit terrestrial environments than their biphasic relatives. Their status as a lungless, direct-developing salamander means that the major physiological constraints on their distribution are often moisture and temperature through effects on evaporative water loss and rehydration. Their high abundance and biomass make these salamanders ecologically contributors to forest ecosystem processes, including as standing stocks of key nutrients and conduits of energy and nutrient transfer (Burton and Likens 1975; Milanovich and Peterman 2016).

WHERE DID THEY COME FROM?

Alternating periods of glacial expansion and retreat have played a key role in shaping the modern distributions of plant and animal populations. In temperate lineages, glacial expansion is generally associated with range contraction away from cold and inhospitable conditions and into refugia (Hewitt 1996). However, this is not always the case. Some temperate species instead follow the opposite pattern more commonly seen in alpine taxa (Galbreath et al. 2009). Such is thought to be the case for the ancestral forms of eastern *Plethodon* lineages in the Appalachian Mountains (Highton 1995; Newman and Austin 2015). During glacial periods in the Late Pliocene these salamanders likely had a continuous distribution at low elevations that encompassed cool, wet temperate forests (Highton 1995; Kozak and Wiens 2006). As glaciers retreated, lowland forests were replaced by warm and dry grasslands. During these interglacial periods, salamander populations that were unable to tolerate the conditions at low elevations could have persisted in relict patches of cool, wet forests at higher elevations (Highton 1995; Newman and Austin 2015). This tendency to maintain a suite of ancestral ecological traits rather than adapt to new conditions—known as phylogenetic niche conservatism—may have played an important role in the initial genetic isolation of montane populations (Wiens 2004). Alternating periods of range expansion and contraction caused by fluctuations in climate are thought to have promoted a subsequent burst of allopatric speciation/lineage splitting within *Plethodon* (Highton 1995; Wiens 2004; Kozak and Wiens 2006). There is additional evidence that niche divergence may have also contributed to the evolution of some *Plethodon* lineages (Wooten et al. 2013).

WHERE DID THEY GO?

Today there are at least 56 recognized species within the genus *Plethodon*. Included among the eastern *Plethodon* are the *P. glutinosus* and *P. jordani* species complexes. The *P. jordani* complex contains seven species that occur at high elevation sites in the southern Appalachians. They are moderate- to large-sized salamanders, and many species possess red coloration, including red cheeks or legs. Species belonging to the *P. glutinosus* complex are large-bodied and characterized by light-colored dorsal and lateral spotting with no red pigment. Each of these complexes consists of morphologically and ecologically similar species, however many of the relationships among species (and in some cases, the placement of species to one or the other group) remains unresolved, partially due to difficulties of untangling the influence of frequent hybridization and introgression (Highton and Peabody 2000; Wiens et al. 2006; Highton et al. 2012). Many of the recently diverged, large eastern *Plethodon* species have not developed strong mechanisms for reproductive isolation and there are numerous examples of hybridization between members of these lineages (Highton 1995; Highton and Peabody 2000). This is exemplified by parapatric populations of *P. shermani* (Stejneger 1906) and *P. teyahalee* (Hairston 1950) in the southern Appalachian Mountains of western North Carolina. *Plethodon shermani* is a moderate- to large-sized montane endemic belonging to the *P. jordani* complex and is characterized by red legs and a black body without white spotting (Figure 1.1). The distribution of *P. shermani* is limited to four allopatric populations (isolates) atop the Nantahala and Unicoi Mountains in southwestern North Carolina and adjoining areas of Tennessee and northeast Georgia (Figure 1.2). Each of these mountain-top isolates is bounded at lower elevations by populations of *P. teyahalee*. Traditionally considered a member of the *P. glutinosus* complex, *P. teyahalee* is a large-bodied salamander characterized by a black body covered with extensive white spotting (Figure 1.1). *Plethodon teyahalee* is broadly distributed across the southern half of the Blue Ridge province of North Carolina, with portions of its range extending into neighboring areas of Georgia, Tennessee, and South Carolina (Figure 1.2).

For many years after their initial descriptions in the literature, researchers struggled with the taxonomic placement of both *P. shermani* and *P. teyahalee* because of specimens found with intermediate phenotypes (see Appendix 1.1 for an overview of the taxonomic uncertainty surrounding these lineages). In 1950, Hairston proposed that these puzzling specimens may have been the result of true hybridization between evolutionarily distinct lineages (Hairston 1950). His supposition would prove to be correct—at intermediate elevations where populations of *P. shermani* and *P. teyahalee* come into contact they readily interbreed and backcross to form hybrid zones. Because these two species are phenotypically distinct and their hybrid offspring typically express traits from both parent lineages (i.e., red pigment on legs and white spotting along the body), individuals of mixed ancestry are often easy to identify (Figure 1.1). Since their initial description, the evolutionary history of these hybrid zones has been an area of great interest to ecologists and evolutionary biologists. In 1970, Highton and Henry proposed that the hybrid zone below the Standing Indian isolate was a relatively recent phenomenon that may have formed following a period intensive logging in the early 1900s, but that secondary contact between *P. shermani* and *P. teyahalee* at the Tusquitee and Unicoi isolates had occurred more than 4000 BP (Highton and Henry 1970). However, recent studies suggest a much longer history of hybridization at all isolates, with one minimum estimate of more than 500,000 BP (Weisrock et al. 2005; Lowe 2016).

WHAT ARE YOU EVEN DOING, COTTON-EYE JOE?

There is still much work to be done to unravel the evolutionary history of these salamanders; however, there is also plenty to learn about how this system operates in its contemporary state. By developing a thorough understanding of what processes regulate the habits and interactions of these salamanders we can better understand the mechanisms that have historically shaped this system and predict how it may respond to changing conditions in the future. There are several key features that make *P. shermani* and *P. teyahalee* well suited for this type of inquiry. First, their status as distinct species is well supported (Highton and Peabody 2000; Highton et al. 2012). Second, they have parapatric distributions and occupy habitats with appreciable ecological differences. Third, each species possesses unique, diagnostic phenotypic traits. And fourth, they can, and do, interbreed to produce phenotypically diagnostic hybrid offspring with widespread backcrossing among hybrids. The result has been the wide distribution of individuals of hybrid ancestry and patterns of genetic and morphological characteristics more indicative of a cline within a single species. Together, this provides us with an opportunity to investigate how particular traits and behaviors are distributed with respect to lineage and determine how those patterns might be influenced by biotic and abiotic conditions. Because we believe the two parent lineages are ecologically distinct, hybridization may be yielding novel combinations of traits available for natural selection to act on, thereby altering the adaptive capacity of this system.

In Chapter 2, I explore how climate has influenced the distribution of genomic and phenotypic traits across a *P. shermani* and *P. teyahalee* hybrid zone in the Coweeta basin. I sequenced genomic samples from salamanders collected from putative "pure" parent populations of *P. shermani* and *P. teyahalee* and hybrid individuals from the Coweeta basin. I use these data to generate genomic clines and estimate admixture and assign a genomic index of hybridization for individuals. I use this index to examine patterns of differentiation and introgression between hybrid ancestry and phenotypic traits using tissue samples collected throughout the basin. I also address the extent to which to color pattern phenotypic traits—which have been used consistently to study the hybrid zone and its dynamics—are predictive of hybrid genotypes at the local to basin-wide scales. Finally, using both the genomic hybrid index data and data from long-term capture-mark-recapture study and unmarked population surveys, I estimate the effects of a suite of climate variables on hybrid index and phenotype scores using Bayesian zero-one inflated beta regression models and ordinal regression models, respectively.

Chapter 3 examines the extent to which *P. teyahalee* and *P. shermani* in the Coweeta basin exhibit behavioral differences regarding their allocations of time to activities on the surface versus those occurring below-ground. I conducted a field study using passive integrated transponder (PIT) tags to uniquely identify and locate individual salamanders across a hybrid zone and document their movement and behavior. I use a multistate mark-recapture model in a Bayesian framework to determine the relationship between hybrid index, behavior, and weather. I estimate recapture probabilities and apparent survival using individual capture histories. I expected *P. teyahalee* and *P. shermani* to exhibit behavioral differences regarding their allocations of time to activities on the surface versus those occurring below-ground. I anticipated that *P. teyahalee* will spend more time in burrows and less time active above ground than *P. shermani*. Furthermore, I expected that hybrid individuals will exhibit intermediate behaviors, which will vary as a function of their phenotype scores. I expected that surface activity will fluctuate with variation in rainfall and temperature.

In Chapter 4, I explore a novel method to assess physiological differences among *P. teyahalee*, *P. shermani*, and hybrids. I used an Aquaflux AF200 Evaporimeter to measure cutaneous evaporative water loss (EWL) of animals in the field, bypassing the standard need to transport animals to a laboratory and acclimate them for weeks to generate similar measurements. I use linear mixed effects models to examine the relationship between ancestry, morphology, habitat, climate, and water-loss. I also discuss the utility of this novel approach for future studies of amphibian physiology. Understanding the physiological limitations of these salamanders and their relationship to climate will help us to better understand what factors regulate their distribution and survival and can help us to make predictions about the adaptive capacity of hybrids.

Finally, in Chapter 5, I use nearly 15,000 records of salamander captures among 67 sites across the Coweeta basin to determine the minimum observed size of a reproductive male and female to estimate the relationship between climate, slope, and aspect on minimum size at first reproduction. Size and age at first reproduction are among the most influential life-history traits of animals. Many animals including some ectothermic species show negative relationships between body size, age at maturity, and temperature; however, many species are also exceptions to this rule including salamanders and other amphibians. A number of hypotheses have been advanced to explain this exception, but none have explored the effect of body size on EWL. Larger amphibians lose water at slower rates (Feder 1983; Feder and Londos 1984), therefore, it is possible that drier climates favor larger body sizes, which results in the evolution of delayed maturity to a larger body size at first reproduction. I used linear models to compare how different climate variables predict minimum *Plethodon* body size at first reproduction across the hybrid zone. I hypothesized that minimum observed size at first reproduction would be negatively correlated with measures of mean annual precipitation or positively correlated with measures of evaporative water loss potential (temperature or vapor pressure deficit. I also predicted that south-facing aspects, which have higher levels of incident light and warmer temperature, and steeper slopes, which have more drained, drier soils, would be positively correlated with increasing minimum observed size at first reproduction.

DESCRIPTION OF THE STUDY SITE

The Coweeta basin is a 1600-hectare forested catchment located in Macon County, North Carolina, and contained within the Nantahala National Forest of the southern Appalachian Mountains. The Coweeta basin is located in the second-wettest region in the continental United States, with mean annual precipitation ranging from 1500 to 2400 mm (Daly et al. 2017). This basin has steep topology, with elevations ranging from 675 to 1592 m above sea level. Climate varies considerably across this gradient and is strongly influenced by orographic effects, with high elevations tending to be cooler and wetter, while low elevations are typically much warmer and dryer.

This site was purchased in 1923 by the United States Forest Service (FS) and became the location of the FS Southern Research Station’s Coweeta Hydrologic Laboratory in 1934. The laboratory was established to study forest hydrology and the impacts of forest management strategies. From 1980 to 2020 this site was also part of the Long-Term Ecological Research Network (LTER) funded by the National Science Foundation (NSF). The Coweeta LTER (CWT) project was largely focused on research examining the impacts of climate, topology, and both natural and human disturbances on ecological processes. This site is one of the longest-running ecological research sites in the United States and has generated a wealth of long-term data—including those collected by climate and precipitation stations located throughout the basin (Miniat et al. 2021).

Coweeta has also been the location of considerable salamander ecology including initial descriptions of the hybrid zone between *P. shermani* and *P. teyahalee* (Hairston 1973), *Plethodon* hybrid zone genetic structure (Highton and Peabody 2000; Weaver 2022), hybrid zone dynamics in relation to climate (Hairston et al. 1992; Walls 2009). Beginning in 2010, the Maerz Lab initiated long-term demographic studies within the hybrid zone and across the wider basin with the aims to better understand how climate affects population and hybrid zone dynamics (Howard 2018) and how forest management may affect salamander population and evolutionary ecology. That ongoing work forms the backdrop for this research.

FIGURES

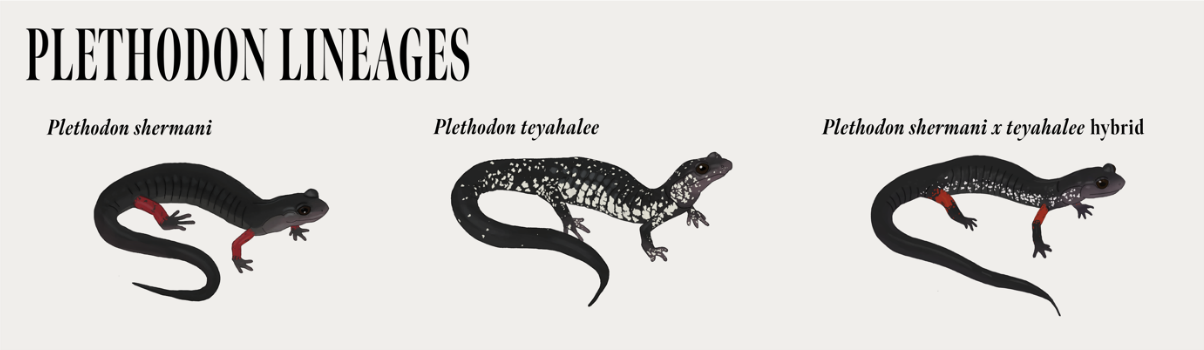

Figure 1.1 Illustrations depicting the general appearance of *Plethodon shermani* (left), *P. teyahalee* (center), and *P. shermani* × *teyahalee* hybrids (right). Illustrations drawn by C. Carter.

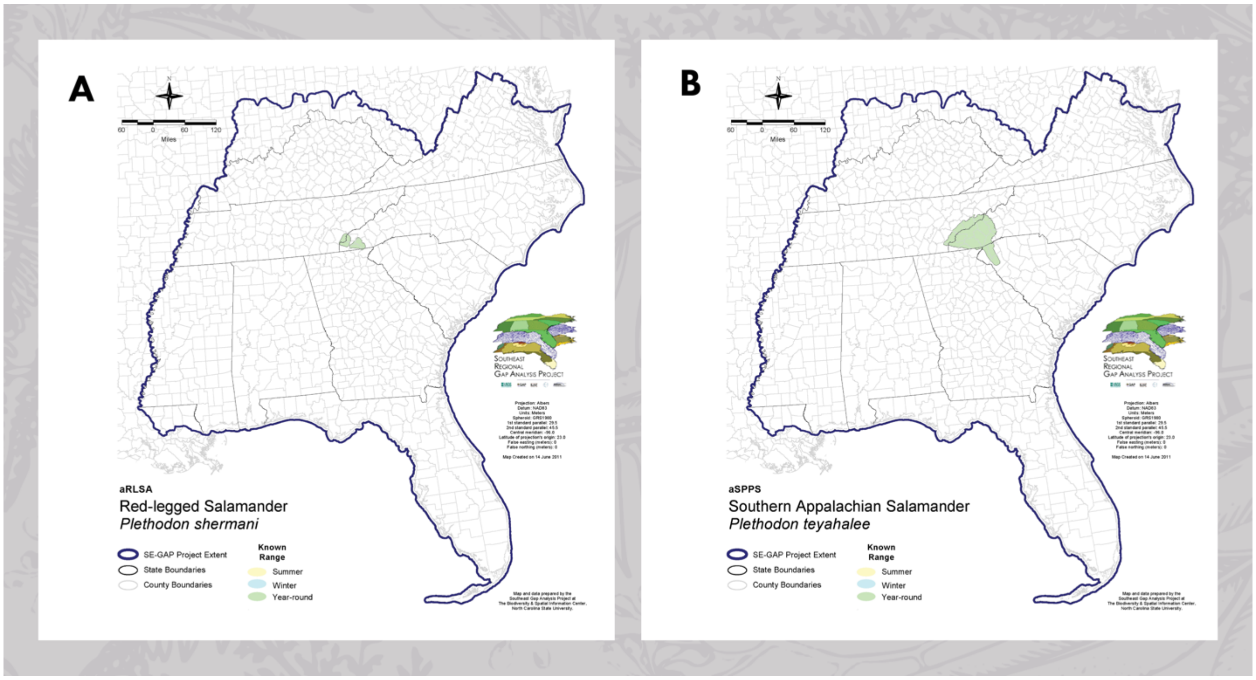

Figure 1.2. Maps showing the distributions of *Plethodon shermani* (A) and *P. teyahalee* (B) created by the Southeast Regional Gap Analysis Project (http://www.basic.ncsu.edu/segap/).

CHAPTER 2:

GENOMIC DATA REVEAL PATTERNS OF HYBRIDIZATION IN APPALACHIAN SALAMANDERS[1]

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[1] Carter, C.M., T.W. Pierson, and J.C. Maerz. To be submitted to *The American Naturalist*.

ABSTRACT

Among ecologically distinct lineages, hybridization has the potential to yield novel combinations of traits upon which natural selection can act, thereby altering the adaptive capacity of populations. We investigate how particular traits and behaviors are distributed across a hybrid zone formed by two lungless salamanders, *Plethodon shermani* and *P. teyahalee*, in the southern Appalachian Mountains and determine how those patterns might be influenced by climate, weather, and ancestry. We collected tissues from salamanders across this hybrid zone, created RADseq style genomic libraries, and used genome-wide SNP data to estimate a genomic index of hybridization. Using this index, we examined patterns of differentiation and introgression and evaluated the relationship between hybrid ancestry and phenotypic traits that are hypothesized to vary with climate. We also used models of genomic clines to identify outlier loci that may be under selection. Models examining the influence of climate on the distribution of hybrids revealed strong relationships between phenotype, genomic ancestry, and climate—particularly mean annual precipitation.

INTRODUCTION

The intrinsic capacity of populations to persist requires that individuals possess traits that are compatible with the range of conditions they will experience at that location. Under a predictable set of conditions, natural selection promotes local adaptation by favoring traits that increase survival and reproduction. While this process enhances fitness under those conditions, it can also reduce the variation of traits that exist within a population, thereby narrowing overall niche breadth (Futuyma and Moreno 1988). When shifting environmental conditions reduce habitat suitability, a population can persist by either adapting to these new conditions or migrating to a new, more suitable habitat. For small species with low dispersal abilities, long-distance migrations are unlikely (Hoegh-Guldberg et al. 2008). In these cases, the ability of populations to adapt to new conditions through evolutionary change will be key to persistence.

The fast pace of anthropogenic climate change may exceed the typical rate of climatic niche evolution for many organisms, suggesting a limited potential for novel adaptations to arise within populations (Quintero and Wiens 2013). Adaptation through phenotypic plasticity alone may also be insufficient, particularly in extreme conditions (Amburgey et al. 2016). Many species that are currently distributed across broad climatic gradients already exhibit extensive climate-associated genetic and phenotypic variation. These locally adapted alleles and their resulting phenotypes may provide variation upon which natural selection can act, enabling evolutionary responses to climate change. Gene flow from populations at climatic extremes can improve fitness in other regions (Bontrager and Angert 2019), but gene flow may be limited by distance, biogeographic barriers, prezygotic isolation, and genetic incompatibilities. Thus, spatial heterogeneity both creates the conditions for local adaptation and limits the movement of these traits from populations in which they evolved to others in which they may prove adaptive in future climates (Aitken and Whitlock 2013). The evolutionary potential of locally adapted genotypes and phenotypes may be further limited by ecological interactions that shape patterns of intraspecific variation. Competition among ecologically similar species can change mean trait values through character displacement (Brown and Wilson 1956) or reduce variance through niche compression (MacArthur and Pianka 1966; Schoener 1974; Wilson and MacArthur 2016).

In cases where ecologically distinct lineages can exchange genes, introgression may serve as an important pathway to enhance the adaptive capacity of a system. Rather than relying on variation that exists within a species or population, hybridizing lineages can exchange traits that have evolved under distinct sets of conditions. Introgression has the potential to yield novel combinations of traits by altering the relationships between co-linked traits and breaking phenotypic syndromes that exist within parent lineages. Hybridization and introgression can also impact patterns of genotypic and phenotypic variation by altering ecological interactions. Genomic data have revealed the ubiquity of hybridization in the evolutionary history of many organisms (Taylor and Larson 2019), and introgressive hybridization is an important mechanism for adaptation to past and current climate change (Lewontin and Birch 1966; Becker et al. 2013). Furthermore, hybrid zones have long been used as "natural laboratories" in which novel combinations of divergent and tractable genotypes and phenotypes can provide more generalizable conclusions about broader ecological and evolutionary processes (Hewitt 1988).

Historically, studies on hybridization have relied heavily upon morphological data for the identification of putative hybrid individuals. In cases where morphologically distinct parent lineages produce hybrid offspring with intermediate phenotypes, the degree of resemblance between offspring and parent lineages has often been interpreted as an indicator of genetic ancestry (Hairston et al. 1992; Walls 2009). However, we now know that morphological phenotypes are not necessarily reliable or precise indicators of parentage, nor do they indicate the extent of hybridization within a system (e.g., identifying F1, F2, later generation, and backcrossed hybrids) (Dong et al. 2019). Furthermore, patterns of asymmetry in selection and introgression may not always be accurately reflected by the distribution of morphological phenotypes (e.g., Pascal et al. 2023). In the modern genomic era, these limitations can largely be addressed using high-throughput sequencing and analysis. Advancements in genomic tools, and the increased affordability of sequencing, have enhanced our capacity to generate precise, population-level estimates of genetic ancestry.

In this study, we investigate the structure of a hybrid zone formed by two lungless, plethodontid salamanders, *Plethodon shermani* and *P. teyahalee*, in the southern Appalachian Mountains using both phenotypic and genomic data. First, we estimate a genomic index of hybridization measuring admixture for individual salamanders across a hybrid zone. Using this index, we examine patterns of differentiation and introgression and evaluated the relationship between hybrid ancestry and morphological traits. Next, because the parent lineages are generally distributed across different elevations with different climates, and because moisture and—indirectly—temperature are critical factors driving the ecology of lungless salamanders (Feder 1983), we examine how genomic and morphological traits were distributed across this hybrid zone with respect to climate. Finally, we use models of genomic clines to examine patterns of introgression and differentiation and identify outlier loci that may be under selection in this system.

METHODS

SAMPLE COLLECTION: Tissue samples were collected from animals with a range of phenotypes from sites distributed across the Coweeta basin as part of several on-going projects. These samples included putative non-hybrid animals belonging to each parent lineage (*P. teyahalee* and *P. shermani*, from low- and high-elevation sites, respectively) and putative hybrid animals from sites at various elevations across several watersheds. Tissues were collected by applying pressure 5 to 10 mm from the distal tip of the tail with clean blunted forceps; the resulting autotomized tissue samples were placed in individual microcentrifuge tubes containing 70% ethanol. Standard measurements were taken from all individuals including snout-vent length (SVL), total body length, and mass. All individuals were also assigned two independent phenotype scores adapted from the criteria outlined by Hairston (1973) and Hairston et al. (1992) based on their presentation of *P. shermani* and *P. teyahalee* traits (Hairston 1973; Hairston et al. 1992). Phenotype scores ranged from 0 to 3, with *P. shermani* scores (PS) assigned based on the amount of red present on the legs, and *P. teyahalee* scores (PST) based on the amount of white spotting on the body (Figure 2.1). For each score, a higher value indicates increased morphological resemblance to that parent lineage.

DNA EXTRACTION & LIBRARY PREP: We removed tissue samples from their original vials and allowed the ethanol to evaporate before beginning the extraction process. For large tissues, a subsample was removed using a sterile razor blade and returned to the original vial for future use and the remainder was set out to dry. After drying, each tissue was placed in a vial with a digest buffer (180 µl of Qiagen DNeasy buffered ATL and 20 µl of Proteinase K), vortexed, and placed in a 56 °C incubator overnight. Tissues were extracted following the Qiagen DNeasy Blood and Tissue Kit protocol with elution in 100 µl of AE buffer. We quantified DNA extracts using a Qubit 3.0 Fluorometer (Life Technologies) and checked the quality using an agarose gel. Our 2RAD libraries were prepared with individual PCRs following the Adapterama III protocol (Bayona-Vásquez et al. 2019), size-selected for 450–550 base pairs (bp), and sequenced for approximately 10 million paired-end, 150-bp reads per sample on an Illumina NovaSeq. Libraries were prepared in the Pierson Lab at Kennesaw State University, size selection was performed on a Pippin Prep at the UT Genomics Core, and Illumina sequencing was performed by the Oklahoma Medical Research Foundation Clinical Genomics Center with approximately 10 million paired-end reads per sample.

GENOMIC DATA ASSEMBLY AND FILTERING: We demultiplexed and assembled sequence data using ipyrad (Eaton and Overcast 2020). We trimmed demultiplex reads to 130 bp, used a clustering threshold of 0.85, used a minimum depth of 6 reads, and required a locus to be present in at least 100 samples for inclusion in the final assembly. We filtered the final assembly using BCFtools (Danecek et al. 2021) to include only biallelic single nucleotide polymorphisms (SNPs) with a minor allele frequency <0.2 and thinned the dataset to include only one SNP per locus.

HYBRID INDEX ANALYSES: We estimated a genomic index of hybridization (HI) for all sampled individuals using the R package gghybrid (Bailey 2020), which uses a Bayesian approach to estimate admixture using Buerkle's likelihood formula (Buerkle 2005). The gghybrid package estimates HI on a scale from 0 to 1 based on the proportion of alleles originating from predefined parental reference samples (Buerkle 2005; Bailey 2020). We designated animals from putative non-hybrid sites as parental reference samples in our analyses, and these animals were assigned a HI of either 0 (*P. teyahalee*) or 1 (*P. shermani*). We required that a SNP have minimum allele frequency difference of 0.7 between parental populations for inclusion in my analyses. We used estimates from gghybrid to explore the relationship between morphological phenotype scores and HI using zero-one inflated beta regression (ZOIB) models in a Bayesian framework using the R package zoib (Liu 2021). The ZOIB model has three components: the beta component and two inflation components (zero and one inflation). The beta component describes the relationship between predictors and the response variable, assuming that the response follows a beta distribution. This component of the model only considers observations that are not 0 or 1 (in this case, hybrid animals where HI is between 0 and 1). The zero-inflation component models the probability of an observation being exactly zero, over and above what would be expected under the beta distribution (i.e., the likelihood of a salamander having 100% *P. teyahalee* ancestry, or H1=0). Like the zero-inflation component, the one-inflated component models the probability of an observation being exactly one, beyond what would be expected under the beta distribution (i.e., the likelihood of a salamander having 100% *P. shermani* ancestry or HI=1). We fit the ZOIB models using Stan (Stan Development Team 2022) and rstan (Stan Development Team 2018) in R (R Development Core Team 2023). Posterior samples were drawn using four independent Markov chain Monte Carlo (MCMC) chains, each with 150,000 iterations, including 1000 iterations for burn-in, and thinned by a factor of 50. A Normal prior distribution (mean 0, precision 1/0.001) was assumed for each regression coefficient, and a Uniform prior ($\sigma \sim$ unif(0, 20)) for the SD of the random effects. We assessed convergence using visual inspection of trace plots and examination of potential scale reduction factors (Gelman and Rubin 1992). We compared model fit using DIC (Spiegelhalter et al. 2002) and WAIC (Watanabe 2010) implemented in the R package rjags (Plummer et al. 2022). .

We were interested in modeling the relationships between metrics of hybridization and site-level geographic and climatic conditions that might exert some selective pressure on this hybrid system. Climate variables included mean daily vapor pressure deficit (VPD), mean daily temperature, and mean annual precipitation; geographic variables included slope, aspect, and elevation. Mean daily temperature and VPD values were obtained from Daymet gridded Daily Surface Weather Data sampled at a 1000 m spatial resolution from 2011 to 2021 (Thornton et al. 2022). Mean annual precipitation values were obtained from PRISM (Parameter-elevation Relationships on Independent Slopes Model) gridded climate data from 1990 to 2020 (PRISM Climate Group (Oregon State University) 2022). Originally in 800 m resolution, PRISM data were resampled to 1000 m using cubic convolution to align with Daymet data. We extracted elevation values from a digital elevation model (DEM) with a resolution of 1 m (United States Geological Survey 2021). Slope and aspect were calculated for each DEM grid cell in QGIS Version 3.26.0 (QGIS Association 2022) Because south facing slopes receive more direct sunlight, slope values were converted to absolute degree deviations from north (0°) to south (180°). All covariates were standardized prior to analyses.

We calculated the Pearson correlation coefficient between pairs of these variables and excluded any highly correlated combinations from our models. We used ZOIB models implemented in a Bayesian framework using the R package zoib to examine the relationships between climate, geography, and HI (Liu 2021). We constructed a global model using the combinations of non-correlated, standardized climate and geographic variables. We included site as a random effect to address the fact that we had multiple individuals from the same site. We used an iterative approach to model selection, systematically dropping covariates and comparing model fit using DIC (Spiegelhalter et al. 2002) and WAIC (Watanabe 2010) implemented in the R package rjags (Plummer et al. 2022). For each model, posterior samples were generated using three independent MCMC chains, each with 200,000 iterations, including 2000 iterations for burn-in, and thinned by a factor of 50. A Normal prior distribution (mean 0, precision 1/0.001) was assumed for each regression coefficient, and a Uniform prior ($\sigma \sim$ unif(0, 20)) for the SD of the random effects. We assessed convergence using visual inspection of trace plots and examination of potential scale reduction factors (Gelman and Rubin 1992).

We used Bayesian ordinal regression models in the R package brms (Bürkner 2017) to examine the relationship between climate variables and the distribution of each set of phenotype scores using a larger dataset that included animals collected from 25 sites across the Coweeta basin as part of several projects between 2010 and 2022. We constructed models using the same combinations of standardized climate and geographic variables included in the ZOIB models and again included site as a random effect. We evaluated model fit using WAIC (Watanabe 2010) and LOO (Vehtari et al. 2017).

We constructed genomic clines using Fitzpatrick's logit-logistic genomic cline function within gghybird (Fitzpatrick 2013). We compared those clines to a neutral model of introgression and identified outliers whose centers or slopes deviated from neutral expectations.

RESULTS

A total of 230 tissue samples were analyzed across 16 sites within the Coweeta basin as part of our genomic analyses. Of these, 47 were collected from 4 high-elevation sites where animals typically exhibit strong presentation of *P. shermani* phenotypes. An additional 52 tissues were collected from animals at 4 low-elevation sites where morphological phenotypes consistent with the traits of *P. teyahalee*. The remaining 131 samples were collected from sites of putative hybrids across 8 sites at intermediate elevations. Our filtered dataset contained 3258 SNPs. In our analysis of genomic clines, we identified 365 SNPs with cline centers biased toward *P. teyahalee*, compared with only 104 loci with *P. shermani* biased cline centers (Figure 2.2). We found that each of the two morphological phenotype scores were correlated with admixture on a broad scale. Genomic hybrid index was more strongly correlated with *P. teyahalee* score than with *P. shermani* score (Table 2.1). When modeled individually, PT score appeared to be a slightly better predictor of HI than PS score based on WAIC (Table 2.2, Models 2 and 1, respectively). However, the best predictor of HI was the additive model that incorporated both sets of phenotype scores (Table 2.2, Model 3). The beta component—modeling non-zero and non-one values of HI—of this model indicated that individuals with higher PT and those with lower PS scores were likely to have a lower HI, suggesting they had a larger proportion of *P. teyahalee* ancestry. The zero-inflated part of our model indicated that the likelihood that HI is 0 increased as PT scores increased, and PS scores decreased. The one-inflation component of our model demonstrated that the likelihood of an animal having an HI of 1 (implying pure *P. shermani* ancestry) decreased with increasing PT scores. The influence of PS scores was not clearly defined in the one-inflated model component. When considered together, the results of this model demonstrate the importance of considering the combined effect of both scores in predicting genomic ancestry (Figure 2.3).

Our comparison of climate and geographic variables revealed that elevation, mean annual precipitation, and temperature were all tightly correlated within this system (Table 2.1). Because of this, we could only include one of these metrics as a covariate in our models. Though elevation has historically been used to describe the variation in this hybrid zone, we argue that climate is more likely to be driving the patterns we observe. However, because the climate variables were so strongly correlated, we were unable to empirically assess their relative contributions to the structure of this system. We elected to include mean annual precipitation as the climate covariate in our models because we believe this metric reflects the conditions with the strongest impacts on salamander ecology.

The best fitting model of the relationship between genomic ancestry, geography, and climate (Model 8) incorporated mean annual precipitation, slope, and aspect (north-south) as predictors (Table 2.3). The beta component of the model indicated a positive relationship between non-extreme HI values and precipitation, and negative relationships with aspect and slope. This suggests that in areas with high rainfall, there is an increased probability of salamanders exhibiting higher hybrid indices indicative of greater *P. shermani* ancestry. Conversely, the probability of salamanders having lower hybrid indices, indicative of more *P. teyahalee* ancestry, increases as the site becomes more north-facing or steeper. The zero-inflation submodel indicated that the probability of a salamander having a hybrid index of exactly zero (pure *P. teyahalee*) decreases as annual precipitation increases, the aspect becomes more north-facing, and slope steepness increases. The one-inflation model component showed varying impacts of the covariates. Specifically, the probability of a salamander having a hybrid index of exactly one (pure *P. shermani*) increases with higher precipitation and steeper slopes, though this probability decreases as the aspect becomes more north-facing. However, there is considerable uncertainty around these effects, especially the effect of precipitation in the one-inflation submodel, and they should be interpreted with some caution. Collectively, these model results demonstrate a pattern in which areas of higher precipitation are associated with increased probability of salamanders having a higher hybrid index, suggesting a higher proportion of *P. shermani* ancestry (Figure 2.4). In contrast, as sites become steeper and more north-facing, the probability increases for salamanders to have lower hybrid indices, indicative of more *P. teyahalee* ancestry, although these effects are smaller than that of precipitation.

Mean annual precipitation, slope, and north-south aspect were again the best predictors in ordinal regression models for both sets of phenotype scores. These predictors were significant in both models, however their relationships to the two response variables differed substantially. In the model of PS scores precipitation and north-south aspect both had a positive effect on sores with a negative effect of slope. This relationship was reversed in the models of PS scores, which showed a negative effect of slope and a positive effect of both precipitation and north-south aspect. We predicted the probability of observing each phenotype score across a range of precipitation values and found that as precipitation increased the probability of observing the highest PS score (3) increased while the probability of observing the lowest PS score (0) declined (Figure 2.5). This pattern is reversed for the highest and lowest PT scores. The probabilities of observing intermediate scores were somewhat different, with PS score of 1 reaching its highest probability at intermediate levels of precipitation and declining toward either extreme. The probability of observing a PS score of 2 had a similar shape, but was shifted to the left, reaching its peak at higher precipitation. Again, this relationship was reversed for intermediate PS scores.

DISCUSSION

We found that phenotype score was generally indicative of *Plethodon shermani* and *P. teyahalee* ancestry at a broad, basin-wide scale; however, among individuals of hybrid ancestry there was significant phenotypic variation within a local site that was only weakly correlated with more limited variation in genomic hybrid index. Stated differently, higher local variability of color phenotypes was indicative of intermediate, mixed ancestry for individuals at that locale, but within a site, phenotype score told us little about an individual's hybrid ancestry.

By extension, we do not advocate—as other have done—for the use a single composite score to characterize the relative ancestry of hybrid individuals or focusing exclusively on the proportion of individuals of "pure" phenotypes to describe patterns of hybridization across larger geographic extents (e.g., Hairston et al. 1992; Walls 2009). At the broader scale, we found that the slope of the relationship between *P. shermani* score and genomic hybrid index was shallower than the relationship between *P. teyahalee* score and genomic hybrid index. However, the best model of hybrid index incorporated both phenotypes. Using composite phenotype scores (e.g., subtracting the *P. teyahalee* score from the *P. shermani* score) obscures important differences in levels of admixture. For example, a salamander with a *P. shermani* score of 2 and a *P. teyahalee* score of 1 (net score equals 1) had an estimated hybrid index of 0.75, while a salamander with a *P. shermani* score of 1 and a *P. teyahalee* score of 0 (net score also equals 1) had an estimated hybrid index of 0.24.

Spatial patterns of phenotypic scores and genomic hybrid indexes indicate that the hybrid zone is under active selection in relation to climate. Both phenotype scores and genomic hybrid indexes were non-randomly distributed across the basin and correlated with climate in a pattern consistent with the distributions of the two parent lineages. Mean annual precipitation was a strong predictor of hybrid index overall; however, the relationship between precipitation and phenotype scores was more complex, particularly at intermediate levels of precipitation where there was considerable overlap in the probability of observing multiple phenotype scores.

Prior studies have demonstrated that phenotypic patterns within the Coweeta Basin and at other sites within the region are correlated with elevation (Hairston et al. 1992; Walls 2009). Elevation was a proxy for climatic variation, with higher elevation sites having lower mean daily vapor pressure deficits, more frequent and higher amounts of precipitation, and lower average daily temperatures, though nighttime air temperatures are not consistently cooler at higher elevations; (Novick et al. 2016). Because mean annual rainfall, mean daily vapor pressure deficit, and mean daily temperature were so highly correlated, we could not distinguish the relative importance of these aspects of climate on phenotypic or genotypic patterns within the Coweeta Basin. This might be resolved by studies over a wider geographic extent with greater potential for climate variation and weaker correlations among climate variables. We note that other studies of *Plethodon* responses to climate change focus on temperature change (Milanovich et al. 2010; Gifford and Kozak 2012) which is easier to predict, as a driver of potential range shifts, introgression, or population declines of *Plethodon*; however, the stronger effects of moisture on plethodontid ecology are well understood (Feder 1983) and, therefore, may be an equal or more important gradient for ecological and evolutionary interactions between *Plethodon* species. Until the relative importance of these aspects of climate are known, we should be cautious with predictions based solely on temperature when forecasting how climate change will affect *Plethodon* ecology and hybrid zone dynamics.

Patterns of outlier loci indicated that both *P. shermani* and *P. teyahalee* alleles appeared to be under active selection but with biased introgression of *P. teyahalee* alleles. Prior studies of high and low elevation sites within the Coweeta Basin and based only on the proportions of "pure" coloration phenotypes have also suggested biased selection for *P. teyahalee* traits (Hairston et al. 1992; Walls 2009). Those studies found no evidence of declining pure *P. teyahalee* at lower elevation. However, despite limited morphological evidence of migration of *P. shermani* traits toward warmer, drier, lower elevation sites, the genomic data in our study reveal biased introgression of a small proportion of *P. shermani* traits across the hybrid zone. Nonetheless, we identified 3.5 times the number of loci with *P. teyahalee* alleles with signals of selection. Hairston et al. (1992) hypothesized that selection for *P. teyahalee* traits at higher elevation sites may have started in the early 20th century in response to widespread forest clearing altering local climates.

Walls (2009) hypothesized that decline in pure *P. shermani* phenotypes at high elevation was caused by rising temperatures. Long-term climate data from the Coweeta Basin indicates that temperature has been increasing since the 1970s, but precipitation has become more variable and extreme (wetter wet years and drier dry years) with increasing drought frequency and intensity (Caldwell et al. 2016). We hypothesize that the more variable climate—as it relates to rainfall—may be selecting for both *P. shermani* and *P. teyahalee* traits across the hybrid zone, but selection for *P. teyahalee* traits may be stronger because of increasing frequency and intensity of periods without rainfall and rising temperatures leading to higher vapor pressures deficits. It is important to note that both Hairston et al. (1992) and, by extension, Walls (2009) relied exclusively on phenotypic data as an indicator of ancestry. While phenotype scores do appear to be broadly correlated with genomic ancestry, there is considerable uncertainty surrounding these relationships. Because of this, we believe that inferences about the structure and dynamics of this hybrid system based on phenotype data be interpreted with some caution. Additionally, more recent work by Lowe (2016) has provided compelling evidence that this hybrid zone is stable and *P. shermani* and *P. teyahalee* are not in the process of merging. This finding raises additional questions about the purported pattern of hybrid zone expansion described by Hairston et al. (1992).

There is much we need to know about adaptive introgression between these and other salamander species. Genomic clinal patterns indicate selection at loci from both parent lineages, but we do not yet know what those regions under selection represent. The role of gene flow (e.g., introgression) for facilitating adaptation to climate change is likely important but is generally missing from management and conservation frameworks. The status of hybrid populations is often unaddressed in legal and regulatory frameworks or on occasion deemed a threat to the persistence of "species."

This research contributes to our understanding of how contemporary evolutionary interactions among species may determine how they respond to climate change. This is key knowledge needed for understanding how organismal responses to climate will lead to future patterns of biodiversity. These results help to shape our understanding of how moisture-sensitive organisms may adapt to changes in precipitation—a component of climate change often overshadowed by a focus on changing temperature.

TABLES

Table 2.1. Pearson correlation coefficient matrix calculated for metrics of hybridization in *Plethodon shermani* and *P. teyahalee* (i.e., genomic hybrid index, phenotype score) and climate and geographic variables measured from sites distributed across the Coweeta basin, Macon Co., NC, USA.

	Mean VPD	Temperature	N-S Aspect	Slope	Elevation	Precipitation	PT	PS
HI	-0.857	-0.842	0.136	-0.069	0.822	0.962	-0.823	0.619
PS	-0.676	-0.671	0.175	0.062	0.663	0.648	-0.532	1
PT	0.753	0.744	-0.167	0.054	-0.732	-0.813	1	--
Precipitation	-0.894	-0.881	0.234	-0.006	0.888	1	--	--
Elevation	-0.964	-0.963	0.472	0.098	1		--	--
Slope	-0.134	-0.141	0.544	1	--	--	--	--
N-S Aspect	-0.407	-0.425	1	--	--	--	--	--
Temperature	0.999	1	--	--	--	--	--	--

Table 2.2. Posterior means and 95% credible intervals from Bayesian zero-one inflated beta models of the relationships between genomic hybrid index of *Plethodon* and phenotypic lineage scores of animals sampled across the Coweeta Basin, Macon Co., NC, USA.

			Mod01		Mod02		Mod03		Mod04	
Model Component	Parameter		Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
logit(mean)	Intercept	b[1]	0.33	[-0.92, 1.69]	1.76*	[0.5, 3.05]	1.23	[-0.04, 2.47]	0.66	[-0.71, 2.07]
	PS	b[2]	0.23*	[0.08, 0.38]	--	--	0.14*	[0, 0.29]	0.38*	[0.06, 0.7]
	PT	b[3]	--	--	-0.33*	[-0.45, -0.22]	-0.31*	[-0.42, -0.2]	-0.09	[-0.37, 0.18]
	PS * PT	b[4]	--	--	--	--	--	--	-0.1	[-0.22, 0.02]
logit(Pr(y = 0)	Intercept	b0[1]	4.62*	[2.97, 6.36]	-13.89*	[-19.99, -9.36]	-9.63*	[-16.31, -4.32]	-5.92	[-19.96, 9.25]
	PS	b0[2]	-3.44*	[-4.19, -2.78]	--	--	-1.89*	[-2.76, -1.09]	-5.07	[-17.19, 2.49]
	PT	b0[3]	--	--	3.74*	[2.62, 5.28]	3.39*	[2.1, 5.05]	2.45	[-1.42, 6.08]
	PS * PT	b0[4]	--	--	--	--	--	--	0.8	[-1.15, 3.87]
logit(Pr(y = 1)	Intercept	b1[1]	-5.97*	[-9.48, -2.84]	10.81	[-0.9, 32.61]	7.25	[-4.72, 25.94]	3.07	[-17.23, 32.23]
	PS	b1[2]	1.01*	[0.07, 2.08]	--	--	0.74	[-0.24, 1.88]	5.05	[-5.56, 18.96]
	PT	b1[3]	--	--	-13.13*	[-34.95, -1.56]	-11.95*	[-30.48, -1.26]	-7.79	[-36.6, 12.03]
	PS * PT	b1[4]	--	--	--	--	--	--	-4.28	[-18.2, 6.26]
	Dispersion	*d*	2.57*	[1.34, 3.91]	2.86*	[1.62, 4.14]	2.76*	[1.58, 3.92]	2.75*	[1.6, 3.92]
	SD of random effect	σ	5.02*	[1.97, 12.12]	5.03*	[1.89, 12.3]	4.52*	[1.73, 10.97]	4.31*	[1.67, 10.32]
	DIC		427960		427883		427858		427857	
	Multi-variate psrf		1		1.05		1.02		1.02	
	WAIC		428012.2		427932.1		427912		427914.7	

Table 2.3. Posterior means and 95% credible intervals from Bayesian zero-one inflated beta models of the relationships between genomic hybrid index of *Plethodon* and climate and geographic conditions across the Coweeta Basin, Macon Co., NC, USA.

			Mod05		Mod06		Mod07		Mod08	
Model Component	Parameter		Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
logit(mean)	Intercept	b[1]	0.09	[-0.36, 0.52]	0.21	[-0.3, 0.76]	0.08	[-0.34, 0.53]	0.17	[-0.32, 0.7]
	Precipitation	b[2]	3.09*	[2.81, 3.38]	2.89*	[2.56, 3.21]	3.01*	[2.71, 3.31]	2.78*	[2.44, 3.1]
	Aspect	b[3]	--	--	0.53*	[0.16, 0.92]	--	--	0.54*	[0.17, 0.93]
	Slope	b[4]	--	--	--	--	-0.17	[-0.42, 0.07]	-0.29*	[-0.57, -0.01]
logit(Pr(y = 0)	Intercept	b0[1]	-6*	[-8.05, -4.3]	-6.78*	[-9.44, -4.7]	-6.08*	[-8.25, -4.36]	-25.51*	[-61.39, -8.02]
	Precipitation	b0[2]	-4.99*	[-6.66, -3.58]	-6.27*	[-8.47, -4.49]	-5.15*	[-6.88, -3.71]	-22.5*	[-52.76, -7.74]
	Aspect	b0[3]	--	--	1.47*	[0.66, 2.32]	--	--	10.01*	[3.43, 22.76]
	Slope	b0[4]	--	--	--	--	0.2	[-0.56, 0.97]	-9.19*	[-22.03, -2.53]
logit(Pr(y = 1)	Intercept	b1[1]	-10.33*	[-18.87, -4.91]	-16.25*	[-40.12, -5.51]	-8.89*	[-16.17, -4.78]	-24.95*	[-60.26, -6.89]
	Precipitation	b1[2]	8.61*	[4.2, 15.58]	12.86*	[4.51, 31.14]	7.66*	[4.21, 13.52]	19.29*	[5.65, 46.18]
	Aspect	b1[3]	--	--	1.64	[-0.44, 5.12]	--	--	5.21*	[1.11, 11.19]
	Slope	b1[4]	--	--	--	--	0.76	[-0.13, 1.7]	1.72*	[0.34, 3.47]
	Dispersion	*d*	4.37*	[3.88, 4.84]	4.34*	[3.79, 4.92]	4.37*	[3.89, 4.87]	4.31*	[3.78, 4.86]
	SD of random effect	σ	0.62*	[0.23, 1.49]	0.85*	[0.3, 2.15]	0.61*	[0.22, 1.5]	0.73*	[0.26, 1.89]
	DIC		427529		427513		427525		427493	
	Multivariate psrf		1.01		1.02		1		1	
	WAIC		427564.2		427553.2		427566.7		427539.1	

Table 2.4. Summary of Bayesian ordinal regression models estimating the relationship between climate and geographic site level covariates on *Plethodon shermani* (A) and *P. teyahalee* (B) phenotype scores. Rhat = 1 for all parameters for all models.

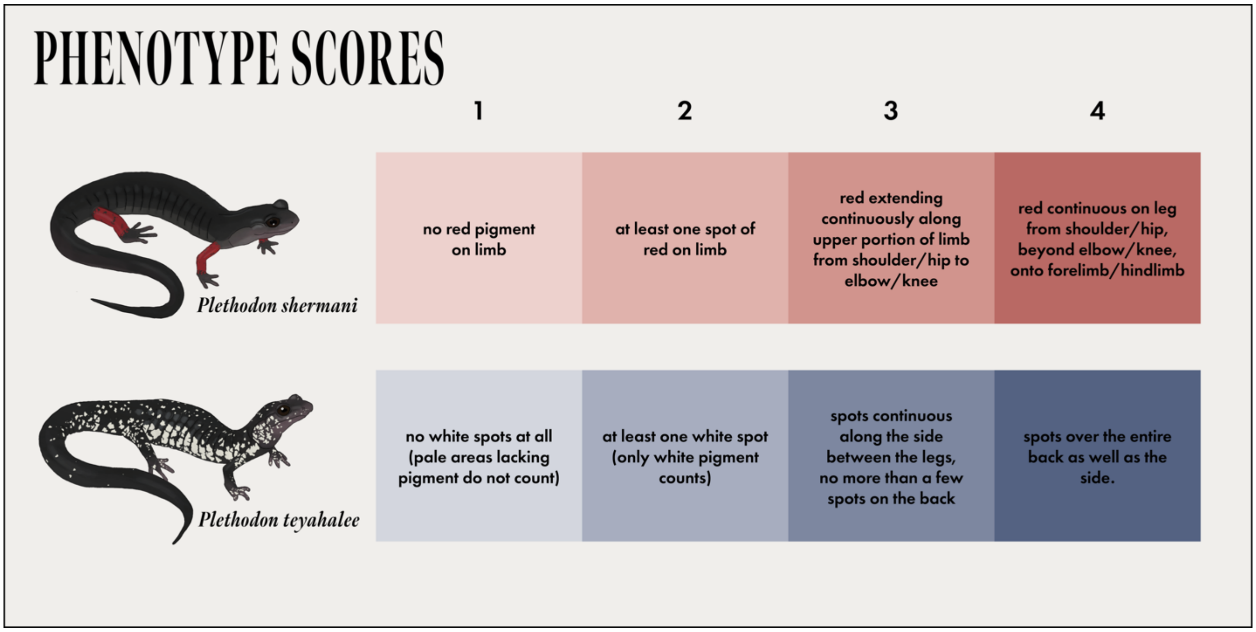
A. *Plethodon shermani*

		Mod09					Mod10					Mod11					Mod12				
		Est.	Est. Error		Bulk ESS	Tail ESS	Est.	Est. Error	95% CI	Bulk ESS	Tail ESS	Est.	Est. Error	95% CI	Bulk ESS	Tail ESS	Est.	Est. Error	95% CI	Bulk ESS	Tail ESS
Group Level Effects (Site: 35 levels)	sd (Intercept)	0.73	0.17	[0.47,1.12]	2698	4414	0.67	0.14	[0.44,0.97]	3852	6507	0.86	0.17	[0.57,1.24]	3470	6371	0.69	0.13	[0.47,0.98]	4409	7546
Population-Level Effects	Intercept [1]	-1.9	0.16	[-2.19,-1.57]	2166	3308	-2.24	0.15	[-2.54,-1.93]	3179	5544	-1.74	0.19	[-2.07,-1.34]	3158	4683	-2.13	0.17	[-2.46,-1.79]	4197	6735
	Intercept [2]	0.26	0.16	[-0.02,0.6]	2146	3324	-0.07	0.15	[-0.38,0.23]	3136	5371	0.43	0.19	[0.09,0.83]	3156	4696	0.04	0.17	[-0.29,0.38]	4151	6721
	Intercept [3]	1.47	0.16	[1.19,1.81]	2204	3507	1.14	0.15	[0.83,1.44]	3185	5642	1.64	0.19	[1.3,2.04]	3196	4912	1.25	0.17	[0.92,1.59]	4157	6842
	Precipitation	0.6	0.1	[0.38,0.77]	2900	4812	0.73	0.08	[0.57,0.87]	5157	8347	0.63	0.09	[0.44,0.8]	5257	7856	0.77	0.08	[0.61,0.92]	7428	9259
	Aspect(N-S)	--	--	--	--	--	--	--	--	--	--	0.31	0.13	[0.07,0.59]	4655	7269	0.23	0.11	[0.03,0.45]	6745	8508
	Slope	--	--	--	--	--	-0.41	0.1	[-0.62,-0.22]	5301	7681	--	--	--	--	--	-0.4	0.1	[-0.61,-0.21]	6889	9417
Family specific parameters (sratio)	disc	1	0	[1,1]	NA	NA	1	0	[1,1]	NA	NA	1	0	[1,1]	NA	NA	1	0	[1,1]	NA	NA
Leave-One-Out Cross-Validation (LOO-CV)	elpd loo	-14691	64.6				-14684	64.6				-14684	64.6				-14682	64.6			
	p loo	31.4	1				31.5	1				32.6	1				31.9	1			
	looic	29382	129.2				29368	129.2				29368	129.1				29363	129.2			

B. *Plethodon teyahalee*

		Mod13					Mod14					Mod15					Mod16				
		Est.	Est. Error	95% CI	Bulk ESS	Tail ESS	Est.	Est. Error	95% CI	Bulk ESS	Tail ESS	Est.	Est. Error	95% CI	Bulk ESS	Tail ESS	Est.	Est. Error	95% CI	Bulk ESS	Tail ESS
Group Level Effects (Site: 35 levels)	sd (Intercept)	2	0.4	[1.32,2.85]	2689	5065	1.07	0.22	[0.71,1.55]	3257	6727	1.76	0.31	[1.24,2.45]	3368	6538	1.03	0.19	[0.71,1.46]	3832	7639
Population-Level Effects	Intercept [1]	-1.25	0.35	[-1.96,-0.57]	1772	3019	-0.25	0.25	[-0.71,0.27]	2120	4112	-1.36	0.31	[-1.99,-0.76]	2829	5051	-0.43	0.25	[-0.9,0.07]	2604	4895
	Intercept [2]	-0.98	0.35	[-1.7,-0.3]	1776	3108	0.02	0.25	[-0.44,0.54]	2138	4053	-1.09	0.31	[-1.71,-0.49]	2843	5038	-0.16	0.25	[-0.63,0.34]	2597	4762
	Intercept [3]	1.35	0.35	[0.63,2.02]	1776	3145	2.35	0.25	[1.88,2.87]	2162	4359	1.24	0.31	[0.61,1.84]	2852	5114	2.17	0.25	[1.69,2.67]	2635	5131
	Precipitation	-0.34	0.14	[-0.61,-0.08]	5080	7314	-1.06	0.11	[-1.28,-0.85]	3643	5550	-0.84	0.16	[-1.14,-0.52]	3929	6728	-1.18	0.12	[-1.42,-0.96]	4286	7473
	Aspect(N-S)	--	--	--	--	--	--	--	--	--	--	-0.78	0.22	[-1.22,-0.37]	4215	7235	-0.37	0.14	[-0.67,-0.1]	4542	8446
	Slope	--	--	--	--	--	0.9	0.15	[0.62,1.21]	3032	6334	--	--	--	--	--	0.82	0.14	[0.56,1.11]	4049	7752
Family specific parameters (sratio)	disc	1	0	[1,1]	--	--	1	0	[1,1]	--	--	1	0	[1,1]	--	--	1	0	[1,1]	--	--
Leave-One-Out Cross-Validation (LOO-CV)	elpd loo	-14030	74.5				-14029	74.5				-14027	74.5				-14027	74.5			
	p loo	34.2	1.4				32.4	1.2				34.1	1.5				32.5	1.2			
	looic	28060	149				28058	149				28055	149.1				28055	149			

FIGURES



PHENOTYPE SCORES

	1	2	3	4
Plethodon shermani	no red pigment on limb	at least one spot of red on limb	red extending continuously along upper portion of limb from shoulder/hip to elbow/knee	red continuous on leg from shoulder/hip, beyond elbow/knee, onto forelimb/hindlimb
Plethodon teyahalee	no white spots at all (pale areas lacking pigment do not count)	at least one white spot (only white pigment counts)	spots continuous along the side between the legs, no more than a few spots on the back	spots over the entire back as well as the side.

Figure 2.1. Phenotype scoring guidelines for *Plethodon shermani* (top) and *P. teyahalee* (bottom) based on the criteria outlined by Hairston (1973) and Hairston et al. (1992).

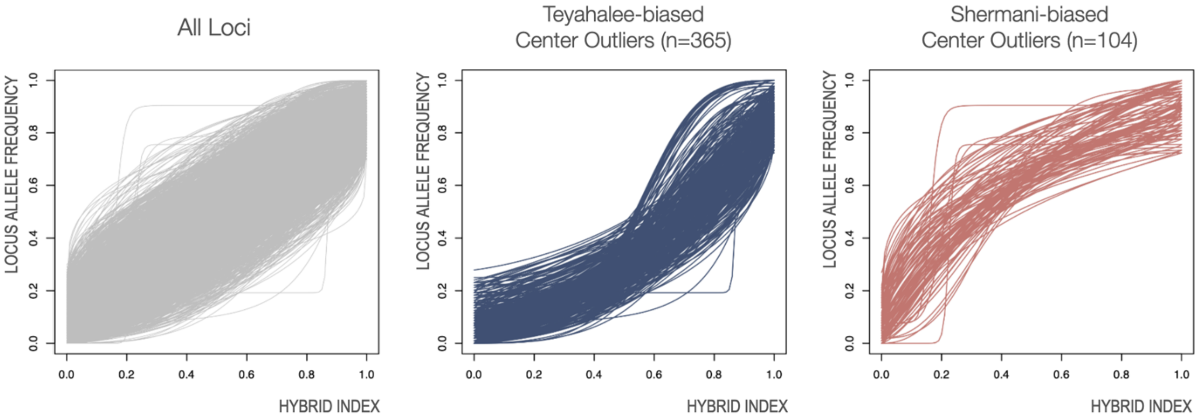


Figure 2.2. Genomic cline centers for all loci (left) and loci with cline center outliers biased towards *P. teyahalee* (center) or *P. shermani* (right).

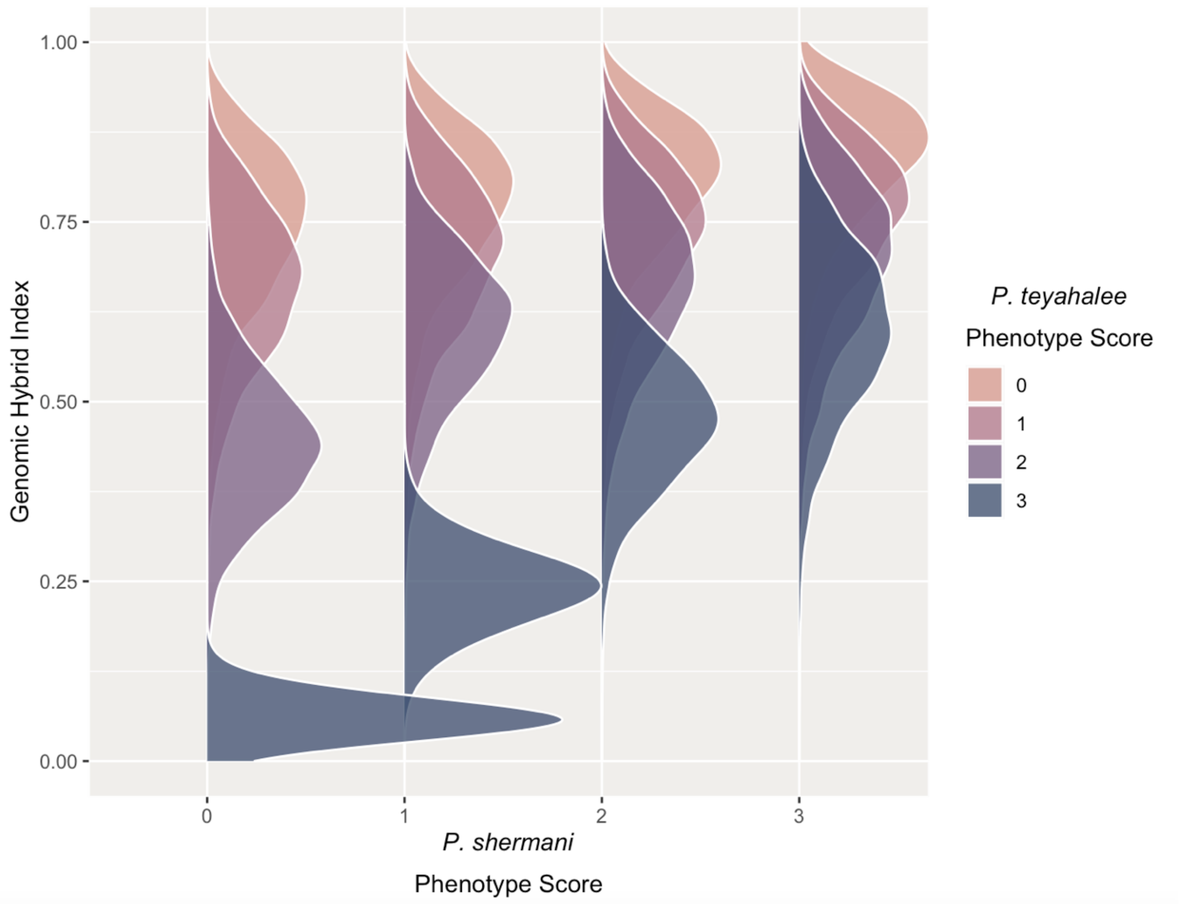


Figure 2.3. Posterior predicted samples from the top Bayesian ZOIB model (Model 3) predicting genomic hybrid index for each combination of *Plethodon shermani* and *P. teyahalee* phenotype scores.

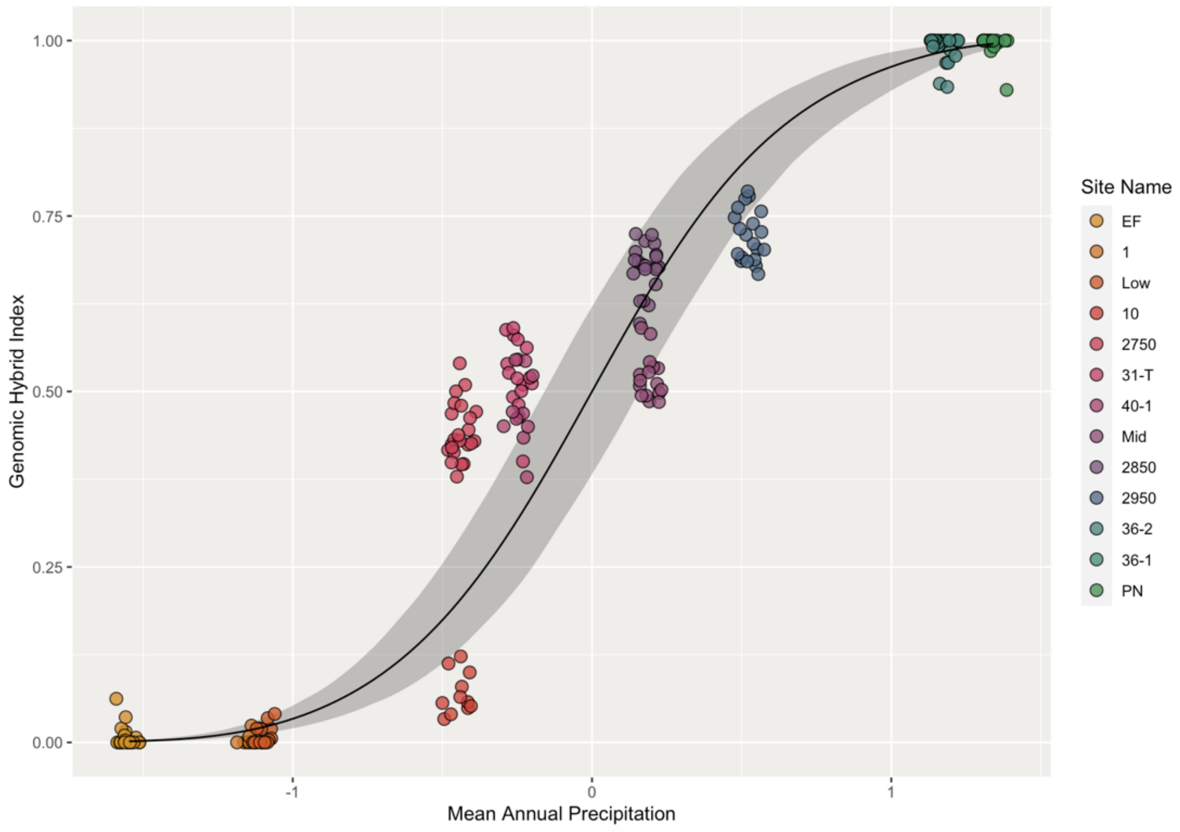


Figure 2.4. Posterior predicted samples from Model 8 predicting genomic hybrid index across a range of mean annual precipitation values observed in the Coweeta basin between 1991 and 2021. Points represent the genomic hybrid index of salamanders sampled from sites across the Coweeta basin.

CHAPTER 3:

ESTIMATING BEHAVIORAL DIFFERENCES IN SURFACE ACTIVITY ACROSS A SALAMANDER CLINAL HYBRID ZONE[1]

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[1] Carter, C.M., H.E. Gaya, and J.C. Maerz. To be submitted to *Journal of Herpetology*.

ABSTRACT

This study investigates the behavioral dynamics of *Plethodon shermani*, *P. teyahalee*, and their hybrids within the Coweeta basin, focusing on above and below-ground activity patterns. Tracking data from 125 tagged individuals revealed that rainfall and body size significantly influenced salamander surface activity. Contrary to expectations, our study revealed no evidence of differences in surface activity propensity between *P. teyahalee*, *P. shermani*, or their hybrids under equivalent weather conditions. Consequently, variation in realized rates of surface activity among these lineages appear to be a direct function of differences in rainfall levels at different sites. Despite prevailing assumptions, our findings do not support the notion that *P. teyahalee* may possess adaptations that allow them to increase time spent on the surface in warmer, drier climates. Where this the case we would expect for *P. teyahalee* to exhibit different behavioral responses to climatic conditions compared to *P. shermani* or their hybrids. Notably, hybrids also did not exhibit different behaviors from either parent lineage in relation to surface activity; their responses were again consistent with patterns in local rainfall conditions. The lack of discernible differences in surface activity across the studied lineages suggests limited adaptive variation with regard to this behavior. These insight prompt further exploration into how these salamanders compensate for environmental constraints on activity and how such adaptations might influence their responses to future climate shifts.

INTRODUCTION

The persistence of a population within a particular environment is contingent upon the ability of organisms to meet their physiological and ecological requirements for survival, growth, and reproduction. This can be particularly challenging for organisms that inhabit environments where conditions are not consistently suitable across time and/or space. Some species manage to survive in these challenging environments through adaptive mechanisms that allow them to adjust their physiology or behavior in response to changing conditions. This adaptability can be particularly important in regions experiencing rapid climate change, where long-term survival may depend on an organism's ability to modify its behavior in response to novel environmental cues.

Salamanders are known to modulate their behavior based on the surrounding environmental conditions, such as temperature and precipitation. As ectotherms, their body temperature, metabolic rate, and overall physiological performance are greatly influenced by ambient environmental conditions. Like many terrestrial animals (Siepielski et al. 2017), an important selective pressure upon terrestrial salamanders stems from their sensitivity to evaporative water loss. Water loss constrains surface activity, which reduces foraging and mating opportunities (Jaeger 1979; Feder 1983). More frequent or prolonged periods without rain and drier soils also reduce survival rates, particularly among smaller juveniles (Peterman and Semlitsch 2014; Howard 2018). Behavior (e.g., surface activity) is the most flexible and immediate way that *Plethodon* can respond to variation in weather and is expected to mediate the influence of climate change upon their populations (Gade et al. 2020).

In some lineages, physiological tolerances evolve slowly due to the maintenance of ancestral ecological traits, a concept known as phylogenetic niche conservatism. Modifying behavior—e.g., altering their microhabitat use or the frequency and duration of surface activity—may alleviate selective pressure for physiological or morphological adaptations, and therefore, be either a driver of or response to niche conservatism in physiological or morphological traits (Muñoz et al. 2014). For salamanders, surface activity can play a crucial role in foraging, reproduction, and thermoregulation, and yet exposes the salamanders to potential risk of desiccation (Feder 1983). Therefore, understanding how environmental conditions influence salamander surface activity and whether behavioral responses vary among species or lineages is an important step in disentangling the complex relationships that govern the survival and distribution of organisms across changing landscapes.

In this study, we focus on a hybrid system that included of two recently diverged Appalachian salamanders, *Plethodon teyahalee* and *Plethodon shermani*, and examine the interplay between behavior and environmental conditions among these lineages. Specifically, we used passive integrated transponder (PIT) tags for individual identification and location tracking of salamanders across the hybrid zone to document their movement and behavior, and then we developed a multistate capture-recapture model within a Bayesian framework to establish the relationship between phenotype, behavior, and climate. Using this approach, we estimate whether these species exhibit different strategies in allocating time to surface activities versus those occurring below-ground, and how these strategies were influenced by environmental factors such as rainfall and temperature. *P. teyahalee*, which generally occurs at drier sites (Carter, Chapter 2), may experience reduced surface activity but exhibit a greater propensity to be active during wet weather as a means of compensating for reduced rain availability. Alternatively, *P. teyahalee* may exhibit greater reluctance to be surface active even during rain events to reduce evaporative water loss. In contrast, *P. shermani*, which generally occur at wetter sites, may experience greater surface activity but exhibit a reduced propensity to be surface active on a given night because wet conditions are less limited. Alternatively, *P. shermani* may be more inclined to be active on a given night because evaporative water loss is less likely to constrain future opportunities for surface activity. We aimed to understand how these strategies differ as a function of ancestry and explore ideas of how selection might shape hybrid traits in intermediate or variable climates. In other words, do salamanders of hybrid ancestry that occupy a range of intermediate climates (Carter, Chapter 2) exhibit intermediate levels of surface activity? Ultimately, this study aims to enhance our understanding of behavioral adaptability in salamanders in the face of changing environmental conditions and the potential consequences for behavioral adaptive introgression to contribute to the persistence of salamanders under future climates.

METHODS

PART 1: CAPTURE, TAG, RELEASE

INITIAL SAMPLING: We conducted night-time surveys at nine sites distributed at various elevations across the Coweeta basin. Three sites were located within the range "pure" *P. shermani*, three were within the *P. shermani* × *teyahalee* hybrid zone, and three were within the range of "pure" *P. teyahalee*. Each site covered an approximately 25- by 25-meter area. During Part 1 of this study, we collected approximately 15 individuals from each site with a snout-vent length (SVL) of >40mm (juveniles and/or adults). The experimental design of this study was partially dictated by permitting restrictions imposed by the North Carolina Wildlife Resources Commission. In an effort to limit the impact of research activities on salamander populations, we were not permitted to include gravid adult females in our study and could only collect from nine sites within the basin. Upon capture, each animal was candled to determine reproductive status and any gravid females were immediately released. Each remaining salamander was assigned a unique identification number. The time, general location of initial capture (site), and a description of the specific habitat (e.g., in a burrow, climbing on downed woody debris, etc.) was recorded for each individual. All salamanders were photographed, measured (SVL and total length), and weighed. We also assigned phenotype scores (0 to 3) for each animal based on their expression of *P. shermani* and *P. teyahalee* traits (Hairston 1973; Hairston et al. 1992; and Carter, Chapter 2). The *P. shermani* scores were assigned based on the amount of red present on the legs, and the *P. teyahalee* scores were assigned based on the amount of white spotting on the body (see Carter, Chapter 2 for greater detail). For each scoring system, a higher value indicates increased morphological resemblance to that parent lineage.

CAPTIVE CARE: Salamanders were placed individually into 1-liter containers (approximately 20 cm long, 15 cm wide, and 5 cm deep) and each container was lined with clean, moist paper towels to mimic wet leaf-litter and keep the salamanders hydrated. Salamanders were housed in these containers until they were released back into their plots at the conclusion of Part 1 of this study (approximately 3 to 5 days after capture). The moist paper towels in each container were replaced daily to ensure the containers remained clean and provide adequate hydration for the salamanders. Salamanders were transported from the Coweeta basin (Otto, North Carolina) to the Maerz Lab (Athens, Georgia) in individual containers in a temperature-regulated vehicle.

PIT TAG IMPLANTATION:

PRE-SURGICAL PREP: Prior to surgery, all instruments (e.g., scalpel, PIT tag, and forceps) were sterilized by soaking them in gluteraldehyde for 30 minutes and then rinsed with sterile water. The person performing the surgery washed their hands with anti-bacterial soap before donning sterile nitrile surgical gloves rinsed with dechlorinated water.

ANESTHETIZATION: Salamanders were anesthetized by immersing them in a pH-neutral, buffered solution of ethyl 3-aminobenzoate methanesulfonic acid (tricaine methanesulfonate or MS-222; supplied by Syndel; Ferndale, Washington) at a concentration of 500 to 1000 mg/L (Cecala et al. 2007; Peterman and Semlitsch 2014). Animals were monitored and considered anesthetized once they no longer displayed reflexive and voluntary responses to gentle pinching of the legs and tail with forceps (approximate time to induction was between 10 and 30 minutes).

SURGERY: Anesthetized animals were placed into a sterilized tray lined with clean paper towels, moistened with sterile water. Prior to implantation, each PIT tag was read using a

Biomark HPR Plus Reader (Biomark; Boise, Idaho) to ensure the tag was functional, and the ID number was recorded for each salamander. PIT tags were implanted using the surgical procedures outlined by Connett and Semlitsch (2012). Using a sterilized scalpel, a small incision was made in the skin anterior to the left hind limb. The PIT tag was gently inserted into the incision and pushed forward toward the body cavity using forceps. PIT tags were 12.5 mm long with a 2.03 mm diameter and a mass of 106 mg (APT12, Biomark; Boise, Idaho). Skin secretions produced by the salamander were sufficient to hold the incision closed and no additional use of sutures or adhesives was required. One centimeter of tail tissue was collected from each animal for genomic analysis. Tissue samples were obtained by applying pressure 1 cm from the distal tip of the tail with clean blunted forceps and placing the autotomized tissue in 70% ethanol for future analysis.

POSTOPERATIVE CARE: Immediately following surgery, salamanders were placed into a shallow dish of dechlorinated fresh water and monitored until they recovered motor function. PIT tags were read again to verify that they were still functional and that the number had been accurately recorded. Salamanders were returned to their containers lined with clean, moist paper towels. Each day until release, salamanders were inspected and had their containers cleaned to ensure full recovery and retention of PIT tags.

RELEASE AND INITIAL MONITORING: After recovering from surgery, each salamander was transported back to the Coweeta basin and released after dark at their initial point of capture. In most cases, animals were tracked approximately one hour after release to document their behavior and movement during the first night; their last known location was marked with a pin flag.

PART 2: LOCATION AND BEHAVIOR TRACKING

SURVEY DESIGN: Following the initial release, we continued to periodically monitor the behavior and movement of tagged salamanders. During this initial phase of the study, the ability to monitor tagged salamanders was tempered by the need to collect and tag additional individuals. Typically, sampling occurred over the course of three nights, with approximately one-third of the sites visited each night. Whenever possible, multiple elevations were sampled each night to avoid confounding elevation and environmental variables. The order that elevations were sampled each night was also randomized as much as possible to avoid confounding elevation and sampling time. We concluded sampling for the season in October 2020 once salamanders became inactive due to low temperatures. In May of 2021, we resumed surveys using the same model (all sites sampled over a period of 2 to 3 nights). We conducted more frequently sampling during the active season (June).

SAMPLING PROTOCOL: At each site, observers recorded the time and weather conditions at both the start and end of sampling. Air temperature, relative humidity, and wind speed were measured using a Kestrel 3500 Pocket Weather Meter (Kestrel Instruments; Boothwyn, Pennsylvania), and soil temperature was measured using a Rapitest digital soil thermometer (Luster Leaf Products, Inc.; Woodstock, Illinois); qualitative scores of sky and moon conditions were also recorded. An observer would begin sampling by locating the pin flag marking an individual salamander's last known location. The observer would then scan the ground immediately surrounding that flag using an RFID system (Biomark HPR Plus Reader and BP Lite Portable Antenna, Biomark; Boise, Idaho) and incrementally expand the radius of the search area until either the salamander was located, or five minutes had passed. If a salamander was not detected within five minutes of searching it was considered unavailable.

When a salamander's PIT tag was detected during a search, its ID number was recorded and a visual search of the immediate area was conducted to determine if the animal was either visibly active on the surface (climbing vegetation, on leaf litter, or sitting in a burrow), or not visible and below the surface. For animals that were visible on the surface, a pin flag and reflector were placed at that location. For animals located below the surface, the detection area (the area of the ground's surface where the antenna was able to register the tag) may vary depending on factors including soil type and distance underground. To mark the location of these animals, we placed a flag at the center point of the detection area. For each animal, we recorded the start and end time of the individual search, if the animal was located, whether it was visible, and the habitat where it was found. We also measured the azimuth and distance between the animal's most recent prior detection and its current location. Salamanders were not handled or intentionally disturbed unless absolutely necessary (e.g., the presence of multiple individuals in close proximity made individual identification with the PIT tag reader impossible).

In instances where an animal was incidentally detected during the search for another animal, a flag was placed to mark its location and the initial search was resumed. For these incidental observations, we recorded the same data outlined above; however, both the start and end times were recorded as the time at which the animal was detected. We also noted on the datasheet that the animal was located incidentally. See Figure 3.2 for a graphical simulation depicting how these sampling methods would be conducted within a hypothetical plot.

ANALYSIS: We developed a multistate Cormack-Jolly-Seber (CJS) model in a Bayesian framework to estimate recapture probabilities (p) and apparent survival (ϑ) of *Plethodon* across the Coweeta basin and investigated the relationships between behavior, ancestry (represented by genomic hybrid index, Carter, Chapter 2), and weather (Cormack 1964; Jolly 1965; Seber 1965; Kéry and Schaub 2012). A multistate model allowed us to assign animals to particular states and determine the probability that an animal transitioned between states. We also calculated state-specific apparent survival probabilities using capture histories where an animal's capture history was a product of both latent state and observation processes. We used the term "apparent survival" (hereafter referred to simply as "survival") because animals could disperse from the study site and this model could not differentiate between mortality and permanent emigration. We know that the behavior of *Plethodon* is strongly linked to precipitation and temperature (Feder 1983), so we constrained our transition probabilities as a function of weather.

WEATHER COVARIATES: Hourly local temperature data was collected from across the Coweeta basin from 2020 to 2021. Temperature data were interpolated to a grid with the Parameter-Elevation Regressions on Independent Slopes Model (PRISM) climate-mapping system (Daly et al. 1994; Daly et al. 1997), allowing for reliable temperature estimates across the entire study area. We extracted the estimated temperature values at each location on each sampling occasion to inform our model. We estimated precipitation values for each sampling occasion using rainfall data collected from nine USFS climate stations distributed across the basin.

MODEL DESCRIPTION: In our global multi-state CJS model, the latent state of an individual was defined as a random variable $z_{i,\,t}$ which indicated whether animal *i* was in state *k* given *[1….K]* possible states, where animals in state *K* were either dead or permanently absent from the plot. Because we were primarily interested in surface activity, our states were defined as "above ground," "below ground," and "dead/gone" (meaning permanently outside of both the horizontal and vertical detection area). The CJS model only models events that occurred following the first capture event (vector f_i), where for any individual *i*, the state at first capture ($z_{i,\, f_i}$) was not *K* (dead/gone). We allowed salamanders to transition between the "above ground" and "below ground" states between occasions. We modeled the probability (π_k) of transitioning between states as a multinomial distribution with:

$$k \sim \text{Multinomial}(\pi_1, \ldots \pi_K)$$

$$\pi_k = \text{plogis}(B0 + B1 \times HI + B2 \times Rain + B3 \times Mass + B4 \times Temp + Bsite) \times \Phi$$

Bsite was the random effects of study plot. We assumed daily survival probability (Φ) was constant across the study period. We further assumed animals could not return to an alive state once they entered the dead/gone state. We modeled detection as a binomial draw with probability p_{ikt}. We modeled p_{ikt} as a linear function with a logit link that varied by individual:

$$\text{logit}(p_{ikt}) = alpha0 + alpha_{individual}$$

We ran five versions of the model with varying combinations of covariates, although all candidate models included an effect of hybrid index, a site-specific effect of rainfall, and a random effect of site (Table 3.1). All analyses were performed using R statistical software (R Development Core Team 2023). We fit this model in a Bayesian framework implemented in JAGS (version 4.3.0) using the R package rjags (Plummer et al. 2022). Prior to analysis, we standardized all continuous covariates by subtracting the mean and dividing by the standard deviation. We ran each model for 100,000 iterations with a burn-in of 20,000 and a thinning rate of 5. We assessed convergence of parameters using the Gelman-Rubin statistic and visual inspection of the Markov chain Monte Carlo (MCMC) chains (Brooks and Gelman 1998). We performed model selection using a full-likelihood approach to WAIC (Watanabe 2010) and chose the model with the lowest WAIC score as the top model. To evaluate goodness of fit, we calculated a posterior predictive fit score that measured the probability that the data produced from the model posterior is more extreme than the observed data for the latent state (z). Values between 0.2 and 0.8 were considered to be an indicator of good model performance (Gelman et al. 1996). After determining the top model, we predicted the probability that an average animal from each site would be surface active across the full range of 3-day rainfall conditions observed across the Coweeta basin. We also predicted the probability that animals from an average site with hybrid index values of either 0.0, 0.5, or 1.0 would be surface active over the same range of rainfall. For both predictions, all other covariates were held constant at their mean.

RESULTS

A total of 126 individuals were tagged during this study. We could not use data for one of our sites because a record rainfall event caused severe flooding, resulting in a washout that prevented us from accessing the site. We were unable to track the animals for several days following their release and most of the animals could not be relocated once tracking resumed. Among the remaining sites, tracking was conducted on 89 nights: 41 nights between mid-September and late-October 2020 and 48 nights between late-May and early-July 2021. We recorded a total of 469 PIT tag detections, with 107 individuals detected at least once during the study. Salamanders were visible on the surface in only 29% of detections, meaning 71% of the time we detected a salamander, the individual was below ground. There were no confirmed fatalities during the study, although 18 individuals were never detected again after their initial release.

Our top model included 3-day rainfall, temperature, and hybrid index as predictors of transitions between above-ground and below-ground states (Table 3.1). The overall posterior predictive fit score for this model was 0.47, indicating that the model's predictions of the number of surface-active salamanders were reasonably close to the observed values for the given dataset. Examination of these scores by occasion revealed that the posterior tended to under-predict the number of surface-active salamanders on occasions 14, 22, and 28, but over-predict that number on occasion 41 (Figure 2.1).

The model estimated a monthly (30 day) apparent survival probability of 0.99 (95% CRI: 0.99–0.99). The mean individual detection probability was 0.43 (95% CRI: 0.41–0.46). This estimate represents the likelihood of detecting an average individual at an average site, given that it is both present and available to be detected. This estimate is highly consistent with prior research on *P. shermani* using a similar PIT tag approach (Connette and Semlitsch 2012). Detection probability varied among sites (Table 2.2). The model estimated a strong positive effect of rainfall on salamander surface activity (median: 0.95, 95% CRI: 0.73–1.19), indicating a heightened likelihood of below- to above-ground transitions following periods of rainfall. SVL was also estimated to have a significant positive effect on the probability of surface activity (median: 0.33, 95%. CRI: 0.12–0.54), indicating larger individuals were more likely to become surface active than smaller individuals under a given set of weather conditions. Though hybrid index was included in the top model, the median effect on transition probability was small, and the credible intervals widely overlapped zero, indicating little to no support of an effect of ancestry on the likelihood of becoming surface active under a given set of weather conditions (median: 0.16, 95% CRI: -0.98–1.34). There was also no support for an effect of ambient temperature on becoming surface active (median: -0.05, 95% CRI: -0.34–0.22). Our results suggest that probability of becoming surface active was the same for any animal at all sites when weather conditions at those sites were similar (Figure 3.3). Comparisons of animals from lineages with differing HI values (0.0, 0.5, and 1.0) simulated from the same average site were predicted to exhibit similar patterns of increasing surface activity with increasing rainfall (Figure 3.4). However, there were differences in the distributions of 3-day rainfall volumes among the sites such that, on average, salamanders at the three high- and mid-elevation sites would have a 50 to 60% increase in surface activity compared to salamander at the two drier, low-elevation sites (Figure 3.3).

DISCUSSION

Our findings indicate that the surface activity of these salamanders is strongly influenced by rainfall, consistent with our well-established knowledge of the importance of moisture on surface activity (Feder 1983) and other studies examining surface activity among these and other closely related species (e.g., Gade et al. 2020). We also found that body size was a positive predictor of surface activity, again consistent with our knowledge that evaporative water loss rates decline with increasing body size, which in turn increases the likelihood and duration of surface activity (Feder 1983; Feder and Londos 1984). This result has important implications for studies of *Plethodon* and other similar species depending on whether investigators do or do not account for body size effects on detection probability. Contrary to expectations, we found no evidence that there are differences in the likelihood of surface activity between *P. teyahalee*, *P. shermani*, or hybrids under similar weather conditions. Rather, our results suggest differences in realized rates of surface activity among *P. teyahalee*, *P. shermani*, and hybrids are a direct function of differences in rainfall levels at different sites. Because *P. shermani* occupy wetter sites with more rain, realized levels of surface activity for *P. shermani* should be substantially higher than those of *P. teyahalee*. The ecological implications of this are potentially high. In the absence of other compensatory traits, *P. teyahalee* surface activity should be more constrained than *P. shermani*, which should lead in turn to reduced growth, fecundity, survival, and, ultimately, abundance. We would also expect the differences in activity levels and associated fitness to create strong selective pressures on life-history traits or other compensatory traits. These results also eliminate one potential adaptive difference that could have either explained the current distribution of these two species or predicted how they may respond to climate change. The expectation that *P. teyahalee* possess traits that make it better-suited to warmer, drier climates is core to the prediction that it may migrate up in elevation in response to climate change, thereby displacing high-elevation endemics like *P.*

shermani. Our results suggest that a behavioral difference related to surface activity is not one of those traits.

When animals—particularly ectotherms—must deal with a range of variable climates, we expect behavior to be an important, primary mechanism that compensates for and alleviates pressure (Muñoz et al. 2014). While we know and this study further demonstrates that salamanders reduce their activity in response to reductions in rainfall, there is no evidence of compensatory or other behavioral changes in response to weather events (e.g., a disproportional increase in the surface activity of *P. teyahalee* relative to *P. shermani* under similar high rainfall conditions). Additionally, if traits have evolved to mitigate the cost of surface activity under harsh environmental conditions in these salamanders (e.g., greater physiological resistance to water loss) we would expect that *P. teyahalee* would exhibit higher levels of surface activity under dry conditions than *P. shermani*, yet no differences in behavioral disposition were detected. There is a need to continue exploring how salamanders such as *P. teyahalee* compensate for—or adapt to—the reduction in surface activity that presumably translates into reduced foraging and other potential costs. Riddell and Sears (2015) demonstrated geographic variation in evaporative water loss resistance between *P. metcalfi*, which occupy wetter, high-elevation sites compared to *P. teyahalee*, and Riddell et al. (2019) demonstrated that temperature can cue *Plethodon* plasticity in skin resistance to evaporative water loss. If *P. teyahalee* exhibit greater resistance to water loss compared to *P. shermani*, our results suggest this does not translate to a compensatory increase in surface activity compared *P. shermani*.

Given that the two parent species did not exhibit differences in expected surface activity, it is unsurprising that we also found no difference in the behavior of hybrid animals. Surface activity of hybrid animals was consistent with local rainfall conditions.

The capacity for introgression to introduce traits across species boundaries and create novel combinations of traits can influence the persistence of organisms through historic and future climate change (Carter, Chapter 1). Our results suggest there is little to no difference in surface activity between salamander species or their hybrid descendant lineages that would be important in understanding how these species or the hybrid lineages might differ in their responses to future climate change.

The persistence of species in a particular environment is contingent upon the ability of organisms to meet their physiological and ecological requirements for survival, growth, and reproduction. This study confirms that rainfall is a major constraint on *Plethodon* surface activity and, therefore, a significant ecological gradient upon which we expect selection to be intense or effects on demography strong. We found no evidence that adaptations for drier environments compensate (even partially) for the environmental constraints on activity and resource acquisition. This may suggest limited adaptive capacity among these *Plethodon* species to compensate for climate changes which would reduce annual rainfall or increase drought frequency or intensity. Consistent with our results, other recent studies have demonstrated reduced abundance of *Plethodon* at drier sites (Howard 2018) and at lower elevations where conditions are warmer and drier (Hocking et al. 2021). We encourage continued investigation into adaptive capacity of *Plethodon* and other salamander species to in response to climate as part of efforts to forecast the impacts of climate change on these animals, while also considering the possibility that there is limited adaptive variation among different species that currently occupy different climates.

TABLES

Table 3.1. Candidate Bayesian multistate models and model selection criteria for hypotheses about the relationship between salamander movement and explanatory variables.

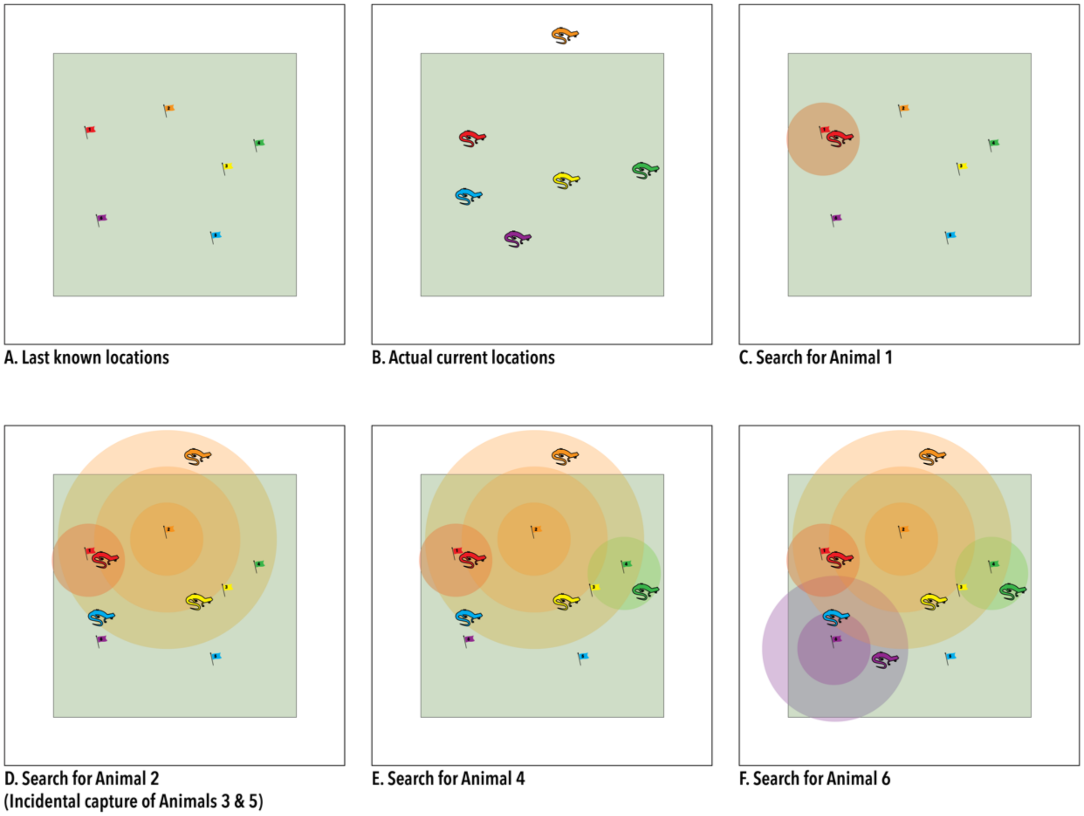
Models[1]	Predictors	WAIC	ΔWAIC
Mod02	Intercept + HI + Rain + Size + Temp	16142	0
Mod04	Intercept + HI + Rain + Size	16158	16
Mod01	Intercept + HI + Rain + Rain × HI + Size + Temp	16163	21
Mod03	Intercept + HI + Rain + Temp	16238	96

[1]All models include 'Site' as a random effect.

Table 3.2. Average detection probability for each site

Site	Detection Probability		Average HI	Mean Annual Precipitation
	Median	95% CI		
31-T	0.469	[0.403, 0.539]	0.528	2287.335
36-1	0.446	[0.381, 0.517]	0.999	2458.992
EF	0.393	[0.329, 0.462]	0.002	1819.523
Mid	0.381	[0.323, 0.442]	0.521	2226.056
40-1	0.360	[0.296, 0.429]	0.472	2125.100
PN	0.351	[0.300, 0.404]	0.997	2498.092
36-2	0.343	[0.272, 0.419]	0.991	2458.992
Low	0.319	[0.241, 0.408]	0.005	1925.385
Overall	0.431	[0.406, 0.457]	--	--

FIGURES



A. Last known locations

B. Actual current locations

C. Search for Animal 1

D. Search for Animal 2
(Incidental capture of Animals 3 & 5)

E. Search for Animal 4

F. Search for Animal 6

Figure 3.1. A graphical simulation depicting how PIT-tag telemetry sampling methods were be conducted within a hypothetical survey plot.

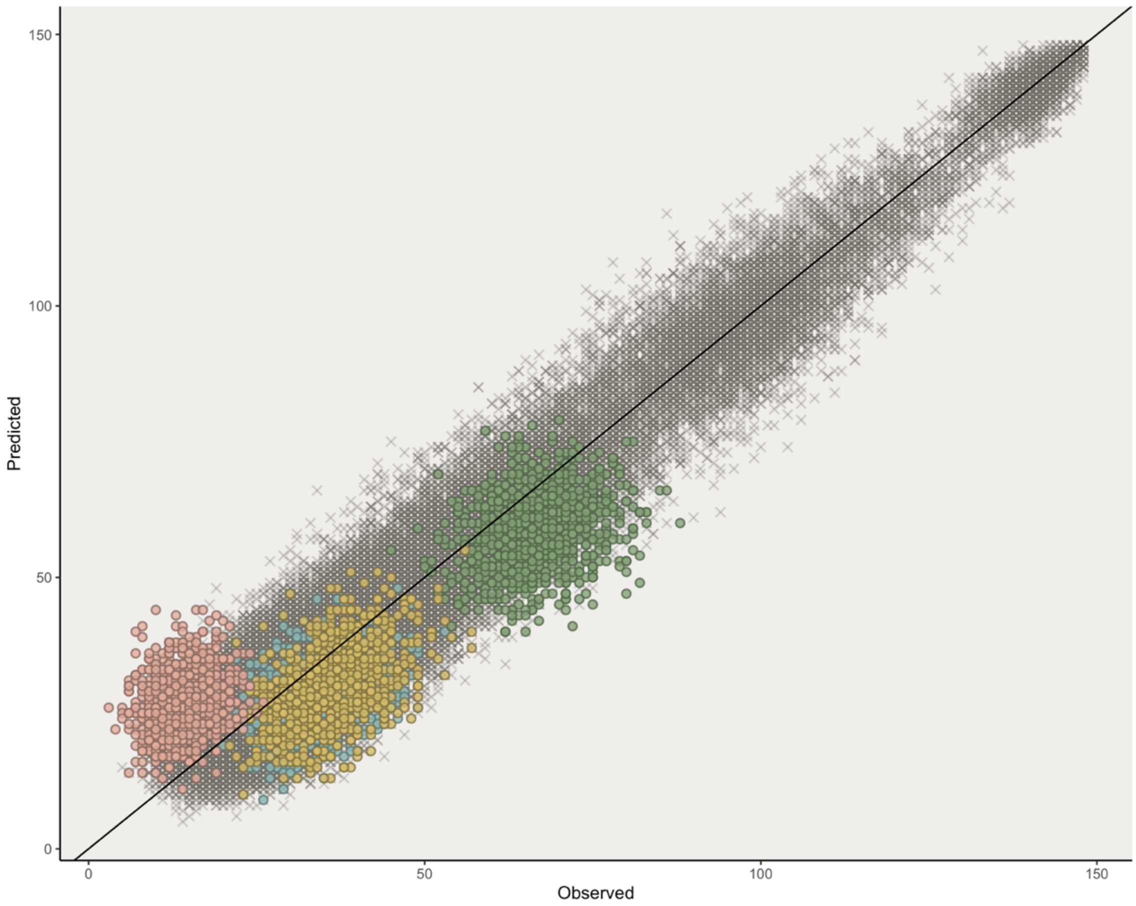


Figure 3.2. Fit of posterior predictions to observed data for each occasion from the last 1000 iterations of the top model (Mod03). Occasions with extreme deviations of predicted vs. observed values are represented by filled circles; values for all other occasions are represented by x's.

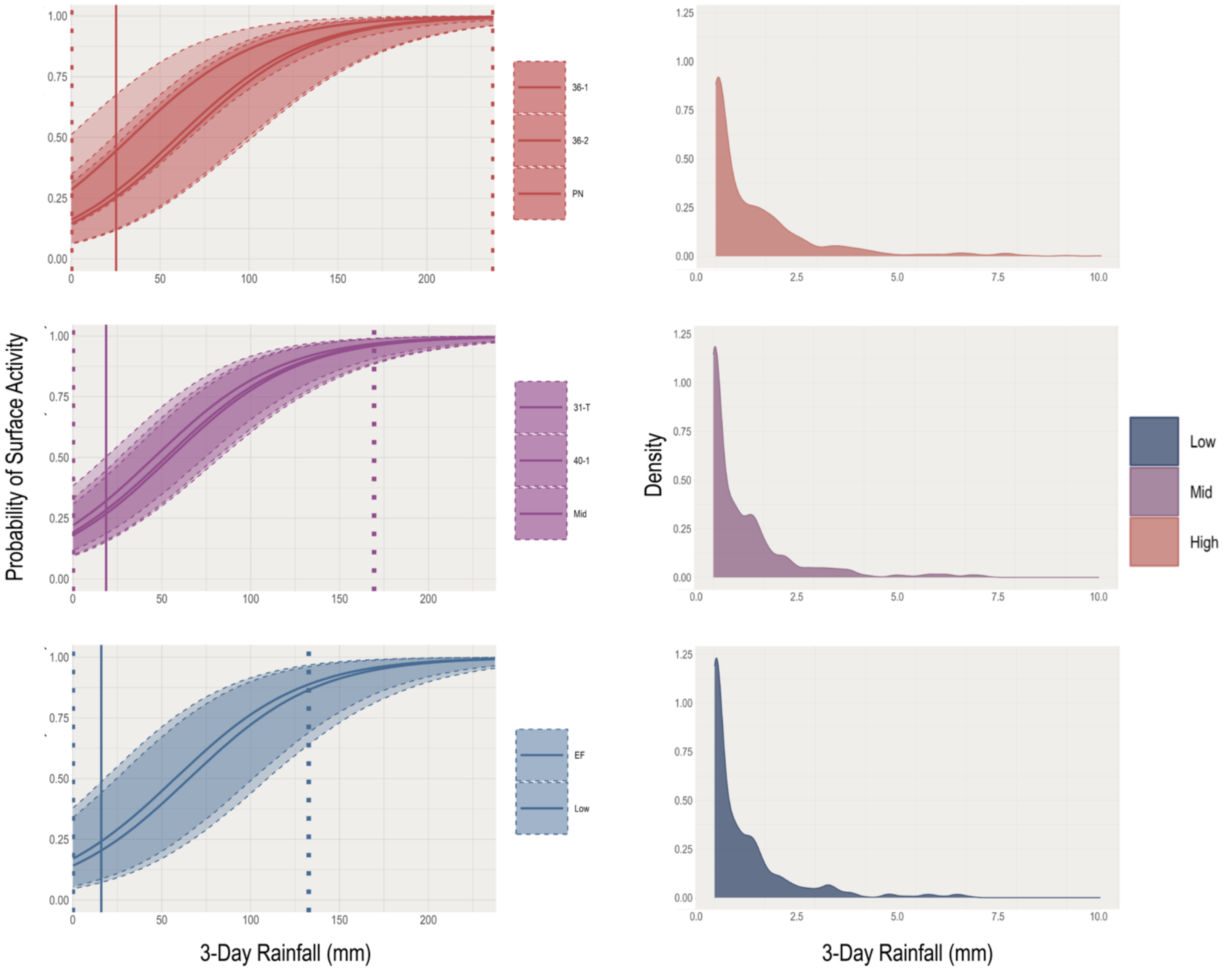


Figure 3.3. The probability that an average salamander from each plot would be surface active across a range of 3-day rainfall values, assuming all other covariates are held constant at their mean (left), and the density of 3-day rainfall volumes experience by sites across the basin. Sites were plotted together based on proximity into “Low”, “Mid”, and “High” elevation groups.

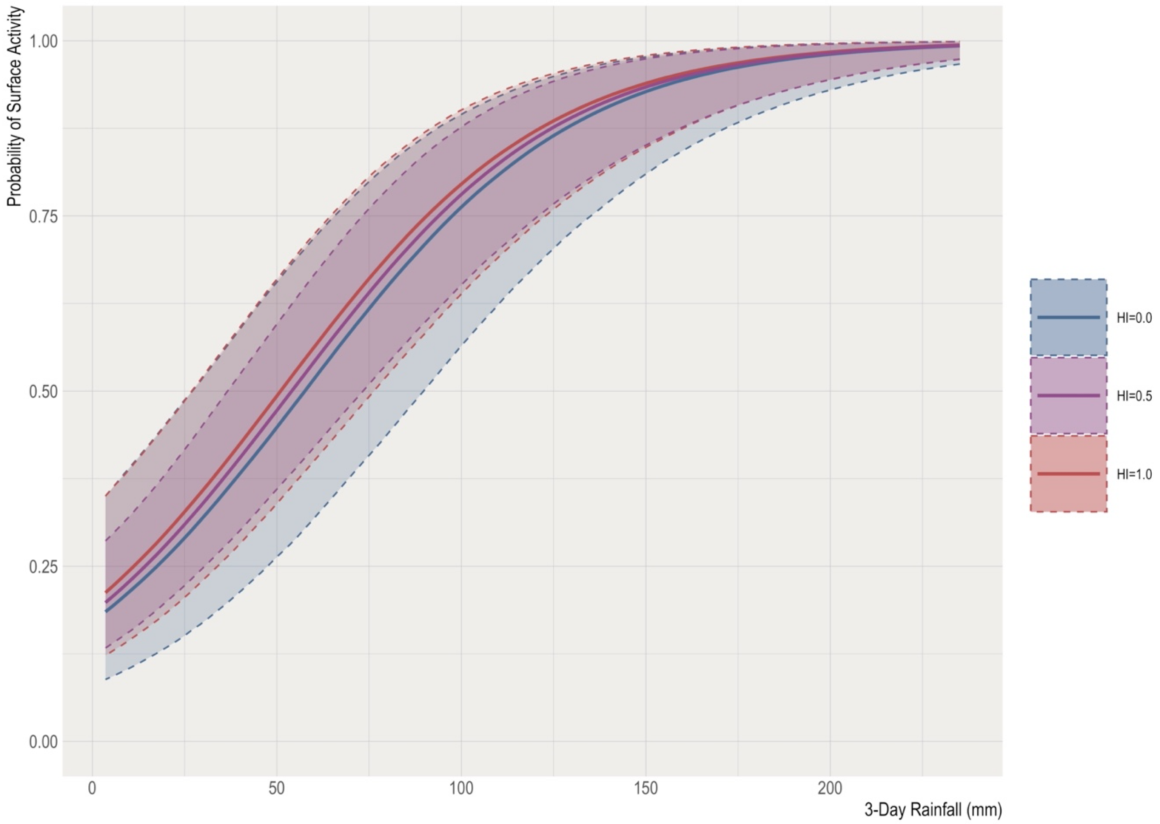


Figure 3.4. The probability that a salamander of a given hybrid index at an average site would be surface active across a range of 3-day rainfall values, assuming all other covariates are held constant at their mean.

CHAPTER 4:

APPLICATION OF A NOVEL TECHNIQUE TO COMPARE EVAPORATIVE WATER LOSS RATES OF TERRESTRIAL SALAMANDERS ACROSS A CLIMATE GRADIENT[1]

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[1] Carter, C.M., E.N. Taylor, and J.C. Maerz. Prepared for: *Herpetological Review*

ABSTRACT

This study investigates patterns in cutaneous evaporative water loss (EWL) across a *Plethodon shermani* and *P. teyahalee* hybrid. Using a sample of 140 salamanders from six different sites within the Coweeta basin, we examined the correlation between EWL and a range of variables including ambient temperature, body mass, acclimation, and mean annual precipitation.

We found a strong positive correlation between ambient temperature and EWL rates and a significant inverse correlation between body mass and EWL, suggesting larger salamanders lose water at slower rates, thereby reinforcing the established understanding of surface-area-to-volume ratios on water-loss rates. These findings are consistent with patterns in temperature and body size seen in other plethodontid salamanders. Contrary to expectations, we found no substantial differences in cutaneous EWL among *P. teyahalee*, *P. shermani*, and their hybrids. Furthermore, differences in mean annual precipitation at an animal's capture sites did not appear to effect EWL. These results suggest that species-specific physiological compensations for drier conditions may be minimal. The insights gathered from this study contribute to our understanding of the relationships between salamander physiology and environmental conditions and raises additional questions about the mechanisms these animals might be using to persist in under challenging conditions. We also describe our use of the Aquaflux AF200 Evaporimeter, for in-field EWL measurements of amphibians and reflect on the suitability of this approach for future work with amphibians.

INTRODUCTION

Physiology, morphology, and behavior are often inextricably linked, and it can be challenging to untangle their individual contributions to the survival of a particular lineage. For example, many animals are physiologically constrained by their thermal and hydric tolerances, but one way they can mitigate the stresses imposed by fluctuating environments is by altering their behavior

(Stevenson 1985). However, when compensatory behaviors limit the exposure of animals to conditions outside of their ecological niche, they also alleviate the selective pressure for physiological traits that might otherwise be adaptive under those conditions (Bogert 1949; Huey et al. 2003) . This process can constrain the evolution of physiological tolerance which may have profound consequences if conditions are altered such that compensatory behaviors are no longer sufficient to meet an organism's physiological and ecological needs (Huey et al. 2003; Muñoz and Bodensteiner 2019). Under these circumstances, if physiological tolerances do not rapidly evolve within the population, the ability of that population to persist in its current environment may be substantially diminished. However, before we can make predictions about how populations may respond to future shifts in climate, we must first understand their thermal and hydric constraints under current conditions.

In this study, we examine the physiological differences among *P. teyahalee*, *P. shermani*, and *P. shermani* × *teyahalee* hybrids using a novel method to measure cutaneous evaporative water loss (EWL) of animals in the field. We use linear mixed effects models to examine the relationship between ancestry, morphology, climate, and water-loss. We also consider the utility of this novel approach for future studies of amphibian physiology. Understanding the physiological limitations of these salamanders and their relationship to climate will help us to better understand what factors regulate their distribution and survival and can help us make to predictions about the adaptive capacity of hybrids.

METHODS

For this study, we measured cutaneous EWL rates of salamanders collected at six sites distributed across the Coweeta basin—two high-elevation sites within the range of "pure" *P. shermani*, two low-elevation sites within the range of "pure" *P. teyahalee*, and two mid-elevation sites within the hybrid zone. For the first phase of these trials, we collected approximately 10 individuals from each site using night-time visual encounter searches. Animals were only collected if their snout-vent length (SVL) measured >45 mm, and all gravid females were excluded. For each individual, time and general location of initial capture (plot) was recorded, as well as a description of the specific habitat (e.g., in a burrow, climbing on downed woody debris, etc.). All salamanders were photographed, weighed, and measured (SVL and total length). We also assigned phenotype scores (between 0 and 3) for each animal based on their presentation of *P. shermani* and *P. teyahalee* traits (for a more detailed description of phenotype scoring, see Chapter 2). The *P. shermani* scores were assigned based on the amount of red present on the legs, and the *P. teyahalee* scores were assigned based on the amount of white spotting on the body. For each scoring system, a higher value indicates increased morphological resemblance to that parent lineage. Captured animals were housed in 1-liter containers (approximately 20 cm long, 15 cm wide, and 5 cm deep) containing rehydrated orchid moss to mimic wet leaf-litter and keep the salamanders hydrated. Salamanders were acclimated to laboratory conditions for 7 days before their cutaneous EWL rates were measured. For phase two of these trails, an additional 10 to 15 salamanders were collected from each site—again using night-time visual encounter searches—and their EWL rates were measured on the same night without acclimation.

Cutaneous EWL measurements were taken in triplicate for each animal using an Aquaflux AF200 evaporimeter (BioX Systems; London, UK) connected to a PC laptop running the manufacturer's associated software. Prior to use, the device was calibrated according to the manufacturer's protocol. The Aquaflux is a closed-chamber device containing a condenser that is cooled to −7.6 °C. Any water vapor inside of the main chamber is crystallized into ice on the condenser, creating a low-humidity environment at the rear of the device where the sensor is located. When the probe is placed against a skin surface, water evaporates from the skin and forms a high-humidity zone at the bottom of the chamber. Water vapor then passively diffuses towards the low-humidity zone where the sensor measures the flux of water vapor. The condenser continually removes humidity from the device chamber, allowing for precise, repeated measurements. The initial measurements taken by the device represent the skin-surface water-loss (SSWL) rate. Once the vapor from the skin's surface is fully evaporated, the flux density levels off and the subsequent measurements represent the steady underlying cutaneous EWL rate. The device also continually records temperature and humidity of the ambient environment during use.

Because stress can cause *Plethodon* to produce large volumes of sticky skin secretions that could inflate water loss measurements, we limited handling of each animal as much as possible. However, because the Aquaflux requires constant contact with the skin in order to accurately measure flux density, it was necessary to immobilize the salamanders during measurements. To accomplish this, animals were placed on an angled metal plate and restrained using a piece of low-density polyethylene plastic attached to a wooden frame. A hole-punch was used to create 6 mm holes in the plastic, which allowed the evaporimeter probe to be placed against the skin of each animal. Once the animal was immobilized, the tip of the Aquaflux probe was placed against the dorsal surface of the animal's body using light and steady pressure. Measurements—including ambient conditions, SSWL, and cutaneous EWL—were automatically recorded by the manufacturer's software. If an animal moved during a measurement or the probe was not flush against the skin for the entirety of a measurement, the flux density would not reach a steady state. In those cases, the measurement was discarded, and a new measurement was taken. The restraint device and the Aquaflux probe tip were wiped down between trials using clean paper towels and distilled water to remove any accumulated skin secretions. After being measured, all animals were returned to their initial site of capture.

ANALYSES

We used linear mixed-effects models to test the impact of specific covariates of interest on cutaneous EWL. Because our objectives were to test hypotheses about the effects of hybrid index, climate, and morphology on water loss—as well as investigating factors influencing the reliability of this novel method—candidate models included combinations of the following covariates: mean annual precipitation, average genomic hybrid index estimated for the site, whether or not an animal had been acclimated, animal's body mass (g), and ambient temperature at which cutaneous EWL was measured. Mean annual precipitation values were obtained from Parameter-elevation Relationships on Independent Slopes Model (PRISM) gridded climate data from 1990 to 2020 (PRISM Climate Group (Oregon State University) 2022). Originally in 800 m resolution, PRISM data were resampled to 1000 m using cubic convolution to align with additional data sources in other studies of this system.

We calculated the Pearson correlation coefficient between pairs of these covariates and did not include any covariates with a Pearson correlation coefficient >0.60 in the same model. All covariates were standardized for analyses, and all candidate models included site as a random effect. We used the lmer function in the R package lme4 (Bates et al. 2014) for all models and conducted model selection using the aictab function in the R package AICcmodavg (Mazerolle 2023). We considered all models within 2 AICc of the top model parsimonious models (Burnham and Anderson 2002). All analyses were conducted using Program R version 4.2.1 (R Development Core Team 2023).

RESULTS

We captured and measured cutaneous EWL of 140 salamanders across six sites within the Coweeta basin. Of these, 61 individuals were acclimated to temperatures between 20 and 24 °C for 7 days. Approximately 18% of individuals were female, 50% were male, and 32% could not be definitively sexed (Table 4.1).

All five candidate models had similar parsimonious support, with the top three models within 2 ΔAIC. All five models contained ambient temperature, body mass as covariates. The top candidate model also included acclimation and mean HI precipitation AICc: 2691.15; AICc weight: 0.27; Table 4.2). The second model contained acclimation, and mean annual precipitation, and third model only included acclimation ($\Delta AIC_c = 0.13$ and 0.33, respectively). All of these models indicated that ambient temperature had a strong positive effect on evaporative water loss rates (Figure 4.1). These models also showed that water loss was negatively correlated with increasing body mass (Figure 4.2). Acclimation was also negatively correlated water loss; however, this effect was only marginally significant in all three models. Though the inclusion of either mean annual precipitation or mean hybrid index improved model fit, neither of these parameters was significant in their respective models (Figure 4.3).

DISCUSSION

The results confirm a significant, positive influence of ambient temperature on the rates of cutaneous EWL. This relationship between EWL and temperature is consistent with prior studies demonstrating that rate of dehydration in salamanders is influenced by ambient temperature, and that dehydration tends to be more rapid at higher temperatures (Spotila 1972; Feder 1983; Feder and Londos 1984). Body mass was found to be inversely related to EWL, with larger salamanders experiencing slower rates of water loss per unit area per hour. This finding, in conjunction with the well-established relationship between surface-area-to-volume (SA:V) ratios and water loss rates, indicate that larger individuals may have a distinct advantage in dry conditions (MacMahon 1964; Spight 1968; Nevo 1973).

Interestingly, hybrid index did not significantly influence cutaneous EWL, suggesting that *P. shermani*, and *P. teyahalee* do not substantially differ in their physiological capabilities to resist water loss under the observed conditions. These results are only partially consistent with Riddell and Sears (2015) who compared acclimated resistance to water loss of *P. teyahalee* with *P. metcalfi*, which typically occurs at cooler, wetter, high-elevation sites. These two species differed in resistance to water loss, with *P. teyahalee* exhibiting greater resistance under some conditions. Moreover, both species showed geographic variation in resistance that was consistent with climate variation along elevation gradients.

In addition to finding no difference between species or hybrids, we also found no effect of mean annual precipitation of the sites where animals were collected. The results of Riddell and Sears (2015) do show variation in resistance to water loss reaction norms within and between species and show that under some conditions resistance to water loss is similar between species and among sites within species. Because of the short acclimation period in the present study and the use of only one acclimation condition, we are unable to make strong inferences about potential differences of these salamanders to adjust water loss rates in response to changing conditions. Given the plasticity of water-loss physiology demonstrated by Riddell and colleagues (Riddell and Sears 2015; Riddell et al. 2019), the results of the present study should be interpreted with caution. Nonetheless, unlike other studies, our study was able to generate measures of water loss rates for animals in the field in relatively "real time" and failed to find any evidence that evaporative water loss rates differed between species or among animals over a wide climate range. When paired with the results in Chapter 3—which indicate no intrinsic difference between *P. shermani* and *P. teyahalee* surface activity relative to weather conditions—the results of the present study suggest that there are few meaningful differences in compensatory changes in EWL rates or behavior between species in response to climate. Therefore, we continue to find no support that salamanders at drier, warmer, low-elevation sites have meaningful physiological or behavioral differences that compensate—at least partially—for the effects of drier conditions in reducing activity.

While this study was not initially intended to investigate a novel method for measuring water loss in salamanders, the present study is the first instance in the literature of using the Aquaflux device to take instantaneous EWL measurements of amphibians in the field. While the Aquaflux device has not, to our knowledge, been previously used on amphibians, it has been used successfully to examine water loss in reptiles (Guillon et al. 2014; Chabaud et al. 2022; Weaver et al. 2022). The Aquaflux approach offers several advantages over conventional methods of measuring water loss. As we have demonstrated, the portable design allows measurements to be taken in the field, rather than requiring animals be transported to a facility housing a flow-through system. This allows for greater flexibility in study design and may better accommodate the needs of researchers doing work in remote areas. Additionally, this method can be used to take targeted measurements of small areas, thereby allowing investigators to compare water loss rates among different regions of an animal's body (Weaver et al. 2022). Overall, we believe the Aquaflux system has the potential to significantly advance ecophysiology research on amphibians, enhance our understanding of hydrologic constraints and adaptations, and, ultimately, allow us to make better models of and predictions for amphibians' responses to shifting climates. Although this method offers exciting avenues for future research, it also presents one important constraint to the present study. Due to the numerous methodological differences between the two systems (e.g., acclimation time, control of ambient conditions), we are unable to directly compare the measurements taken by the Aquaflux AF200 evaporimeter to those of traditional flow-through systems commonly used to measure water loss rates in salamanders (e.g., Riddell and Sears 2015). It would be valuable to have measurements taken simultaneously using both methods in order to confirm their accuracy and reliability.

TABLES

Table 4.1. Site-specific summary of study's conditions and salamander characteristics across sites. For each site, the table includes: average Hybrid Index and precipitation, the number of unique salamanders, their distribution by sex, range of body sizes, number of salamanders in each acclimation state, and the range of ambient temperatures experienced during the collection of EWL measurements.

Site	Median HI	Mean Annual Precipitation (mm)	Range of Body Size			Sex			Not Acclimated		Acclimated	
			SVL (mm)	Total Length (mm)	Mass (g)	No. ♀	No. ♂	No. Unknown	No. Individuals	Ambient Temp (°C)	No. Individuals	Ambient Temp (°C)
1	0.001	1915.026	54-84	112-174	2.6-11.1	3	14	3	10	22.5-25.1	10	22.4-22.9
31	0.528	2125.1	53-76	82-158	2.3-7.8	5	11	8	14	24.5-25	10	23.9-24.8
36	0.998	2458.992	46-66	74-139	1.9-5.6	3	7	13	12	23.9-24.1	11	26.2-27.7
EF	0.002	1819.523	48-88	90-163	1.9-10.6	4	11	10	15	22.7-23.5	10	22.7-23
Mid	0.521	2226.056	56-80	103-146	2.5-7.3	5	17	3	15	23.5-23.8	10	26.7-27.3
PN	0.997	2498.092	56-68	92-143	3.0-5.6	5	10	8	13	23.8-24	10	24.7-25.5

Table 4.2. Model selection results for 5 candidate models of Cutaneous Epidermal Water Loss rates in *Plethodon shermani*, *P. teyahalee,* or *P. shermani X teyahalee* hybrid lineages within the Coweeta Basin, Macon County, NC. MAP refers to PRISM interpolated mean annual precipitation from 1991-2020.

Models[1]	Predictors	K	AIC	ΔAIC	AICcWt	LL	CuWt
Mod01	Ambient temp + Mass + Acclimation + HI	7	2691.15	0	0.27	-1338.45	0.27
Mod02	Ambient temp + Mass + Acclimation + MAP	7	2691.28	0.13	0.25	-1338.51	0.52
Mod03	Ambient temp + Mass + Acclimation	6	2691.48	0.33	0.23	-1339.64	0.75
Mod04	Ambient temp + Mass + MAP	6	2692.63	1.48	0.13	-1340.22	0.88
Mod05	Ambient temp + Mass + HI	6	2692.71	1.56	0.12	-1340.26	1

[1] All models include 'Site' as a random effect.

Table 4.3. Summary of the linear mixed-effects model examining factors influencing evaporative water loss (EWL) in *Plethodon shermani*, *Plethodon teyahalee*, and *P. shermani X teyahalee* hybrids. The model tests the effects of ambient temperature, body mass, acclimation status, and mean site hybrid index, while considering the variation across different sites.

	Parameter	Variance	SD		
Random effects	Intercept	6.889	2.625		
	Residual	25.139	5.014		
	Parameter	Estimate	SE	t	p
Fixed effects	Intercept	86.874	1.129	76.967	<0.001
	Ambient Temperature	7.699	0.359	21.428	<0.001
	Mass	-0.969	0.321	-3.019	0.003
	Acclimation	-0.942	0.553	-1.703	0.089
	Mean HI	-0.670	1.129	-0.593	0.582

FIGURES

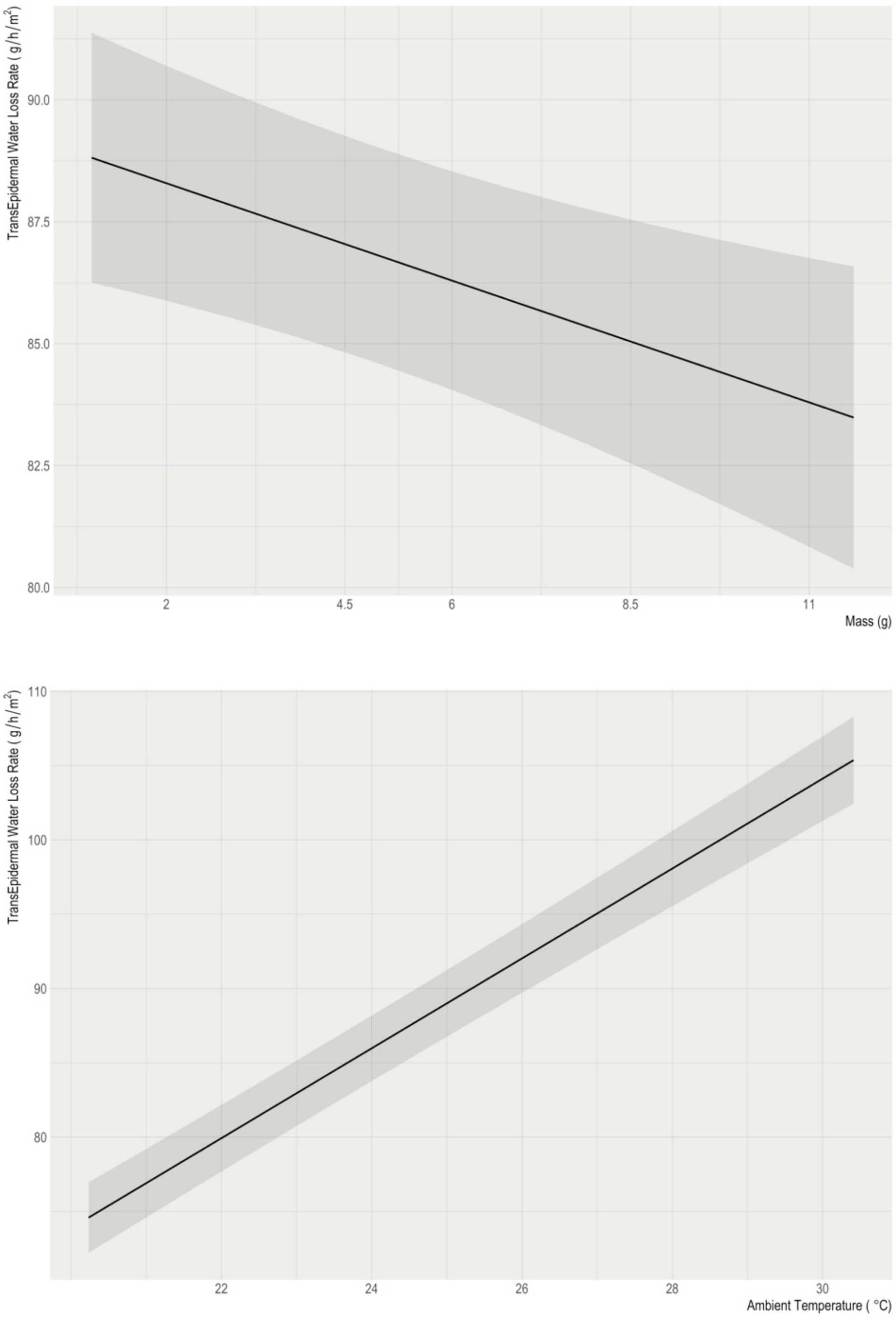

Figure 4.1. Predicted rates of evaporative water loss as a function of body mass (top) and ambient temperature (bottom) by a linear mixed effects model. The model included ambient temperature at the time EWL was measured (C°), body mass (g), acclimation (0, 1), and mean hybrid index as fixed effects and site as a random effect.

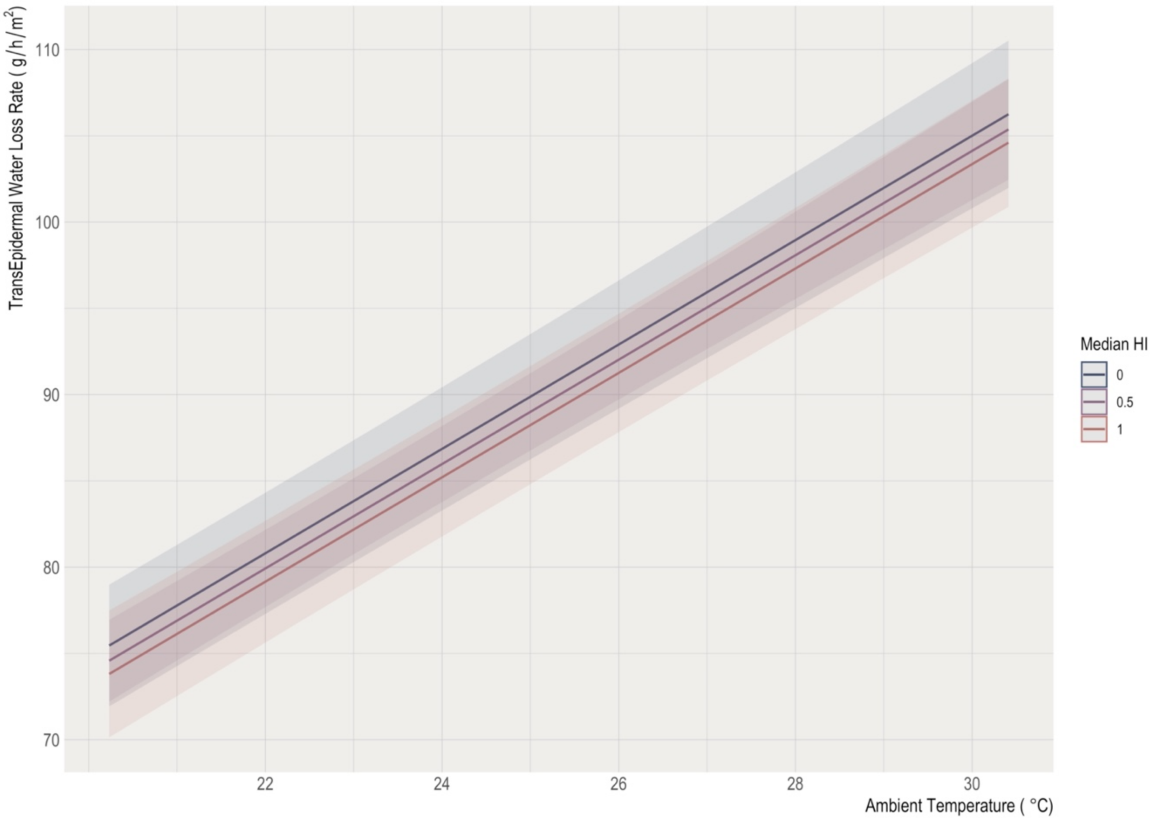

Figure 4.2. Predicted rates of evaporative water loss as a function of ambient temperature (bottom) for three levels of genomic hybrid index (0, 0.5, and 1) estimated by a linear mixed effects model. The model included ambient temperature at the time EWL was measured (C°), body mass (g), acclimation (0, 1), and mean hybrid index as fixed effects and site as a random effect.

CHAPTER 5:

BODY SIZE AT MATURITY AMONG LARGE *PLETHODON* ALONG A CLIMATE GRADIENT[1]

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[1] Carter, C.M and J.C. Maerz. Prepared for: *Herpetological Review.*

ABSTRACT

This study investigates the role of hydroclimate and topography on the minimum observed size at first reproduction in *Plethodon* salamanders, utilizing nearly 15,000 records of salamander captures across 67 sites in the Coweeta basin. Our analysis revealed that minimum size at first reproduction in both females and males was negatively correlated with mean annual precipitation at the site, and, for any given mean annual precipitation, male size at first reproduction was smaller than that of females. Additionally, a modest positive correlation was observed between slope and minimum size at first reproduction. This pattern suggests a potential delay in maturity corresponding to larger body sizes in drier conditions, with a remarkable difference in size at first reproduction over the precipitation gradient within a relatively small geographic distance. Our results raise key questions about plasticity versus fixed thresholds for body size at first reproduction within and across sites, and about the influence of past hybridization on the capacity for evolution of different body sizes at first reproduction. Future studies will need to examine growth data from drier sites and delve into the potential impact of climate-driven variation on amphibian life-history evolution.

INTRODUCTION

Climate is a fundamental pressure shaping the behavior, morphology, and life history of organisms. Nearly all species are distributed spatially across a range of climates and different lineages must have evolved a means to contend with the variation in temperature and moisture. This is widely recognized in phenomena such as Bergmann's and Allen's rules, whereby body size or robustness increases with decreasing average temperature among some taxa (Bergmann 1847; Allen 1877; Mayr 1956) or in examples of counter-gradient adaptations where intrinsic growth rates have evolved to partially compensate for climate effects on growth (Adams and Church 2008; Olalla-Tárraga et al. 2010)

Among amphibians, the strongest effect of climate should be on traits that affect evaporative water loss (EWL) or rehydration. Because of their highly permeable skin, amphibians are prone to high rates of EWL, which can constrain activities such as foraging and, in turn, affect the performance of individuals. EWL rates should be higher in climates with high vapor-pressure deficits (VPDs), lower rainfall, and warmer temperatures. Amphibians can accommodate climate-driven variation in EWL pressure via behavioral changes (e.g., altered surface activity), physiological changes (e.g., metabolic rates, skin resistance to water loss), or morphological changes (e.g., body size and surface-area-to-volume-ratio).

Change in body size or shape may seem like a relatively simple and straightforward means to mitigate EWL, but a larger body size is most likely achieved by delaying maturation. Delayed maturation has demographic consequences that might constrain the evolution of larger body sizes or the abundance of amphibian species in drier climates.

The objectives of this study were to compare minimum observed size at reproduction of "populations" of *Plethodon shermani*, *P. teyahalee*, and populations of hybrid ancestry across a hydroclimate gradient in order to determine whether size at maturity is evolving in response to climate. Specifically, we tested the hypothesis that minimum size at first reproduction was either positively correlated with mean daily VPD or negatively correlated with mean daily or annual precipitation. We then used data from known-age growth-rates to calculate the minimum age at first reproduction as a function of climate. Our analysis reveals that delaying maturity to increase body size appears to be a key adaptive response to drier climates but also indicates that this response has significant demographic effects which may explain declining abundance of large *Plethodon* species within drier climates.

METHODS

We used a data set composed of all individual *Plethodon* captured among 67 sites across the Coweeta basin between 2010 and 2022 as part of multiple populations studies that differed in objectives and duration. These include a 13-year mark-recapture study across three sites (described in Howard 2018), repeated “unmarked” surveys between 2015 and 2022 among 64 sites (Howard 2018) (and J. Maerz unpublished data), an ongoing study of salamander responses to forest vegetation management between two watersheds from 2018 to 2022 (J. Maerz and K. Grab, unpublished data), and a telemetry and EWL study among 9 sites (Carter, Chapters 2 and 3). For each site, we identified the smallest measured snout-to-vent length (SVL) of a confirmed mature male and a confirmed mature female animal. Males were determined to be mature if they had a clearly swollen vent or the presence of a mental gland. Mature females were identified by the presence of yolked eggs of any size detected through the body wall. Because we required the presence of yolked eggs to confirm reproductive mature, some adult females that were not reproductive at the time of capture will have been excluded from our final analyses.

We ultimately constructed 16 candidate linear models using all combinations of non-redundant site covariates. Climate variables included mean daily vapor pressure deficit (MDVPD), mean daily temperature (MDT), and mean annual precipitation (MAP); topographic variables included slope, aspect, and elevation. Mean daily temperature and MDVPD values were obtained from Daymet gridded Daily Surface Weather Data sampled at a 1000 m spatial resolution from 2011 to 2021 (Thornton et al. 2022). Mean annual precipitation values were obtained from PRISM (Parameter-elevation Relationships on Independent Slopes Model) gridded climate data from 1990 to 2020 (PRISM Climate Group (Oregon State University) 2022). Originally in 800 m resolution, PRISM data were resampled to 1000 m using cubic convolution to align with Daymet data. We extracted elevation values from a digital elevation model (DEM) with a resolution of 1 m (United States Geological Survey 2021). Slope and aspect were calculated for each DEM grid cell in QGIS Version 3.26.0 (QGIS Association 2022).

These covariates were the same as those used in prior studies of salamander behavioral and EWL responses in prior chapters (Carter, Chapters 2 and 3). Because our objectives were to test hypotheses related to climate and size at maturity, we did not include a null candidate model and all models included at least one hydroclimate covariate. All models included sex as a factor in combination with the other hydroclimate covariates (tMDT, MAP, or MDVPD). MDVPD was calculated using each days mean temperature and mean relative humidity and then average across all days over the 10-year period and converted to a raster for the basin. Because south-facing slopes receive more direct sunlight, slope values were converted to absolute degree deviations from north (0°) to south (180°). We examined correlations among all covariates and did not include any covariates with a Pearson correlation coefficient >0.60 in the same model. All covariates were standardized for analyses. We used the glm function in Program R for all models and conducted model selection using the aictab function in the R package AICcmodavg (Mazerolle 2023). We considered all models within 2 AIC_c of the top model parsimonious models (Burnham and Anderson 2002). All analyses were conducted in R version 4.2.1 (R Development Core Team 2023).

RESULTS

There were 14,775 *Plethodon* captured among the four studies. Because the amount of sampling effort and salamander abundance differed between sites, the numbers of animals observed at each site varied greatly. For example, the number of salamanders captured per site ranged from 1 to 3,880. A total of 1,628 confirmed reproductive adults were captured among 30 of the 67 sites. This included 22 sites where at least one reproductive female was captured and 29 sites where at least one reproductive male was captured, resulting in a final sample size of 51 site × sex combinations.

Elevation was strongly correlated with mean annual precipitation (1991–2020) and mean daily precipitation, VPD, and temperature (2011–2020) (Table 5.1). Because we were specifically interested in hypotheses related to climate, we chose not to include any models with elevation. All precipitation, temperature, and VPD covariates were also highly correlated and were therefore never included within the same candidate model. Aspect and slope were not generally correlated with any precipitation, temperature, or VPD covariates, but were instead moderately correlated with each other (Table 5.1). Specifically, south-facing sites tended to have steeper slopes, which reflects the known topography of the Coweeta basin. This left us with 16 potential candidate models.

Two similar models of minimum observed size at first reproduction had parsimonious support ($\Delta AIC_c < 2$). The top candidate model included an interaction between sex and mean annual precipitation and an added effect of slope (log-likelihood: -164.433; AICc: 342.776; AICc weight: 0.436; Table 5.2). The next best-fit model included only an interaction between sex and mean annual precipitation ($\Delta AIC_c = 0.707$). Both models show that for both females and males, minimum observed size at first reproduction was negatively correlated with mean annual precipitation at the site (Figure 1). For a given mean annual precipitation, male size at first reproduction was smaller than female size at first reproduction. Slope was also positively correlated with minimum size at first reproduction; however, the effect of slope was relatively modest compared the effects of sex and mean annual precipitation and was marginally statistically significant (Figure 5.1).

DISCUSSION

Our results show a clear pattern of increasing minimum observed size at first reproduction with declining mean annual precipitation. The difference in mean predicted minimum size at first reproduction was nearly 20 mm across the relatively steep rainfall gradient that spanned a relatively small geographic distance (~4.5 km). This is a remarkable difference in size at first reproduction for animals that range between 43 and 85 mm as adults. Without growth curves for individuals from the different sites, we cannot know how long maturity is delayed in order to achieve larger body sizes with declining mean annual precipitation; however, we can tentatively speculate. Females at our wettest sites had a mean predicted size at first reproduction of ~53 mm compared to ~73 mm at our driest sites. Males at our wettest sites had a mean predicted size at first reproduction of ~45 mm compared to ~67 mm at our driest site. Connette (2012) estimated growth rates for a population of *P. shermani* at 1200 m elevation within the Nantahala Mountains (Clay County, North Carolina) ~16 km from our study site. Based on his growth curves, *P. shermani* at this site, *P. shermani* would grow to 45 mm SVL in 2 to 3 years and 52 mm by 3 to 4 years. The smallest reproductive male observed at this site was 43 mm and the smallest reproductive female was 49 mm. Similarly, Howard and Maerz (Howard 2018 and J. Maerz, unpublished data) generated growth curves fitted to known age *P. shermani* × *teyahalee* from 11 years of capture-recapture data among three sites within the Coweeta basin between 850 and 960 m elevation with intermediate rainfall (Figure 5.2). They also estimated it would take 2 to 3 years for an individual to grow to 45 mm SVL and 3 to 4 years to grow to 52 mm SVL. The smallest reproductive males observed among these sites were between 41 and 52 mm SVL, and the smallest reproductive females were between 53 and 58 mm SVL—indicating that males typically take 2 to 4 years to first reproduction and females take 3 to 6 years. These results suggest that a modest change in climate associated with a 4 to 8 mm increase in minimum size at first reproduction could increase time to maturity by 1 to 2 years. Therefore, by extension, the 20 mm difference in mean predicted size at first reproduction between our driest and wettest sites within the Coweeta basin could represent a 3- to 5-year difference in age at first reproduction. We caution that we do not have growth rate data from any study for immature individuals larger than 55–60 mm. It is possible that immature individuals sustain more rapid growth beyond 55–60 mm than is represented by the growth rates of reproductive individuals. However, it is also likely that drier conditions at sites with larger size at first reproduction will limit foraging activity (Feder 1983; Carter, Chapter 2) and, therefore, growth. It will be important for future research to generate growth data for *Plethodon* from drier sites; however, surface activity and abundance at those sites are generally low. This makes the collection of such data challenging and may also create a greater potential for observing larger individuals in dry sites. Furthermore, because body size is correlated with both survival and climate, higher mortality among smaller adults may create additional bias towards sampling larger adults in drier climates. Our prior work modeling surface activity in this system has given us some capacity to gauge the likelihood that these factors are creating a sampling bias (Carter, Chapter 3). We find that, though larger individuals have a higher probability of being observed in drier sites, the effect of body size is small relative to the effect of precipitation on surface activity across this hydroclimate gradient. However, we recommend that future studies address this potential source of bias using novel approaches such as passive integrative transponder (PIT) telemetry to facilitate sampling.

A key question emerging from our results is the extent to which thresholds for body size at first reproduction are fixed versus plastic within and across sites. Previous research has demonstrated that amphibians exhibit plasticity in response to climate among a wide suite of traits (reviewed by Urban et al. 2014) and has documented environmentally-driven (e.g., competition-driven) variation in size and age at first reproduction in amphibians. However, we are currently unaware of any study addressing climate-associated plasticity in size at maturity in amphibians—or any other ectothermic vertebrates, for that matter. Furthermore, in the absence of a mechanistic understanding of the proximate (genetic and hormonal) factors responsible for regulating size at first reproduction, we are unable to determine whether the patterns of variation in observed size at first reproduction represent climate-driven variation caused by a single reaction norm or genetic differences among sites. Critically, this knowledge gap hinders our ability to fully understand the extent to which past hybridization—and the existence of adaptive introgression related to climate—contributes to the capacity for evolution of different body sizes at first reproduction. In other words, we do not know whether *P. shermani* and *P. teyahalee* have similar reaction norms for size at first reproduction or whether the two species have different reaction norms and compressed capacities to adapt to different climates. It is possible that compression of adaptive responses would constrain the distributions of the two species across a hydroclimate gradient. The demographic effects of delayed maturation to a larger size might also explain why *P. teyahalee* is restricted to lower, drier elevations compared to *P. shermani*. If the two lineages have different maturation reaction norms to climate, then *P. teyahalee* may be demographically outcompeted in wetter climates by *P. shermani*, which reaches maturity years earlier and at a smaller size. However, hybrid lineages and adaptive introgression between these two species (Carter, Chapter 2) may allow broader body-size life-history adaptation of lineages to climate. If this is the case, then hybrid zones may determine the responses and persistence of salamander lineages under future climate conditions.

TABLES

Table 5.1. Pearson correlation coefficients among potential site covariates. MAP refers to PRISM interpolated mean annual precipitation from 1991-2020. MDP, MDT, and MDVPD refers to Dayment interpolated mean daily precipitation, temperature, and vapor pressure deficit between April 1 and October 31, 2011-2020. Mean daily vapor pressure deficit was calculated using each days mean temperature and mean relative humidity and then average across all days over the ten-year period and converted to a raster for the basin.

Covariate	Slope	Aspect	MDVPD	MDT	MDP	MAP
Elevation	0.223	0.121	-0.916	-0.917	0.918	0.824
MAP	0.066	-0.033	-0.809	-0.792	0.786	1
MDP	0.167	0.118	-0.994	-0.995	1	--
MDT	-0.124	-0.065	0.999	1	--	--
MDVPD	-0.118	-0.036	1	--	--	--
Aspect	0.514	1	--	--	--	--

Table 5.2. Model selection results for 16 candidate models of minimum observed size at first reproduction for female and male *Plethodon shermani*, *P. teyahalee*, or *P. shermani X teyahalee* hybrid lineages within the Coweeta Basin, Macon County, NC.

Model	K	AICc	ΔAICc	ModelLik	AICcWt	LL	Cumulative.Wt
Sex*MAP + Slope	6	342.776	0.000	1.000	0.436	-164.433	0.436
Sex*MAP	5	343.483	0.707	0.703	0.306	-166.075	0.741
Sex*MAP + Aspect + Slope	7	345.122	2.3456	0.310	0.135	-164.258	0.876
Sex*MAP + Aspect	6	345.335	2.559	0.278	0.121	-165.713	0.997
Sex*MDVPD + Slope	6	361.174	18.398	1.01E-04	4.41E-05	-173.633	1.000
Sex*MDVPD	5	361.481	18.705	8.68E-05	3.78E-05	-175.074	1.000
Sex*MDP + Slope	6	362.477	19.701	5.27E-05	2.30E-05	-174.284	1.000
Sex*MDT + Slope	6	362.808	20.032	4.47E-05	1.95E-05	-174.449	1.000
Sex*MDT	5	363.055	20.279	3.95E-05	1.72E-05	-175.861	1.000
Sex*MDP	5	363.433	20.657	3.27E-05	1.42E-05	-176.050	1.000
Sex*MDVPD + Aspect + Slope	7	363.868	21.092	2.63E-05	1.15E-05	-173.632	1.000
Sex*MDVPD + Aspect	6	363.951	21.175	2.52E-05	1.10E-05	-175.021	1.000
Sex*MDP + Aspect + Slope	7	365.156	22.380	1.38E-05	6.02E-06	-174.276	1.000
Sex*MDT + Aspect + Slope	7	365.501	22.725	1.16E-05	5.06E-06	-174.448	1.000
Sex*MDT + Aspect	6	365.522	22.746	1.15E-05	5.01E-06	-175.807	1.000
Sex*MDP + Aspect	6	365.815	23.039	9.93E-06	4.33E-06	-175.953	1.000

FIGURES

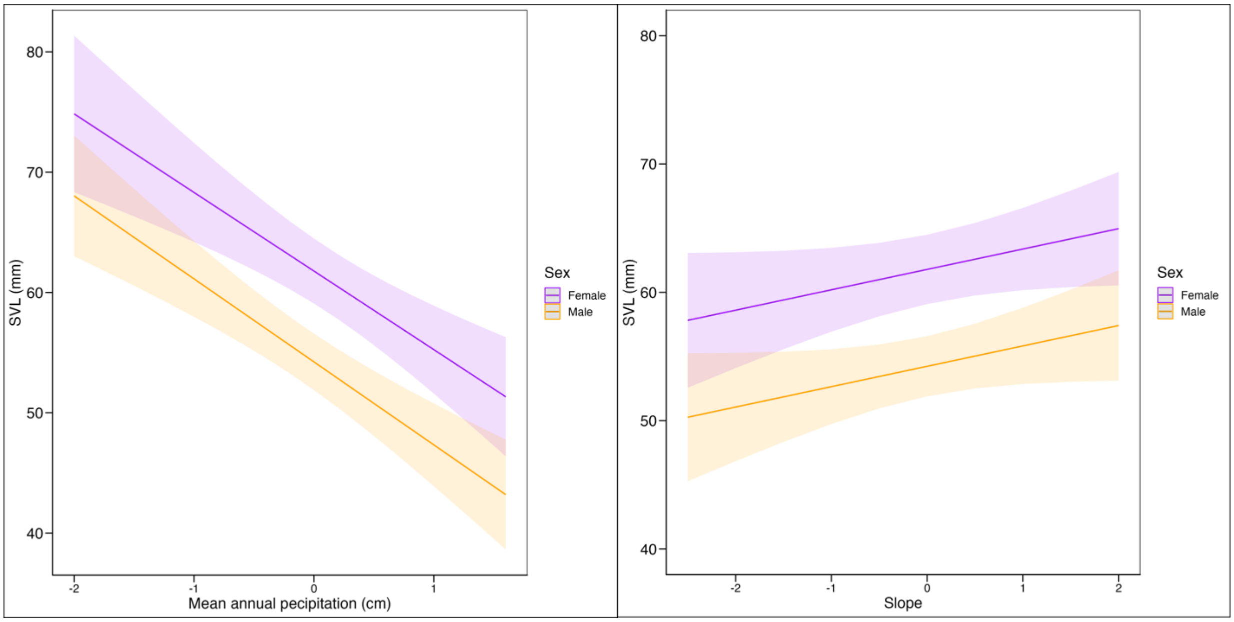

Figure 5.1. Predicted relationships between mean annual precipitation (PRISM, 1991-2020) and minimum observed size at first reproduction for female and male *Plethodon shermani*, *P. teyahalee*, or *P. shermani X teyahalee* hybrid lineages within the Coweeta Basin, Macon County, NC.

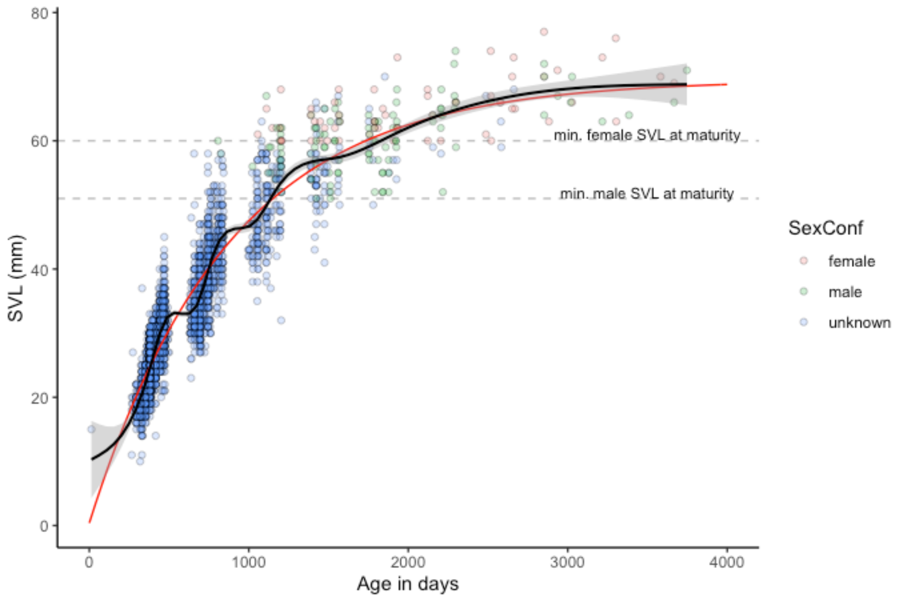

Figure 5.2. Growth curves developed by Howard (2018) and Maerz (unpublished data) fitted to known age *P. shermani* × *teyahalee* from 11 years of capture-recapture data among three sites within the Coweeta basin between 850 and 960 m elevation with intermediate rainfall. The red line is a fitted Von Bertalanfy function and the black line is mean trend reflecting periods of no growth during winters. The midpoint of each period represents ~1 year.

CHAPTER 6:

CONCLUSIONS

The goal of this dissertation is to describe genomic and morphological patterns between two hybridizing salamander lineages in relation to climate and then evaluate pattern of behavior, physiology, and life history in relation to ancestry and climate. The results of this work provide strong evidence that a spatially-complex hydroclimate gradient—created by variation in steep topography—functions as a powerful selective force shaping the structure and distribution of evolutionary interactions between two parent lineages and the distribution of traits in this system. In Chapter 1, I demonstrate that climate, specifically precipitation, is tightly correlated with the distribution of genotypic variation across the hybrid zone. The patterns of hybrid genotypes and phenotypes in addition to the existence of outlier loci that indicate this system is evolutionarily active and may be undergoing adaptive introgression. These results are consistent with other recent studies of similar hybrid zones in the region (Lowe 2016; Weaver 2022). Within the center of the hybrid zone there is wide variation in morphological phenotypes that is weakly correlated with genomic hybrid index which may indicate differences in the selective pressures acting on those traits. We propose that evolution within this hybrid zone may be an important area for creating novel genotypes and phenotypes that may be important for future evolutionary responses to environmental change.

In Chapters 3-5, I aimed to identify variation in behavioral, physiological, or life history traits that might differ between the two parent species and vary within hybrids that might explain the clinal genotypic and phenotypic patterns in relation to climate. Prior to this work, only variation in color patterns had been examined within the hybrid zones of *P. teyahalee* and *P.*

shermani. Similar patterns of color morphology have also been studies between *P. teyahalee* and *P. jordani*, but few studies have examined other phenotypic traits. Several studies have found evidence of competition between members of the *P. jordani* and *P. glutinsosus* complexes. Nishikawa (1990) compared movement and resource use in *P. teyahalee* and *P. jordani* and proposed that these species exhibited patterns of intraspecies territoriality. This study found that while both species had small, fixed home ranges with limited overlap among individuals they differed in the relative amount of space they occupied and the extent to which they returned to and defended burrow sites. *P. teyahalee* had smaller home ranges appeared to reuse and defend burrow sites—a behavior not observed in *P. jordani*. Hairston (Hairston Sr. 1980; Hairston Sr. 1981) conducted several experimental field manipulations of *P. jordani* and *P. teyahalee* (then called *P. glutinosus*) populations and found that competition between these species had a significant impact on their abundances, though the strength and symmetry of these impacts was variable. with *P. jordani* had a negative impact on the abundance of *P. teyahalee*. Riddell and Sears (2015) describe geographic variation in surface activity and resistance to water loss for *P. metcalfi* and *P. teyahalee*. In Chapter 2, I demonstrated that surface activity varies as a function of recent rainfall amount and body size—findings that supports the strong established role of hydric constraints in regulating salamander surface activity. However, my work failed to find any support for behavioral differences in surface activity between *P. shermani*, *P. teyahalee*, or hybrid salamanders that one might expect to compensate for significant variation in climate. *P. teyahalee* tend to experience reduced surface activity because of reduced rainfall at sites where the species occurs, but for a given set of weather conditions and body size, their behavior was indistinguishable from *P. shermani*. In Chapter 3, I used a novel method to indicate that *P. teyahalee* and P. *shermani* do not appear to differ in evaporate water loss rates under concurrent field conditions. This is not consistent with other work suggesting there may be adaptive variation in evaporative water loss rates among *Plethodon* in response to geographic variation in climate (Riddell and Sears 2015). However, this result is consistent with my behavioral findings that, for a given body size, individuals of both species and hybrid individuals show similar responses to weather. This is what one would expect if evaporative water loss rates were similar across species and sites.

One finding that was important in both my behavioral studies and evaporative water loss study was that body size was negatively correlated with evaporative water loss and positively correlated with surface activity. Increased resistance to water loss and reduced evaporative water loss leading to increased periods of activity is well established for plethodontids and other amphibians (Spotila 1972; Feder 1983; Feder and Londos 1984; Riddell and Sears 2015). This prompted us to explore whether variation in body size might be a key adaptation to climate among salamanders at our study site. Like most vertebrate ectotherms, growth is relatively rapid until maturity at which time growth can slow dramatically as energy is diverted from growth to reproduction. As a result, a key mechanism for achieve a larger body size is to delay maturation and reproduction. We demonstrated that even across the relatively short geographic extent of our study, there was significant variation in minimum observed size at first reproduction almost certainly linked to substantial variation in years to maturity among sites. Importantly, this variation in minimum observed size at first reproduction was strongly correlated with climate: both mean annual precipitation and slope. Decreased mean annual precipitation and increasing slope, which we associate with more drained soils, were both correlated with increasing size at first reproduction. The implications of this result are significant. On the one hand, increasing body size is a relatively simple and intuitive adaptation to drier environments, allowing animals to reduce evaporative water loss and increase activity in drier environments. However, it is also complex because increased size almost certainly requires delayed maturation, which has significant life history and demographic consequences. Age and size a maturity has been characterized as the most important life history traits because body size affects all aspect of an organism's biology and fitness, and the relationships between climate and size and age at first reproduction have long been and remain a dynamic and active area of research. In most cases, animals get larger and have delayed maturity in cooler climates, but numerous studies have found exceptions to this pattern, particularly among salamanders that are often larger in warmer, drier climates. We believe this pattern warrants wider exploration within and among *Plethodon* spp. to determine whether it is general, and it for studies of the mechanisms, plasticity, and demographic consequences of this phenomenon. If robust, this pattern will be important to forecasting *Plethodon* responses to climate change. In addition, it creates a target for future studies of the hybrid zone to determine whether there is greater capacity for life history variation among hybridizing lineages that is present in non-hybridizing lineages.

LIMITATIONS

There were several key constraints to what this dissertation work could achieve. While my analysis of genomic clines reveals evidence of asymmetrical introgression, the lack of a reference genome for *Plethodon* prohibits the identification of traits linked to outlier loci and, therefore, renders inferences about the selective pressures shaping these patterns impossible at present. Arguably, the establishment of a reference genome for *Plethodon* would do the most to advance this research and ecological genomics of this group of diverse and influential salamanders. I strongly advocate that future work should focus on the assembly and annotation of a *Plethodon* reference genome, thereby allowing for the inference of the genomic architecture of introgressed alleles and their putative function.

Another constraint is the sometimes-limited representation of the range of climatic and topographic conditions within the basin represented in the datasets used in this study. For example, tissues sequenced for Chapter 2 were primarily collected from wet high-elevation sites or dry low-elevation sites and sites with intermediate precipitation near the center of the hybrid zone. Because temperature, vapor pressure deficit, precipitation, and elevation are tightly correlated along this gradient, it is difficult to identify their relative contributions to patterns in ancestry. Our dataset only included one site (site 10) where these climatic and topological variables deviated substantially from this pattern. This site is steep and relatively dry given its elevation (~1025 m), and while the mean annual rainfall at site 10 was similar to that of the site 2750 (2076 mm and 2080 mm, respectively), its mean HI was over 6.5 times lower (0.67 and 0.44, respectively). This deviation from the pattern seen across our other sites highlights the need for more thorough coverage of the full range of climatic and topographic conditions in the basin. Obtaining samples from dry sites presented a further challenge because animals at these locations spend a large portion of their time underground. Therefore, it was difficult to generate large sample sizes from these areas. For the same reasons, we are currently limited in our ability to conduct demographic research on populations at drier sites. As a result, studies must often rely on asymmetrical numbers than favor wetter sites over their dry counterparts, increasing uncertainty of estimates from drier sites and potentially biasing some models.

A constraint of Chapters 3 and 4 was the limited range of conditions during which data collection occurred. It is possible that differences in the probability of surface activity or water-loss rates as a function of hybrid index might only be apparent under either extreme or prolonged dry conditions. This issue could be addressed in future studies of water loss rates by acclimating animals to dry conditions in the laboratory for longer periods of time. Some additional limitations of Chapter 4 include the narrow timeframe and range of conditions under which I was able to acclimate animals, the inability to regulate the temperature at which animals were measured, and a lack of direct comparisons to traditional methods for measuring water-loss rates. The use of a novel approach for measuring water loss was necessitated by unforeseen obstacles preventing the use of a flow-through system in a laboratory setting. While imperfect, the use of the evaporimeter in the field provided valuable physiological data, and future work should explore ways to minimize the drawbacks described here.

REFLECTION

The importance of gene flow (e.g., introgression) for facilitating adaptation to climate change is evident. Predicting adaptive capacity requires understanding how interactions within and among species shape the distribution of genotypic and phenotypic variation. Future work should continue to explore the "natural laboratories" of hybrid zones and make comparisons to similar systems where species co-occur without gene flow to better understand how introgression alters the distribution of genomic, behavioral, morphological, and life history adaptations to climate.

A system particularly well-suited for this kind of inquiry lies less than 40 km away from Coweeta. At Cheoah Bald, *P. cheoah*—which is morphologically identical and sister to *P. shermani*—also co-occurs with *P. teyahalee*. However, these two species are sympatric along a broad elevation–climate gradient with limited genetic evidence of introgression (Highton and Henry 1970; Highton and Peabody 2000). Laboratory courtship trials suggest a partial proximate cause is sexual isolation during courtship as a prezygotic barrier to hybridization between *P. cheoah* and *P. teyahalee* at Cheoah Bald (Reagan 1992). Therefore, despite the close relationship and remarkable morphological and ecological similarities between *P. shermani* and *P. cheoah*, they appear to have completely different ecological and evolutionary interactions with *P. teyahalee*. This comparative context presents a relatively unique opportunity to evaluate the role of introgression in promoting adaptation to climate change. Through hybridization, *P. shermani* have access to *P. teyahalee* traits that are linked to drier, warner conditions. If changes in the hydroclimate of this region pose a disproportionate risk to the survival of *P. shermani* the spread of these traits through introgression could increase the adaptive capacity of animals with hybrid ancestry and allow *P.*

shermani's genome to persist under future climate conditions. In contrast, the adaptive potential of the high-elevation endemic *P. cheoah* may be constrained to the limited variation that already exists within this range-restricted species, potentially resulting in a different resilience to climate change than we would predict for *P. shermani*.

This dissertation makes important contributions to the body of research examining how evolutionary interactions among populations influence species' adaptive capacity. In light of the rapid pace of anthropogenic climate change, research exploring the role of evolutionary processes generally—and of hybridization specifically—in determining biodiversity resilience to climate change will be an important next step. In order to maximize the conservation potential of these research contributions, there is a pressing need for analogous work that focuses on managing wildlife populations for adaptive capacity. The preservation of hybrid lineages and the landscape processes that allow natural introgression to occur may provide a critical tool for promoting the persistence of organisms with a shifting climate. At the same time, there is must also be corresponding changes to the regulations that govern conservation efforts. Because populations of hybrid origin have no legal recognition at best and are deemed conservation threats at worst, it will be necessary to develop new frameworks that recognize the importance of hybrid zones to the persistence of biodiversity under a changing climate.

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APPENDIX 1.1:

OVERVIEW OF TAXONOMIC HISTORY & SYSTEMATIC UNCERTAINTY SURROUNDING *PLETHODON SHERMANI* AND *P. TEYAHALEE*

“Will we ever resolve plethodontine phylogeny?
My guess is that we will not.”
David Wake (2017)

PLETHODON SHERMANI

This species was first described in 1906 from a specimen collected by entomologist Franklin Sherman, Jr., on August 24, 1904 (Stejneger 1906). The holotype specimen was deposited in the USNM by C. S. Brimley. Sherman initially described the collection location as “on Nantahala Mountain, between Andrews and Aquone.” A year later, Sherman expressed some uncertainty about the precise location in a letter referenced by Stejneger in a footnote of his species description. In 1908, Brimley accompanied Sherman on an expedition to collect additional animals between Franklin and Aquone. During this trip, Sherman remarked that he believed that Wayah Bald Mountain was actually the location where the original specimen had been collected (Brimley 1912).

In 1941, Bishop classified *shermani* as a subspecies of *P. glutinosus* because of white spotting in some *shermani* specimens—a trait he believed represented intergradation between the two lineages (Bishop 1941). Bishop’s assertion was rejected by Grobman (1944) who again elevated the lineage to species level as *P. shermani* (considered a member of the “Metcalfi group”). This classification was upheld by Hairston & Pope (1948) who asserted that there were structural differences in the spotting of *P. shermani* and *P. glutinosis* specimens, but later revised by Hairston (1950) who classified *shermani* as a subspecies of *P. jordani.* Highton (1973) affirmed this classification; however, *shermani* was removed from synonymy of *P. jordani* by Highton and Peabody (2000) and continues to retain specific status as *P. shermani*.

PLETHODON TEYAHALEE

This species was initially described as *Plethodon jordani teyahalee* from a specimen collected in the Snowbird Mountains by Hairston (1950). In 1970 Highton and Henry reclassified *teyahalee* as a subspecies of *P. glutinosus* and Highton later elevated it to species level in 1984 (Highton and Henry 1970; Highton 1984). According to Highton (1989), *P. teyahalee* is genetically homogenous throughout its range and differs from most *P. glutinosus* and *P. jordani* members at 6 allozyme loci:

- 2 of those loci have unique alleles
- 2 loci have common alleles w/ *P. cylindraceus*
- 2 loci have common alleles w/ *P. shermani*

In this same paper, Highton suggested that *P. teyahalee* might have hybrid origins in the Pleistocene, though may still have arisen via allopatric speciation via isolation of a hybrid population (Highton 1989). Following the publication of Highton's allozyme data, Hairston (1993) declared that because the type specimen of *P. teyahalee* appeared to be the hybrid offspring of representatives from the *P. jordani* and *P. glutinosus* complexes, the name *teyahalee* could no longer be applied to any species belonging to either group, per the International Code of Zoological Nomenclature. Hairston collected new type specimens from a location thought to be outside of any hybrid zone and called them *P. oconaluftee*. Though some scientists have adopted this new name for the species, the revision has been dismissed as unnecessary by many (e.g., Highton and Peabody 2000; Highton et al. 2012).

QUICK SUMMARY OF GENERAL TAXONOMIC AND PHYLOGENETIC UNCERTAINTY

There is general consensus that the eastern *Plethodon* form a well-supported clade. Highton and Larson (1979) classified the eastern members of the genus into four broad groups: two large-bodied groups (*P. wehrlei* and *P. glutinosus*) and two smaller-bodied groups (*P. cinereus* and *P. welleri*). There has been some additional work suggesting that a fifth group containing the *P. websteri* species complex—formerly considered a member of the *P. welleri* group—should be recognized (Feist et al. 2019). The relationships both among and within these species groups are not well resolved. Historically, the *P. glutinosus* "group" has been considered to contain the smaller *P. glutinsosus* "species complex" and the *P. jordani* "species complex" (Highton and Peabody 2000). Within each of these complexes, species are ecologically similar, and many have parapatric distributions. However, both allozyme and mtDNA trees do not produce monophyletic clusters for either complex (Highton et al. 2012).