How Salamander Species Can Hybridize Extensively Yet
Remain Distinct: Insights from Habitat Data, Molecules, and
Behavior

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Finally I need to thank my parents, my brothers, and most of all, my wife, Julie Lowe. Without their boundless support and encouragement this work would not have been possible.

DEDICATION

To my mother and father, Ron and Lindsay Lowe, whose love and support made this dissertation possible. Thank you for fostering my fascination with the natural world.

ABSTRACT

    This project explores a situation where two distinctive species hybridize extensively at zones of contact yet appear not to be collapsing into a single species. Lack of impending merger is inferred when regions made up of organisms of mixed ancestry ('hybrid zones') are not increasing in extent but instead are stable. There is growing recognition that this phenomenon is common nature. Several forces have been proposed to function in preventing lineage merger in these situations. Selection for different ecological conditions ('exogenous selection') is widely acknowledged to maintain species limits, especially when the contacting lineages are associated with different habitats. Genome incompatibilities (when hybridization leads to the breakdown of co-adapted gene complexes, or 'endogenous selection') have also been cited as contributing to hybrid zone stability. Positive assortative mating, where individuals preferentially mate with similar individuals, has also been put forward as a process that could prevent the merger of species.

    Two species of lungless salamanders, *Plethodon shermani* and *P. teyahalee*, have parapatric distributions in the southern Appalachian Mountains and form hybrid zones where their distributions meet. I investigated hybrid zone stability and potential factors maintaining species limits between these salamanders. In Chapter 1, I use coalescent simulations to identify a lower bound on the timing of secondary contact, which in turn leads to an expected hybrid zone extent under a model of neutral diffusion. By comparing observed and expected hybrid zone extent, I show that the two species are not in the process of freely merging despite extensive introgression of molecular markers. In Chapter 2, I evaluate predictions of ex- and endogenous selection using ground-level temperature data and character clines fitted to molecular and morphological data. I find evidence for exogenous selection but not for endogenous selection. In Chapter 3 I present data from courtship trials performed in the laboratory that reveal that despite frequent interspecific hybridization, these two species show a preference for conspecifics. These findings shed light on *Plethodon* hybrid zone dynamics and the maintenance of species limits for hybridizing species.

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CHAPTER 1

ASSESSMENT OF HYBRID ZONE FIT TO MODEL OF NEUTRAL DIFFUSION

ABSTRACT

Whether lineages that meet and form broad hybrid zones ultimately merge or remain distinct is a key question in systematic and evolutionary biology. For example, determining whether lineages are in the processes of merging is important for species delimitation and for inferring processes that generate and maintain species lineages. Two southern Appalachian salamanders, *Plethodon shermani* and *P. teyahalee*, form broad hybrid zones (relative to their dispersal abilities) where their distributions meet. Some researchers have suggested that these two species form a hybrid swarm and that *P. shermani* is being genetically consumed by *P. teyahalee*, whereas others have concluded that these two species maintain divergent evolutionary histories despite hybridization. Here, I use morphological and molecular data and recently developed methodologies to address this question more directly. Specifically, coalescent theory is used achieve a lower-bound estimate of the timing of hybridization initiation, and that estimate is used to estimate the expected extent of hybrid zones under a process of neutral diffusion, which I compare with observed hybrid zone extents. My analyses find the two species are not in the process of freely merging despite extensive introgression of molecular markers. These analyses also render findings that have bearing on other hypotheses regarding the evolutionary history of these species. Finally, I discuss several non-mutually exclusive factors that may be serving to maintain species limits for these species.

INTRODUCTION

Hybrid zones, areas where two parapatric lineages are in contact and exchanging genes, have been of interest to evolutionary biologists for more than a century (Bateson 1913, Du Rietz 1930, Huxley 1939). Early researchers made a distinction between wide, possibly ephemeral zones of intergradation, and relatively narrow, seemingly stable hybrid zones (e.g., Du Rietz 1930). Often, early researchers did not specify the criteria that differentiated "narrow" and "wide" hybrid zones (e.g., Dobzhansky 1940), and those

that did indicated that the designation was based on comparisons with other hybrid zones formed by similar taxa (e.g., Huxley 1939). Lineages that come into contact in wide ephemeral hybrid zones have often been considered subspecies, while those that form stable, narrow hybrid zones have generally been considered species (but see Dobzhansky 1937, Mayr 1940). Further, ephemeral hybrid zones are most likely a product of secondary contact, while stable hybrid zones can arise from either primary or secondary contact (Hewitt 1988). Several seemingly stable hybrid zones have been the subject of long-standing research programs (e.g., *Bombina* [Szymura & Barton 1986], *Gryllus* [Rand & Harrison 1989]); however, little progress has been made toward understanding the factors that maintain hybrid zone stability.

Whether lineages exhibiting incomplete reproductive isolation merge or remain distinct is a key question in systematic and evolutionary biology, given the implications for species delimitation and inference of processes that generate and maintain species lineages. As such, determining whether lineages that form hybrid zones are in the processes of merging is important. Surprisingly little work has been put towards developing methods for distinguishing between stable and ephemeral hybrid zones. One method is to determine whether the observed hybrid zone is narrower than expected under a model of unimpeded gene flow. Endler (1977) developed a "neutral diffusion" model for estimating the time required for hybridizing lineages to merge given no barriers to gene flow or selection against hybrids (i.e., an ephemeral hybrid zone). When solved for hybrid zone width (w), the equation calculates the expected hybrid zone width under neutral diffusion. The required terms are time since initial hybridization (T_H), the species' mean dispersal before reproduction (d), and generation time (T_G) (Endler 1977, Hafner et al. 1983).

Estimating timing of secondary contact is often difficult. It has been suggested that historical events such as changes in climate or water level can be used to estimate the timing of secondary contact (Hewitt 1988; e.g., Carling et al. 2010); however, the assumption of hybridization immediately following such an event is unverifiable. If the aim is to rule out neutral diffusion across the hybrid zone, the actual timing of secondary contact is not necessary; all that is needed is a lower bound estimate of timing of

secondary contact that is sufficiently old to result in an expected hybrid zone width that is much larger than the observed hybrid zone width. This method was used in a recent study examining hybridization between gull species (*Larus* spp.; Gay et al. 2008). The authors used an early field observation of a phenotypically intermediate gull (Dawson 1908). Although this observation only provides a lower bound estimate of timing of secondary contact of roughly 100 years, the great dispersal capabilities of these birds results in an expected hybrid zone under a neutral-diffusion that is sufficiently broader than the observed hybrid zone, which allowed the authors to reject the hypothesis of neutral diffusion. However, for many systems, early observations of phenotypically intermediate individuals are either absent or insufficiently old relative to inferred dispersal potential.

Here, I address whether two salamander species, *Plethodon shermani* and *Plethodon teyahalee*, are in the processes of merging into a single evolutionary lineage, or whether they maintain divergent evolutionary histories despite the extensive hybridization that occurs between them along the slopes of the southern Appalachian Mountains. I employ a method for evaluating the relative stability of hybrid zones using molecular data when the date of the first observed hybrid is either unknown or not of sufficient antiquity to rule out neutral diffusion (sensu Gay et al. 2008). This method builds on recently-developed coalescent-based methods (Joly et al. 2009) and identifies incongruent haplotypes in gene trees that cannot be attributed to incomplete lineage sorting, thus rendering a lower bound estimate of time since hybridization initiation. I demonstrate that gene flow across this hybrid zone does not conform to a pattern of neutral diffusion and therefore environmental, behavioral, and/or genetic factors are maintaining hybrid zone stability.

METHODS

STUDY SYSTEM

I evaluated the stability of a hybrid zone formed between two species of slimy salamander (Plethodontidae: *Plethodon glutinosus* species group [Highton 1962]). *Plethodon shermani* (Stejneger 1906) occurs atop four disjunct "sky islands" (hereafter isolates) in the Blue Ridge province of the southern Appalachian Mountains. This

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species is of moderate size, and generally has red legs and lacks white spots (the northwestern population lacks red legs and exhibits some white spotting; Fig. 1.3). It has long been known that *P. shermani* hybridizes with larger *Plethodon* species (principally *Plethodon teyahalee*) with which they contact at mid-elevations (summarized in Highton and Peabody 2000; Weisrock et al. 2005). In addition to large size, these lower-elevation *Plethodon* are characterized by black legs, and extensive white spotting (Fig. 1.3). These two species are not sister taxa (Kozak et al. 2009; Blankers et al. 2012, Highton et al. 2012), and therefore hybrid zones between these two species must have formed through secondary contact.

A long-term morphological study conducted along an elevational transect at a zone of contact between *P. shermani* and *P. teyahalee* revealed the hybrid zone to be wide and spatially dynamic over time (Hairston et al. 1992). Additionally, a study of allozyme variation across hybrid zones formed between *P. teyahalee* and the four *P. shermani* isolates revealed great variability in hybrid zone extent and marker cline accordance among the various isolates (Peabody 1978; summarized in Weisrock et al. 2005). Though these observations are consistent with a stable hybrid zone, I aimed to evaluate the ephemerality of *P. shermani-P. teyahalee* hybridization in a more objective fashion.

ESTIMATING EXPECTED HYBRID ZONE WIDTH UNDER MODEL OF NEUTRAL DIFFUSION

Formula for Expected Hybrid Zone Extent: To evaluate whether *P. shermani* and *P. teyahalee* are in the process of merging, I compared observed hybrid zone extent with that expected under a model of neutral diffusion. (I will use the term 'extent' where most other authors have used the term 'width,' as 'extent' seems more appropriate for describing hybrid zones that occur in 3-dimensions.) If the observed extent is narrower than expected under a model of neutral diffusion, I can infer that factors (e.g., outbreeding depression, divergent ecological selection, assortative mating) are limiting gene flow between the hybridizing taxa. To generate this estimate of minimum expected extent, I employed Endler's (1977) neutral diffusion model (solved for *w*):

$$w = 1.68 \cdot d \cdot \sqrt{T_H/T_G}$$

where *w* is hybrid zone extent in meters, *d* is an estimate of mean dispersal prior to reproduction in meters, T_H is time since hybridization initiation (years), and T_G is mean time to reproduction (years). The constant 1.68 is derived from plotting values of *w*/*d* against $\sqrt{T_H/T_G}$ from simulations using numerous values of *d* (Endler 1977).

Mean Dispersal Distance (*d*), Generation Time (*T_G*): For *Plethodon glutinosus*-group salamanders, dispersal estimates can be found in the literature, though these are for the span of the study (thus require scaling up to mean time to reproduction). Further, those estimates are for horizontal movements, while the *P. shermani-P. teyahalee* hybrid zone is on a slope (thus three-dimensional) and the ecological axis is vertical. I converted estimates of dispersal abilities from previous studies to estimates of elevational dispersal distance per generation. Elevational dispersal ability *d* was calculated using the following formula:

$$d = R \cdot T_A \cdot T_G \cdot m$$

where *R* is an estimate of short-term dispersal rate (in meters per day), T_A is annual activity period (in days per year), T_G is generation time (i.e., mean time to reproduction; in years), and *m* is the slope of the transect. The short-term dispersal rate used was 9.4404×10^{-2} (Madison 1969); this is estimate is from a study of an ecological equivalent of *Plethodon shermani* (*Plethodon jordani*), and is comparable to unpublished estimates for *P. shermani* (Connette & Semlitsch 2013). The annual activity period used was 154 days (early May through early October; Petranka 1998, pers. obs.). The generation time (mean time to reproduction) used was five years. This value is based on estimates for ecologically equivalent congeners *P. jordani*, *P. metcalfi*, and *P. glutinosus* (Hairston 1980; Highton 1962). Further, it is bracketed by exceptional estimates for *P. shermani* (9.8 years; Hairston 1983) on one end and by estimates for ecologically disparate congeners on the other (e.g., three years for *P. cinereus* [Sayler 1966], *P. ouachitae* [Pope & Pope 1951], and *P. grobmani* [Highton 1962]). The slope of the transect (0.0895) was estimated by dividing the difference between transect top and bottom elevations by the

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Euclidian distance between the transect top and bottom; topographic maps were used to determine these values.

Lower-bound Estimate of Timing of Secondary Contact (T_H): Estimating T_H is often difficult. As described in the introduction, for highly vagile taxa an early record of a hybrid individual can provide a lower bound timing of secondary contact that results in a rejection of the neutral diffusion model (Gay et al. 2008), but if the taxa of interest have weak dispersal ability another method is likely required. There is an early observation of an animal of admixed *P. shermani-P. teyahalee* ancestry (1936; based on phenotype [Bailey 1937]), but the extremely low vagility of these animals precludes using this as a lower limit of the initiation of secondary contact in an effort to rule out neutral diffusion (sensu Gay et al. 2008), and therefore necessitates the method for secondary-contact estimation described in this paper.

   In situations of secondary contact, a time-calibrated phylogeny provides an opportunity to identify the timing of resumed gene flow. Specifically, instances of individuals possessing haplotypes that form a clade with those of heterospecifics and are divergent from those of conspecifics ('gene-tree incongruencies' [hereafter GTI]; see "bug 5" in Fig. 1.1a) could be the result of interspecific gene flow. However, they may instead be due to incomplete lineage sorting (hereafter ILS; Neigel & Avise 1986; Pamilo & Nei 1988) of ancestral polymorphism. When speciation occurs, for any given gene, each daughter lineage is expected to retain haplotypes more closely related to haplotypes present in its sister lineage than to some haplotypes in its own lineage (i.e., GTI due to ILS). If the taxa of interest are suspected to have diverged some time ago (e.g., are appreciably morphologically divergent), GTI with very small pairwise genetic divergences with respect to sequences from heterospecifics (e.g., approaching zero; "bugs 5 & 6" in Fig. 1.1a) can be assumed to be the product of post-divergence gene flow (McGuire et al. 2007). Further, if GTI exhibit considerable pairwise genetic divergence with respect to heterospecifics (e.g., "bug 9" in Fig. 1.1a) and display a random geographic distribution (i.e., not adjacent to a contact zone; Fig. 1.1b), ILS is a likely scenario (Barbujani et al. 1994; McGuire et al. 2007). Unfortunately, these methods are

neither sufficiently rigorous nor sufficiently precise to allow me to identify the oldest GTI attributable to ILS.

Given estimates of speciation timing and population sizes, coalescent theory can predict the lower limit of expected pairwise divergences for GTI due to ILS. Here I use a method (drawing heavily from Joly et al. [2009]) that utilizes coalescent theory to differentiate these two phenomena. Specifically, using estimates of time since speciation, mutation rate, and population size, I simulated gene trees to the same dimensions as an empirical gene tree, but under the constraint of no post-divergence gene flow. From those trees I simulate sequence data sets, generate pairwise divergence values, and generate a 95% confidence interval for lowest GTI sequence divergence (which can only be due to ILS). The most divergent GTI in the empirical gene tree with a divergence lower than the 95% confidence interval is the lower-bound estimate of the timing of secondary contact (illustrated as a flow chart in Fig. 1.2). The specifics of this method are detailed in the three sections immediately below.

Molecular Data Collection: For the purpose of estimating parameter values, I compiled sequence data for four nuclear (nuDNA) markers for *Plethodon shermani* and *Plethodon teyahalee* (Table 1.1). These samples were derived from ten populations composed of animals that overwhelmingly correspond to the pertinent species (hereafter parental localities; five parental populations per species). These populations correspond to the ends of five transects spanning hybrid zones formed between these two species. From these samples, genomic DNA was extracted from tissue using Viogene Genomic DNA Extraction kits (Viogene, Inc., Taipei, Taiwan). PCR products were purified using Viogene gel extraction kits (Viogene, Inc., Taipei, Taiwan), ExoSAP-IT (USB Corporation, Cleveland, Ohio), or AMPure SPRI bead purification (Beckman Coulter Genomics, Danvers, MA). Cycle-sequencing reactions used BigDye terminators (v3.1 Cycle Sequencing Kit, Applied Biosystems Inc., Foster City, California). Subsequent to the cycling reactions, samples initially cleaned using SPRI beads were then cleaned with AgenCourt CleanSEQ (Life Technologies, Carlsbad, CA). The resulting samples were then sequenced in the forward direction on an ABI 3730xl automated sequencer (Applied

Biosystems Inc.). The software programs SEQUENCHER (v4.8; Gene Codes Corporation, Ann Arbor, MI) and GENEIOUS (v6.1.2; Biomatters Ltd., Auckland, NZ) were used to edit sequences. Diploid data were converted to constituent haplotypes and tested for recombination using the software PHASE (v2.1.1; Stephens et al. 2001, Stephens & Donnelly 2003, Stephens & Scheet 2005) as implemented in DNASP (v5; Librado & Rozas 2009).

Population Genetic Parameter Estimation and Time-calibrated Phylogeny: The nuDNA sequence data described above along with ND2 mitochondrial sequence data (mtDNA) from Weisrock et al. (2005) were used to estimate population size parameters θ for the ancestral and two daughter branches of the *P. shermani-P. teyahalee* species tree (using the program BP&P [v.2.1; Yang & Rannala 2010]). As both cytoplasmic and nuclear sequence data were used, a heredity scalar was used to account for the difference in effective population sizes. Sequences were pooled for each species, and across the *P. shermani* isolates. I acknowledge that this leads to an error in the population size parameters θ. However, I contend that this procedure results in a conservative estimate of the extent of the hybrid zone under a model of neutral diffusion, because lumping genetically divergent populations will result in a larger population size estimate, and therefore a longer coalescence time. As the coalescent time is being used to identify the cutoff point at which GTI can no longer be attributed to ILS, with a longer coalescent time, GTI's will be biased such that ILS cannot be ruled out, when in fact they are old enough to be so. Importantly, for testing the hypothesis of neutral diffusion as I do here, these conditions are expected to increase the probability of a type I error (rejection of stability when the hybrid zone is in fact stable) but not of a type II error (a false acceptance of stability). Thus, any rejection of the null hypothesis of neutral diffusion is conservative.

To create a gene tree in which GTI could be identified and evaluated, I reconstructed a time-calibrated phylogeny in the program BEAST (v1.8.2; Drummond & Rambaut 2007) using the mtDNA data from Weisrock et al. (2005). This phylogeny was inferred from an unpartitioned data set. With the limitations of downstream applications in mind, the

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Akaike information criterion (AIC) as implemented in the software program JMODELTEST (v2.1.4; Guindon & Gascuel 2003, Posada 2008, Darriba et al. 2012) was used to identify the most suitable model of evolution. The constant population size coalescent model was applied. Clade ages and standard deviations from Wiens et al. (2006; Plethodontidae root age = 66 mya) were applied to eastern *Plethodon* and "Clade A" of the *Plethodon glutinosus* group (including *Plethodon shermani* sequences that appear to be the product of introgression from "Clade A" species) (see Wiens et al. 2006 for "Clade A" membership). Analyses were performed using both a strict and relaxed clock and the two models were compared using Bayes factors. This analysis also yielded a mutation rate estimate μ (accessed from BEAST.log files using the program TRACER [Rambaut et al. 2014]) and an estimate of the absolute divergence time (T_D) between the *P. shermani* and *P. teyahalee* clades (gleaned from the time-calibrated phylogeny using the program FIGTREE [Rambaut & Drummond 2012]). The estimates of μ and T_D were used to calculate divergence time in mutational units (τ; Rogers & Harpending 1992) using the formula $\tau = 2\mu T_D$.

Simulations and Identification of Most Divergent GTI Not Due to ILS: Estimates of τ and θ were used to simulate 1000 trees under a model of no post-divergence gene flow using the program MCCOAL (Rannala & Yang 2003; distributed in the BP&P package). For my purposes, this program simulates trees under the multispecies coalescent model. Importantly, the simulated trees were constrained to have terminals of the same number and species assignment as observed in the tree inferred from the actual mtDNA sequence data. Further, these trees were simulated under a model of no post-divergence gene flow, thereby creating trees where all GTI are due to ILS.

The program SEQ-GEN (v1.3.3; Rambaut & Grassly 1997) was then used to simulate one data set per simulated tree of the same dimensions as the original mtDNA data set. These simulations were performed under the same model of evolution used to infer the empirical phylogeny, and used estimates of nucleotide frequencies, nucleotide substitution rates, gamma shape, and number of gamma categories elucidated in the BEAST analysis. These data were converted to Nexus format using the 'read.dna' and

'write.nexus.data' functions in the R (v3.1.0) package APE (R Development Core Team 2014; Paradis et al. 2004). In PAUP* (v4.0b10; Swofford 2002), the pairwise genetic distances were calculated. R was used to prune the file to a single column of interspecific pairwise divergences. The 'rollapply' function in the R package ZOO (v1.7-12; Zeileis et al. 2015) was used to extract the minimum interspecific pairwise divergence from each of the 1000 data sets, and this was used to identify a one-sided 95% confidence interval of minimum branch lengths of incongruencies due to incomplete lineage sorting. In the time-calibrated phylogeny, any observed incongruencies falling outside this interval were interpreted as post divergence gene flow events, and the oldest of these was used as the lower-bound estimate of timing of secondary contact T_H. I consider this estimate to be highly conservative, not only on account of populations being lumped (see above), but also due to the likelihood of earlier hybridization events that may have resulted in GTI that have been lost through stochastic processes.

<div align="center">CALCULATING OBSERVED HYBRID ZONE EXTENT</div>

Data Collection: Along with the tissue sample collection described above, I collected tissue samples from sites between the parental localities (hereafter hybrid zone localities; four or five such localities per transect). Phenotypic characters were also scored for salamanders from these and the parental localities using the protocol of Hairston et al. (1992). This entails assigning the degree of red on the legs and white spotting on the dorsal and lateral surfaces a score between 0 (absent) and 3 (maximally developed).

Sequence data were collected using the methods described above. For hybrid zone characterization, sequence data were generated for eight nuclear loci (Table 1.1), four being unpublished anonymous markers developed by colleagues (Kozak, KH, unpublished data) and four being previously published non-anonymous markers (3 protein coding, 1 intron). The resulting sequences were investigated by eye to identify a single base position that differed between the two parental types (single nucleotide polymorphisms, or SNPs); the respective species were not fixed for these differences, but differed greatly in allele frequency (allele frequency difference between the top and bottom of the transects ranged from 0.3 to 0.78; mean = 0.58). Subsequently, all

individuals were scored for their diploid genotype at this position.

Cline Fitting & Hybrid Zone Extent Estimation: I used the program CFIT-7 (Gay et al. 2008, Lenormand & Gay 2008) to describe patterns of hybrid zone structure along the various transects. This analysis software fits sigmoid clines to data under a likelihood framework, allowing for (1) the estimation of individual character clines within a single hybrid zone and assessment of their accordance (coincidence and concordance; Fig. 1.4), (2) the estimation of hybrid zone cline parameters given its constituent character clines, and (3) the degree to which different hybrid zones accord to a common independent variable (e.g., distance, temperature). Furthermore, clines can be fit using a minimal model or models of varying degrees of parameter richness. In particular, with the addition of two parameters (starting point and slope), an exponential "tail" can be fit to the data, modifying a simple sigmoid cline (e.g., Fig. 1.4) to have differently shaped distal extent. As an extension, these tails can be mirrored on both sides of the cline (also only two additional parameters) or each end can be fitted separately (four additional parameters).

I first used CFIT-7 to determine the best cline model for each transect. I chose to fit a unimodal cline to these data as my collecting localities are centered on the previously inferred zone of admixture (Highton & Peabody 2000), which is many times wider than the dispersal ability of these salamanders and therefore must be maintained by extensive late-generation hybridization and back crossing (Jiggins & Mallet 2000, Schilthuizen 2000, Gay et al. 2008). In model-fitting the morphological data the following seven parameters were invariably fit: cline center, cline slope, minimum phenotypic score, difference between minimum maximum phenotypic scores, and phenotypic score variance coefficients for the center and both ends of the cline. For my purposes, cline center is the point along the hybrid zone where salamanders exhibit a hybrid index of 0.5 (i.e., equal influence from both parental species), and the slope is the rate of hybrid index change at the center. The latter five parameters are required if the inherently quantitative phenotypic data are to be analyzed along with the genetic data (which are discrete--each individual is either heterozygous or homozygous for one of the two alleles). The five

following models were evaluated: no additional parameters, three models with two additional parameters (right tail, left tail, symmetrical right and left tails), and four additional parameters (right and left tails fit independently). The three variance coefficients were always allowed to vary independently. CFIT-7 runs produce log likelihood (lnL) scores, which can be used to evaluate the fit of models. As some of the models being compared are not nested, I used the lnL scores to compute corrected Akaike information criterion (AICc) scores (Pettengill & Moeller 2012). The model with the lowest AICc score was selected as the best-fitting model. This model-fitting technique was also used to determine the best model for the molecular clines with one difference: minimum phenotypic score, difference between minimum and maximum phenotypic score, and the three variance coefficients were not estimated (see above).

For these analyses I used elevation as the independent variable. These and all other CFIT-7 analyses were runs with different random seeds, the number of chains and replicates were increased dramatically relative to the default values, and all analyses were run multiple times with different priors to assess convergence. The scores were scaled to 0 and 1, and the extent spanning the values of 0.2 and 0.8 was used as hybrid zone extent. (Numerous authors have employed this convention as a measure of hybrid zone extent [e.g., May et al. 1975, Endler 1977, Hafner 1982].) Cline extents for both morphological and molecular data sets were estimated for each transect. Cline extents for combined data sets were also estimated where the morphological and molecular data sets were demonstrated to be in accordance; for these, the more complex model identified for the two data sets was applied.

RESULTS

CHARACTERIZATION OF *PLETHODON SHERMANI-P. TEYAHALEE* HYBRID ZONES

Preliminary model-testing analyses identified two transects across which morphological and molecular clines were accordant; the remaining three exhibited different morphological and molecular clines (two were coincident but not concordant, one was neither coincident nor concordant; Table 1.2). The estimated cline extents are presented in Table 1.2. Cline-fitting analyses in CFIT-7 recovered a broad range of

character cline extents ranging from 60 elevational meters to indefinite (i.e., failed to cross the 0.2 and/or 0.8 hybrid index thresholds). Clines for morphological data were consistently narrower than for molecular data, with the greatest observed extent being 275 elevational meters. The three molecular clines that crossed both the 0.2 and 0.8 thresholds exhibited extents ranging from 385 to 1170 elevational meters.

EXPECTED EXTENT OF *PLETHODON SHERMANI-P. TEYAHALEE* HYBRID ZONES UNDER MODEL OF NEUTRAL DIFFUSION

The most suitable model of evolution for the ND2 dataset (as determined via AIC) was TIM3+G, and the model employed in the BEAST and SEQ-GEN analyses was GTR+G. The time calibrated phylogenetic analysis failed to reject a strict clock; therefore the strict clock tree was used. Estimates of θ, *Plethodon shermani-Plethodon teyahalee* divergence timing, and τ are presented in Table 1.3.

The oldest GTI observed in the tree outside the 95% confidence interval of smallest divergence expected due to ILS (and the estimate of minimum time since secondary contact) was 565,200 years. Employing this estimate along with my estimate of mean dispersal at reproduction (6.05 elevational meters) in Endler's formula (1977) results in a hybrid zone extent estimate of 3,417 m, an order of magnitude greater than all five of the observed morphological cline extents and two of the molecular cline extents. Therefore, overall I reject a neutral diffusion model in this system, though in at least two cases the molecular clines do fit a pattern of neutral diffusion.

DISCUSSION

Hybrid zones are often considered windows into the process of speciation (Harrison 1990, Wilczynski & Ryan 1999; Kingston et al. 2014). This is attributable to the fact that allopatric speciation with complete reproductive isolation prior to secondary contact is insufficient to explain the diversity of life on earth (Bowen et al. 2013, Egan & Funk 2009, Gay et al. 2007). Over evolutionary time, recently diverged allopatric lineages frequently come into secondary contact before complete intrinsic reproductive isolation has been achieved. Therefore understanding what factors allow such lineages to remain

distinct, or alternatively merge into a single lineage is of great interest. Before that can be addressed however, discerning which path hybridizing lineages are on is necessary. In the present study I explore whether two species of salamanders that hybridize extensively along the slopes of the southern Appalachian Mountains are in the process of merging into a single evolutionary lineage. Building on previous work (Joly et al. 2009), I present a method for determining that is applicable in a wide range of situations.

Determining Expected Hybrid Zone Extent under a Model of Neutral Diffusion

In order to rule out neutral diffusion across a hybrid zone, one must first estimate the expected hybrid zone extent under a model of neutral diffusion. A minimum expected extent that is sufficiently large to rule out neutral diffusion can be derived for highly vagile taxa using historical observations of animals of intermediate morphology (e.g., Gay et al. 2008), or (theoretically) for systems for which there is existing information of sufficiently ancient secondary contact. However, few hybrid zone studies will be able to adequately rule out neutral diffusion using these classes of information. Discerning gene flow in the fossil record is not generally possible, though in some instances pollen from pollen cores may provide an interesting opportunity with vascular plants (e.g., Lishawa et al. 2013). Further, the relatively recent advent of modern natural history collections precludes their use for identifying a lower-bound estimate of timing of secondary contact that is sufficiently old to rule out neutral diffusion for all but the most vagile organisms. I suggest that a protocol employing molecular data and coalescent theory can provide a more fruitful lower-bound estimate of the timing of gene flow resumption, regardless of the species' dispersal ability or fossil record.

Key to my purposes, these methods provide a lower limit for expected GTI due to ILS, thereby identifying the oldest GTI to which the sole possible explanation for is post-divergence gene flow. Though the distinction between ancestral polymorphism and gene flow at secondary contact is often obvious at a qualitative level, these methods allow me to rigorously test these hypotheses. Further, it must be reiterated that I am not implying that this lower bound estimate of timing of secondary contact is an accurate estimate of the actual timing of secondary contact; it is very likely that secondary contact long

predates this estimate, but existing GTI predating this estimate could either be due to historical gene flow or ILS. In some instances, such as when the taxa of interest diverged recently or when few GTI are observed, these methods may be unable to identify a sufficiently old GTI that cannot be attributed to ILS.

Many of the issues surrounding these methods and opportunities for improvement are discussed at length in Joly et al. (2009). One issue in particular is that the number of samples included in the phylogenetic analysis is proportional to the age of the oldest GTI that cannot be attributed to ILS. In other words, a sparse tree may fail to recover a GTI that is of sufficient antiquity to allow for a rigorous evaluation of hybrid zone stability. Therefore, all efforts should be made to maximize the number of sequences used in the phylogenetic analysis.

A major caveat regarding this method is that it does assume that the lineages have remained in contact since the time period identified by the GTI attributable to hybridization. Unfortunately, it is likely that this assumption is violated in some instances. Extremely dense sampling could demonstrate that GTI have accumulated continuously since the first GTI attributable to hybridization, but it is just as likely that in a continuous contact scenario the coalescent process has removed GTI, creating a discontinuous distribution of GTI. This discontinuous pattern would be indiscernible from a pattern of intermittent hybridization. If contact has been sporadic, the calculations described here would result in a greater expected hybrid zone extent under a model of neutral diffusion than actually is the case. This would increase the likelihood of a type II error (accepting stability when the species are actually merging). Therefore I suggest caution be used when employing these methods.

Hybrid Zone between *Plethodon shermani* and *Plethodon teyahalee*

I found that the character clines across these transects were generally not accordant, and in all instances the molecular clines were of greater extent than the morphological clines. Though in-depth discussion of these findings is beyond the scope of this paper, I feel that the selectively neutral nature of these nuclear markers has allowed them in at least two instances to spread unimpeded across the hybrid zone and through the

respective species. I chose to collect data for nuclear markers as I felt this character type would best characterize the hybrid zones, as they should be free of diversifying selection while being variable at this shallow divergence level on account of a lack of purifying selection (including synonymous sites in protein-coding genes). However, given the age (at least half a million years) and nature of the hybrid zones, it appears the spread of alleles across the hybrid zones has to varying degrees resulted in deviations from the underlying hybrid zone patterns exemplified by characters more likely to be under selection (e.g., allozymes [Weisrock et al. 2005], phenotypic characters).

The remaining eight character clines and the two combined character clines were found to be considerably narrower than expected under a model of neutral diffusion, leading to the conclusion that the hybrid zone between *P. shermani* and *P. teyahalee* can be described as stable. There are several factors that may be working to maintain the hybrid zone and prevent the merger of the species. One conspicuous candidate factor is divergent ecological selection stemming from different environments ('exogenous selection'; Moore 1977). As this hybrid zone occurs across an elevational gradient, the *P. shermani* phenotype may have a selective advantage at higher elevations while the *P. teyahalee* phenotype has a similar advantage at lower elevations. Further, under this scenario, animals of mixed ancestry may have a selective advantage in areas of intermediate environmental condition (May et al. 1975). Alternatively, the breakdown of coadapted gene complexes may bestow animals of mixed ancestry with reduced fitness. This 'endogenous selection' (Moore 1977) would confer reduced fitness to hybrid and backcrossed individuals independent of environmental context (Barton & Hewitt 1985). Finally, assortative mating can work alone or in concert with the factors described above to maintain species limits where gene flow has resumed following secondary contact (Kondrashov & Shpak 1998; Kirkpatrick & Ravigné 2002). Identifying which factors are at play in this system will be the subject of future studies. Regardless of which factors are contributing to the maintenance of species limits in this system, the most salient contribution coming out of this study is that *P. shermani* and *P. teyahalee* are not in the process of merging. This is remarkable considering previous authors have suggested the species may be a hybrid swarm and/or are in the process of merging (Hairston et al. 1992,

Highton & Peabody 2000).

Though I treated all of the transects as belonging to a single hybrid zone, as they are divided among four separate *P. shermani* isolates, some points of contact may individually conform to a model of neutral diffusion while others do not. Therefore isolate-specific analyses should be performed. Effectively, this would entail performing the analyses described above independently for each of the four isolates, including phylogeny and parameter estimation, using only data for the isolate of interest. Nevertheless, as pooling the samples leads to a more conservative lower bound estimate of the timing of secondary contact, the conclusion that the species are not in the process of merging is robust. Better estimates of lifetime dispersal ability and generation time would also improve the accuracy of this method. Finally, I feel performing this analysis under a range of parameter values would be useful in determining the sensitivity of the methodology to each parameter.

CONCLUSIONS

With the exception of molecular clines across two transects, the character clines investigated across zones of contact and hybridization between *Plethodon shermani* and *Plethodon teyahalee* are narrower than would be expected under a model of neutral diffusion. Therefore I conclude there are factors at play functioning to prevent the merger of these two lineages. In doing so, I demonstrate the efficacy of a method incorporating independent, diverse characters and coalescent theory to predict the extent of a hybrid zone under a model of neutral diffusion with which an observed hybrid zone extent can be compared. The *P. shermani-P. teyahalee* system serves as a valuable case study, as these species exhibit exceptionally poor dispersal ability, and therefore represent a scenario under which such evaluation is most problematic. Determining the relative stability of a hybrid zone is the first step in characterizing a hybrid zone; once unimpeded merger is ruled out, additional avenues of inquiry are opened up. These include investigations into the mechanisms maintaining stability, differential gene flow contributions from the parental forms, and the relative fitness of the parental and various hybrid classes.

17
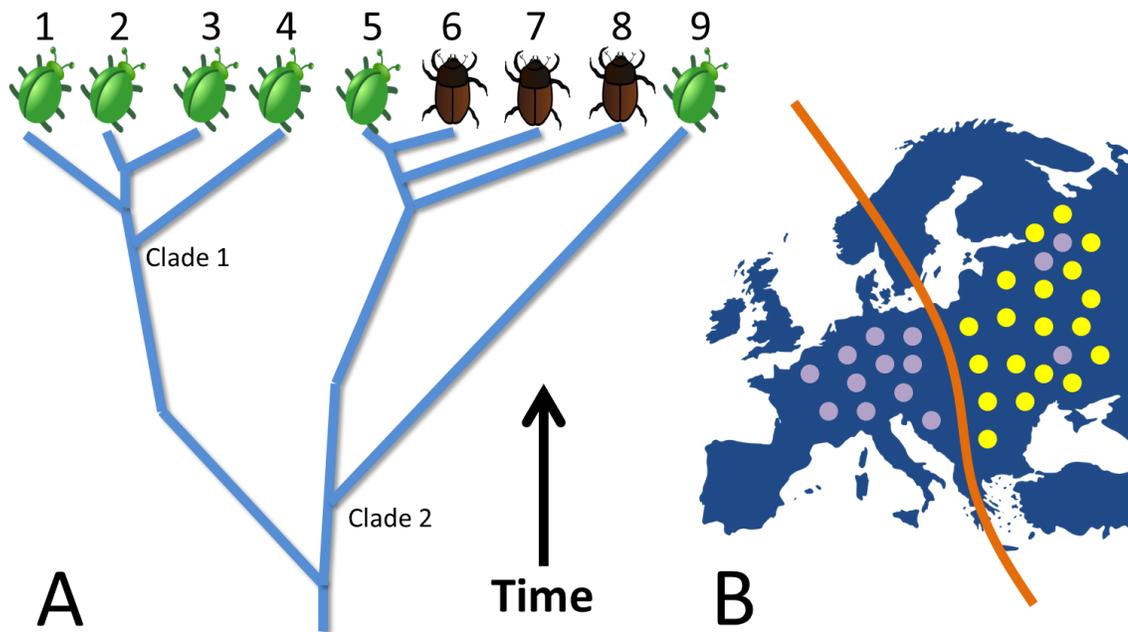
Figure 1.1. Illustrations depicting hypothetical situations where either gene flow subsequent to secondary contact or ILS can be suspected visually. (**A**), a gene tree, wherein the "bug 5" possesses a haplotype belonging to Clade 2 and nearly identical to that of "bug 6" while being morphologically identical to the "bugs" of Clade 1 (despite belonging to a very divergent haplotype clade). Having the morphology of one species but the gene of another is a gene tree incongruency (GTI). In this scenario, gene flow subsequent to secondary contact is the most likely explanation for the GTI. Alternatively, "bug 9" is very divergent from samples heterospecifics with which it forms Clade 2; this is situation is compatible with incomplete lineage sorting (ILS). (**B**), two parapatric and morphologically diagnosed species ("east" and "west") are separated by the orange line. The colored dots (purple and yellow) indicate genetic samples attributable to two different haplogroups. These two haplogroups largely correspond to the two species, however, three purple haplotypes (generally attributable to the "west" species) show up within the distribution of the "east" species. As these incongruent haplotypes are randomly distributed and not clustered at the contact zone, the most likely explanation for this pattern is ILS.
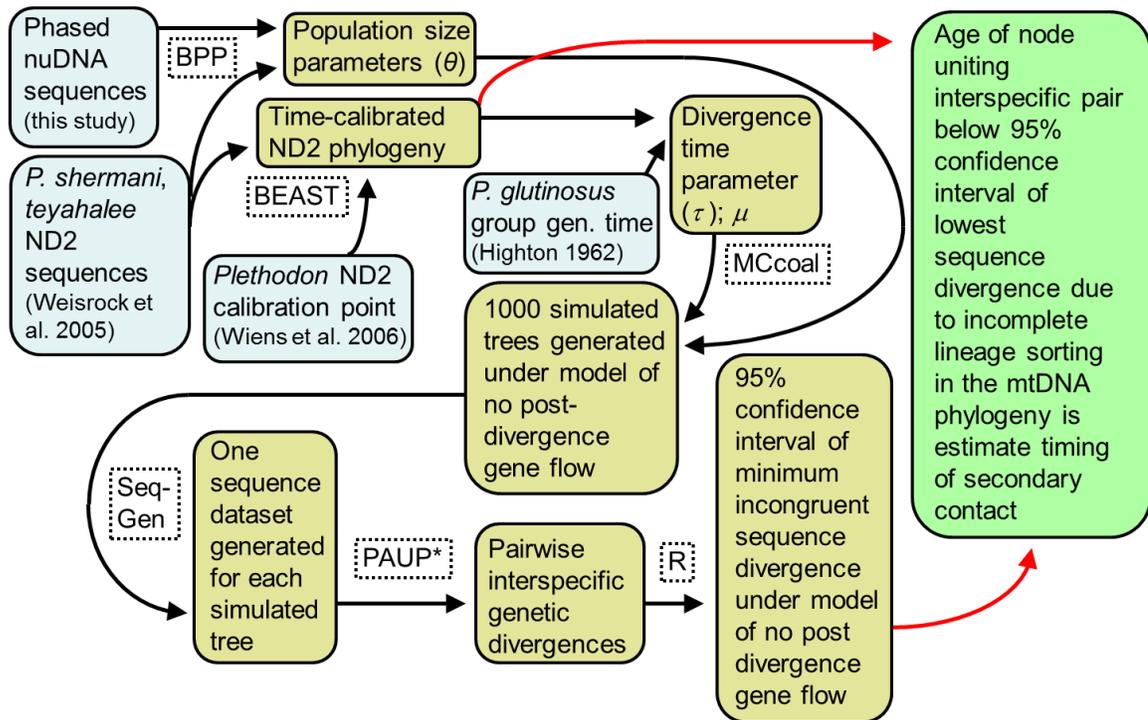
Figure 1.2. Flow diagram depicting method used in determining timing secondary contact and resumption of gene flow between *Plethodon shermani* and *Plethodon teyahalee*. Diagram begins at the upper left. Items boxed by dotted line are software programs. Items in pale blue, tan, and green shapes are required data, intermediate steps, and end of flow diagram, respectively. Red lines indicate information used to identify lower-bound estimate of timing of secondary contact.

Figure 1.3. Photographs of salamander species included in this study. A: Standard *Plethodon shermani*, Macon County, NC. B: Standard *Plethodon teyahalee*, Blount County, TN. C: *P. teyahalee* with red pigment on front left leg, Graham County, NC. D: *P. shermani* lacking red legs, Graham County, NC. All photos taken by Benjamin Lowe.
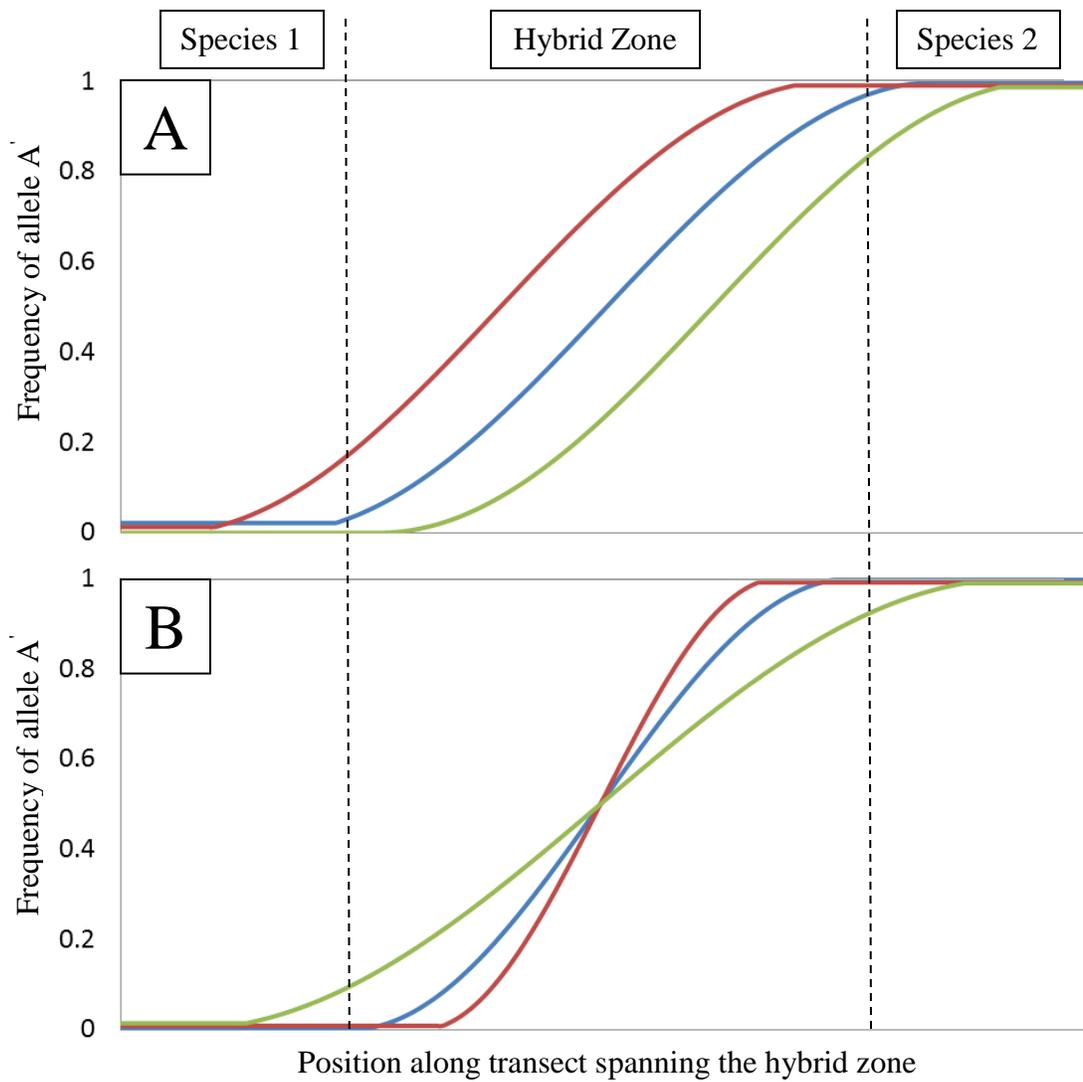
Figure 1.4. Schematic illustrating types of character cline accordance. (**A**), concordance, where cline extents are equal, regardless of position of centers. (**B**), coincidence, where clines share a common center, regardless of cline extent.

Marker	Locus category	Coding/non-coding	Transects*	Primer Source
ND2†#	Mitochondrial	Coding	NA	NA
C3#	Anonymous	Non-coding	FC, SM, SI, TR, WB	Novel locus
Pglut9	Anonymous	Non-coding	FC, SI, WB	Novel locus
Pglut10	Anonymous	Non-coding	SI, TR	Novel locus
Pglut19	Anonymous	Non-coding	FC, SI, WB	Novel locus
CXCR4#	Non-anonymous	Coding	SM, SI, TR	Novel locus‡
MC00#	Non-anonymous	Non-coding (intron)	FC, SM	Chatfield et al. 2010
POMC#	Non-anonymous	Coding	SM, SI, WB	Vieites et al. 2007
SLC8A3	Non-anonymous	Coding	SM, SI, TR	Rovito 2010

Table 1.1. Markers used in this study. Anonymous and non-anonymous markers from nuclear genome. All nuclear markers used in estimating hybrid zone extents.

*See Table 1.2 for explanation of transect abbreviations.

†Sequenced by Weisrock et al. 2005; retrieved from GenBank.

#Also employed in θ estimation.

‡Internal primers designed from locus of Roelants et al. 2007.

Transect	Accordance	Data	Model	Elevational extent
Fires Creek (FC)	Neither coincident nor concordant	Morph	C & S	85
		Mol	C & S	385
Snowbird Mountain (SM)	Coincident & concordant	Morph	C & S	60
		Mol	C & S	∞
		Comb	C & S	495
Standing Indian (SI)	Coincident	Morph	C & S	275
		Mol	Right tail	∞
Tellico Road (TR)	Coincident	Morph	C & S	120
		Mol	C & S	1170
Wayah Bald (WB)	Coincident & concordant	Morph	C & S	155
		Mol	Right tail	485
		Comb	Right tail	260

Table 1.2. Empirical cline extent estimates. Morph = morphological data set. Mol = molecular data set. Comb = combined morphological and molecular data set. "C & S" = only two parameters estimated (center and slope). "Right tail" = two parameters in addition to center and slope estimated (resulting in an independently modeled right tail). Elevational extent in meters. "∞" indicates given sampling regime, elevational extent effectively extends indefinitely.

Parameter	Estimate
Plethodon shermani θ	5.425×10^{-3}
Plethodon teyahalee θ	3.137×10^{-3}
Ancestral *P. shermani-P. teyahalee* θ	3.116×10^{-3}
Mean time to reproduction for *P. shermani*, *P. teyahalee* (T_G)	5
Mutation rate (μ)	1.074×10^{-8}
Timing of *P. shermani-P. teyahalee* divergence (T_D)	2.9391×10^{6}
Timing of *P. shermani-P. teyahalee* divergence in mutational units (τ)	$1.26263736 \times 10^{-2}$
Timing of *P. shermani-P. teyahalee* secondary contact (T_H)	5.652×10^{5}
Nucleotide frequencies (a,c,g,t)	0.338,0.241,0.072,0.299
Substitution frequencies (a-c,a-g,a-t,c-g,c-t,g-t)	0.138,2.648,0.06487,0.124,1,0.128
Gamma categories	4
Gamma shape parameter (α)	0.33

Table 1.3. Parameter estimates. All parameters refer specifically to Eastern *Plethodon* ND2 dataset unless otherwise stated. All *T* are in years.

CHAPTER 2

ASSESSING THE DEGREE OF CONCORDANCE AND COINCIDENCE OF HYBRID ZONES WITH EACH OTHER AND WITH ENVIRONMENTAL ECOTONES

ABSTRACT

Hybrid zones between species with incomplete reproductive isolation that have come into secondary contact are a common phenomenon, but evolutionary forces that prevent the merger of the species are rarely known. Likely candidates include selection from the environment (exogenous selection), genomic incompatibility (endogenous selection) and mating preference (positive assortative mating). Hybrid zones between two plethodontid salamander species, *Plethodon teyahalee* and *Plethodon shermani*, have long been of interest to salamander and speciation biologists alike. The hybrid zones between these species are "stable" (the species are not in the process of freely merging); however, the factors that maintains them remains unknown. The distribution of the montane *P. shermani* is comprised of four allopatric populations, each of which contacts and forms a hybrid zone with the lower-elevation *P. teyahalee*, making this system a repeated natural experiment that may offer unique insights into how these stable hybrid zones are perpetuated. To explore this phenomenon, I collected color pattern and DNA sequence data from salamanders, as well as temperature data, across five transects spanning hybrid zones formed between these two species. I used these data to evaluate expectations of exo- and endogenous selection. These analyses reveal interesting variation in character cline center and breadth among the five transects, with multiple independent lines of inquiry implicating exogenous selection as a force maintaining stability in of these hybrid zones.

INTRODUCTION

Hybrid zones, areas where genetically divergent populations meet and interbreed to produce individuals (and sometimes populations) of mixed ancestry, have long been of interest to evolutionary biologists. Hybrid zones that show signs of stability (i.e., not

progressing toward a single, homogeneous population) are of particular interest, as they may shed light on speciation in the face of gene flow. Stable hybrid zones can be maintained through selection. The most frequently invoked selection mode is ecological, where the parental species are adapted to different ecological conditions and hybrid zones occur in areas of ecological transition ("ecotones") (Slatkin 1973, Endler 1977, Moore & Price 1993 and references therein). The selection maintaining these hybrid zones is driven by the environment, and it is termed 'exogenous selection' (Moore 1977). The widths of hybrid zones maintained by exogenous selection are independent of dispersal ability, and are dictated by the width of the ecotone bridging the parental species' respective habitats (Barton & Hewitt 1985). Given that ecotones are frequently non-linear and patchy in distribution, an expectation of this model is a mosaic of parental and hybrid genotypes, where the hybrid zone parallels the patchwork of contacting habitats (Rand & Harrison 1989; Fig. 2.1). Additionally, under the exogenous selection model, animals of hybrid ancestry may have a selective advantage within the hybrid zone if neither parent is as adapted to intermediate environmental conditions as individuals of mixed ancestry. This phenomenon has been termed "bounded hybrid superiority," given that elevated fitness is geographically limited and animals of hybrid ancestry have lower fitness in the parental habitats (May et al. 1975).

Another form of selection implicated in maintaining stable hybrid zones is selection against hybrids resulting from the breakdown of coadapted gene complexes (i.e., advantageous epistatic relationships; Mayr 1963, Lynch 1991). Under this form of selection, gene combinations that coevolved while the parental species were allopatric, breakup due to hybridization upon secondary contact. In contrast to hybrid zones maintained by exogeneous selection, this form of selection against hybrids is independent of environmental conditions; hybrids are less fit than parentals wherever they occur (Barton & Hewitt 1985, Gava & de Freitas 2002). Instead, the width of hybrid zones maintained by this type of selection is strictly a function of the organism's dispersal ability and the strength of selection against hybrids. Specifically, greater dispersal ability leads to a wider hybrid zone, while greater selection against hybrids reduces hybrid zone width. As such, hybrid zones maintained by this type of selection have been termed

"tension zones" (Key 1968), and since the source of the selection is within the organism, it has been termed 'endogenous selection' (Moore 1977). Tension zones are freer to move than exogenously maintained hybrid zones (Barton 1979), and are particularly prone to movement when parental populations experience demographic changes (Smith et al. 2013). There is also a tendency for tension zones to form in density troughs (regions of low population density; Barton 1979, Moore & Price 1979). Given that density troughs often coincide with ecotones, the two types of hybrid zones can be superficially similar in structure (Endler 1977, Barton & Hewitt 1985, Barton & Hewitt 1989).

Distinguishing between these two types of selection has been problematic in natural systems. Nevertheless, each hypothesis has predictions that can be tested empirically. Character clines across hybrid zones maintained by exogenous selection should vary in width, with characters under strong selection (e.g., phenotypic characters, protein-coding genes) exhibiting narrower clines and those under no selection (e.g., neutral molecular markers) having wider clines. Within exogenously maintained hybrid zones, different characters are not expected to share the same center, as selection unique to a character will impose a specific center (Hewitt 1988). Alternatively, for hybrid zones maintained by endogenous selection, the width should partially reflect the dispersal ability of the organism, with low-vagility organisms exhibiting narrow hybrid zones, even if selection against hybrids is weak. In systems where hybrid zones are exogenously maintained, independent instances of secondary contact and hybridization should result in clines for a particular character that are centered on a common ecotone (be 'coincident'; Fig. 2.2), while the different cline widths for that character should be positively correlated with its respective ecotone width (e.g., where the ecotone is wider, the cline should be wider). By contrast, the widths of hybrid zones maintained by endogenous selection should not vary with ecotone width, and should exhibit similar widths (be 'concordant'; Fig. 2.2) unless the populations have diverged from one another with respect to their co-adapted gene complexes through genetic drift (in which case the widths would vary randomly). Furthermore, independent hybrid zones maintained by endogenous selection should only coincide with a common ecotone center if it is also a density trough.

Here, I investigate hybrid zones formed between two species of *Plethodon* salamander occurring in the Unicoi and Nantahala mountains of southwestern North Carolina. One species, *Plethodon teyahalee*, occupies the lower elevations and populations across the study area are presumably connected through gene flow. A second species, *Plethodon shermani*, forms four disjunct, mountain-top populations (isolates) which are presumably genetically isolated from each other. Though closely-related (both species belong to the *Plethodon glutinosus* group; Highton & Larson 1979), these two species are not sister taxa. Nevertheless, they hybridize, backcross, and form hybrid zones at intermediate elevations where they come into contact. Highton & Peabody (2000) collected allozyme data across hybrid zones formed between these two species, and reanalysis of these data by Weisrock et al. (2005) revealed a clinal pattern of variation across the hybrid zones comparable to those observed for color pattern characters.

In this study, I use molecular, morphological, and environmental data (elevation and temperature) to characterize several hybrid zones between *P. shermani* and *P. teyahalee*, and to test the relative importance of endo- and exogenous selection in maintaining the hybrid zones between these species. Within hybrid zones, I evaluated the coincidence and concordance of morphological and molecular clines. As there is an ecotone across the hybrid zones (they occur across an elevational gradient accompanied by biotic and abiotic variation), I predict that exogenous selection is at play, and therefore expect character clines within transects to differ in width (hereafter "extent," as the pertinent ecotone is elevation-dependent), depending on whether the character is under selection or not. I evaluated the accordance (coincidence and concordance; Fig. 2.2) of the character clines between hybrid zones with respect to elevation and temperature. I hypothesize that the various hybrid zones would not be coincident with regard to elevation (coincidence here could be construed as evidence in support of endogenous selection), but would be coincident with regard to temperature, implying that the various hybrid zones are centered on a common ecotone. Finally, because an understanding of thermal variation across the transects may help explain hybrid zone structuring, I evaluated the thermal conditions across the hybrid zones from the perspective of *Plethodon* suitability by calculating "activity-hours" for all locations for which I had temperature data logger data.

ABIOTIC, MORPHOLOGICAL & MOLECULAR DATA COLLECTION & PROCESSING

Field Sites: I investigated five transects spanning hybrid zones between *Plethodon teyahalee* and *Plethodon shermani* in the Nantahala (4) and Unicoi (1) mountains. These transects are divided among the four *P. shermani* isolates (Highton & Peabody 2000): Unicoi (1: SM), Standing Indian (1: SI), Tusquitee (1: FC), and Wayah (2: TR, WB) (Fig. 2.3; Table 2.2). I first selected a site at the bottom of the mountain where previous work and/or personal observation identified the *Plethodon glutinosus* group salamander as *P. teyahalee* (the first parental site). I proceeded up the mountains using available roads, identifying four additional sites at regular elevational intervals within the hybrid zone, and a sixth site within the distribution of *P. shermani* (also based on previous work and/or personal observation; the second parental site). These six sites along each transect will hereafter be referred to as the primary sites. Given that the transects were influenced by the vagaries of suitable habitat distribution and mountain roads, they were not linear or evenly spaced, in either elevational or Euclidean distance.

Abiotic Data: Elevation was calculated for each primary site using a Garmin eTrex Vista HCx GPS unit. Fine-scale temperature data along each transect was generated using iButton Thermochron temperature data loggers accurate to 0.5°C (Maxim Integrated Products, Sunnyvale, CA; model: DS1921G). Two data loggers each were placed at the six primary sites along each transect. Data loggers were secured within PVC caps secured to the north side of tree trunks (to minimize direct solar influence) approximately 0.6 m above the ground. Temperature readings were collected every two hours across two active seasons. Data were inspected for anomalies, particularly mid-day spikes indicating direct solar influence. Average daily maximum and minimum temperatures were calculated from data collected between June 14th through October 27th, 2009 and May 14th through October 6th, 2010. Though these spans are within the active season of these salamanders, I acknowledge that the uneven coverage across the two years precludes insights based on the absolute values. However, as the coverage is complete

across all primary sites, it is possible to make important insights by comparing these estimates.

While I expect a general trend of decreasing average temperature measurements with increasing elevation, due to the myriad other variables that contribute to temperature, I also expect instances of higher elevation sites with warmer temperatures than one or more lower elevation sites along the same transect (i.e., deviations from monotony). To quantify this, I estimated Spearman's rho (i.e., Spearman's rank order correlation coefficient) for both high and low temperature estimates across all transects. A Spearman's rho of -1 indicates monotony, and with increasing non-monotony values approach (or exceed) 0.

Using the entire temperature data set (only pruned to achieve the largest complete-coverage data set), I calculated activity-hours across this time period. This entailed counting the readings taken between the hours of 7:30 PM and 5:30 AM (reflecting the nocturnal habits of these salamanders) for which the temperature was within the span of 12.5° and 20.5° C. These temperatures correspond to minimal and maximal activity temperatures observed for *Plethodon glutinosus* group salamanders generally (Feder et al. 1982). Again, though the time frame is arbitrary (precluding insights based on the absolute estimates), I argue that these estimates do reveal relative temperature suitability across these sites for *P. glutinosus* group salamanders generally.

Morphological Data: I collected morphological data for salamanders encountered at six to seven sites along each transect, using the morphological scoring system of Hairston et al. (1992). This entailed scoring the extent of red on the legs and white spotting on the torso. As these species differ from other sympatric plethodontids in being distinctly unpalatable (Hensel and Brodie 1976, Brodie et al. 1979), predators may exert selection on these conspicuous traits. I assumed the *P. shermani* condition to be maximally red legs and unspotted torso, and the *P. teyahalee* condition to be a maximally spotted torso with no red on the legs. In practice parental populations never achieve morphologically "pure" status; whether this is due to gene flow, ancestral polymorphism, convergence, or an erroneous assumption remains uncertain. But, these characters do show a clinal

pattern across the hybrid zones and have historically been used in studies of this system (e.g., Hairston et al. 1992 and citations therein). I summed the two scores (red on leg, spots on torso) to generate a hybrid index score. All within-hybrid-zone data were taken from live animals in the field, while measurements from sites representing putatively parental populations were taken from freshly preserved specimens.

Molecular Data: I collected salamander tissue samples from six or seven sites across each transect. For sites within the hybrid zones, these generally consisted of tail tips, while tissue samples from sites with putatively parental populations consisted of liver samples. DNA was extracted using Viogene® Blood & Tissue Genomic DNA Extraction kits and PCR amplified using standard protocols. I sequenced eight nuclear DNA markers (Table 2.3), four unpublished anonymous markers developed by colleagues (Kozak, KH, unpublished data) and four previously published non-anonymous markers (3 protein coding, 1 intron). The resulting sequences were investigated by eye to identify single nucleotide polymorphisms (SNPs), and subsequently all individuals were scored for their diploid genotype at this position.

CLINE FITTING & HYPOTHESIS TESTING

I used the program CFIT-7 (Gay et al. 2008, Lenormand & Gay 2008) to describe and compare patterns of hybrid zone structure along the various transects. This analysis software fits sigmoid clines to data under a likelihood framework, allowing for (1) the estimation of individual character clines within a single hybrid zone and assessment of their coincidence and concordance, (2) the estimation of hybrid zone cline parameters given its constituent character clines, and (3) and the degree to which different hybrid zones are associated with a common independent variable (e.g., distance, temperature). Furthermore, clines can be fit using a minimal model or models of varying degrees of parameter richness. In particular, with the addition of two parameters (starting point and slope), an exponential "tail" can be fit to the data, modifying a distal extent of the cline. As an extension, these tails can be mirrored on both sides of the cline (also only two additional parameters) or each end can be fitted separately (four additional parameters).

These analyses allow me to evaluate several expectations of endogenous and exogenous selection. Across individual hybrid zones, clines for phenotypic characters may be narrower than those for molecular characters if exogenous selection is at play (Arnold 1992); with endogenous, they should have similar extents or vary randomly. Where hybrid zones are maintained by endogenous selection, the clines should be narrow with respect to estimates of dispersal ability from the literature; alternatively, they should be wider and be correlated with ecotone extent where exogenous selection is operating (Barton & Hewitt 1985). Further, they may be associated with an area of low population density (Hewitt 1988), and should not coincide with an area with elevated population density. Finally, exogenous selection should result in the various hybrid zones being centered on a common climatic zone within the ecotone (i.e., be coincident; no such expectation if endogenous selection is at play) (Endler 1977). The predictions for each hypothesis are listed in Table 2.1.

Model Fitting: I first used CFIT-7 to determine the best cline model for each data set. I chose to fit a unimodal cline to these data as my collecting localities are centered on the previously inferred zone of admixture (Highton & Peabody 2000), which is many times wider than the dispersal ability of these salamanders and therefore must be maintained by extensive late-generation hybridization and back crossing (Jiggins & Mallet 2000, Schilthuizen 2000, Gay et al. 2008). In model-fitting the morphological data, cline center and slope were invariably fit. The five following models were evaluated: no additional parameters, three models with two additional parameters (right tail, left tail, symmetrical right and left tails), and four additional parameters (right and left tails fit independently). The addition of tails to the model allow the cline to deviate from a simple sigmoid curve (e.g, combine a steeper slope at the center of the cline with a shallower slope at the ends). CFIT-7 produces log likelihood (lnL) scores, which can be used to evaluate the fit of models. As some of the models being compared are not nested, I used the lnL scores to compute corrected Akaike information criterion (AIC_c) scores (Pettengill & Moeller 2012). As models with marginally worse fit could result in dramatically different cline shape, all models with a $\Delta AIC_c < 2$ with respect to the best model were retained and

incorporated into cline charts to demonstrate this difference. This model-fitting technique was also used to determine the best model for the molecular clines.

For these analyses I used elevation as the independent variable, as different transects were not being compared. These and all other CFIT-7 analyses were runs with different random seeds, the number of chains and replicates were increased dramatically relative to the default values, and all analyses were run multiple times with different priors to assess convergence. In addition to lnL scores, these analyses also yielded parameter values for each model, which were used to create morphological and molecular character clines for each transect in MATHEMATICA (Wolfram Research, Inc. 2008).

Assessing Morphological and Molecular Accordance within Transects: For the purposes of assessing accordance of character clines within hybrid zones and justifying the comparison of different hybrid zones using composite clines (clines generated from the combined morphological and molecular data), I performed cline-fitting analyses for each transect to assess whether I could reject cline accordance of the morphological and molecular data sets. I evaluated the following three models: morphological and molecular data share a common center and slope (different slopes is a prediction of the exogenous selection hypothesis), share only a center (i.e., are coincident, permitting comparison of composite clines among hybrid zones), share neither center nor slope (precluding the comparison of composite character clines). For these analyses I used elevation as the independent variable, as different transects were not being compared. This model testing exercise involved comparing likelihood ratio test (LRT) scores between analyses where parameters are constrained and those where parameters are allowed to vary. The model fitting analyses described above sometimes supported different levels of parameterization for morphological and molecular data sets—specifically, differences regarding the tail parameters. The choice of tail parameters affects the slope parameter values. Therefore, the same parameters need to be employed for data sets being compared. As overparameterization should be less of a problem than underparameterization, for the purpose of comparing the three models listed above, for each transect, the more complex of the two models determined in the model fitting

analyses was implemented for both data sets. I performed LRT to identify the best fit among the three nested models (Felsenstein 1981, 1988; Irwin et al. 2009). These analyses were performed to determine whether I could reject concordance between molecular and morphological data sets, as a prediction of exogenous selections is narrower clines for characters under selection (i.e., morphological characters). Coincidence between putatively neutral characters (i.e., nuclear SNPs) and characters under selection, is not a prediction of exogenous selection, as this type of selection may place the cline center for a character under selection at a position unique to that character (Hewitt 1988).

I also conducted similar analyses with the purpose of determining the accordance of the two molecular marker types (anonymous and non-anonymous). For these analyses I generated and evaluated cline data using the methods and models stated above. I applied the model that was found to best fit the molecular data to both data sets. These analyses were performed to evaluate the validity of lumping of molecular data as I did in the morphology/molecular analyses above.

Assessing Fit of Character Clines to a Common Independent Variable: I conducted analyses with the methodology of those above to test how well the different transects accord to a common cline. The most parameter-rich model allowed each of the five transects to have independent centers and slopes, and the two constraint analyses forced all the transects to share either a common center or both a common center and slope. These analyses were performed with elevation as the independent variable. For these analyses, I also calculated Akaike information criterion (AIC) values for evaluation of model fit.

It is possible that hybrid zones between these species are maintained by exogenous selection, but that signal is obscured by genetic divergence, as most of the *Plethodon shermani* populations are genetically isolated. However, the *P. shermani* atop the WB and TR transects belong to the same isolate (Wayah; Highton & Peabody 2000) and therefore are theoretically connected through gene flow. Therefore, I also performed analyses as above incorporating solely these two transects to see if I can reject

accordance in a comparison not complicated by allopatry. Within-transect accordance analyses showed that both the morphological and molecular data sets were coincident for each transect, though the clines of TR had different extents, so I incorporated both morphological and molecular data in these analyses and only compared the coincidence model with the unconstrained model. This afforded the strongest power for discerning coincidence, though it precluded the opportunity to rigorously compare hybrid zone extents. Additionally, one transect (FC) was found to be anomalous with respect to the other transects in that the morphological and molecular clines were not coincident. Therefore I also performed analyses assessing the coincidence of the other four transects.

While I intended to perform iterations of these analyses using the temperature data as the independent variable, temperature estimates for the primary sites did not consistently decrease with increasing elevation, and higher elevation sites were frequently warmer than sites down slope. I considered reordering the sites by temperature, effectively evaluating a "mosaic hybrid zone" model, however, the degree to which some of the transects would be rearranged in accomplishing this deterred me from attempting this. Therefore I took a qualitative approach to evaluating the relationship between temperatures at cline center and temperatures observed across the hybrid zones. For each inferred cline center, I used the center's elevation and the temperature and elevation of the two bracketing primary sites to interpolate the temperature of the cline center (assuming a linear temperature cline between the bracketing primary sites). For both the high and low temperature data sets, I plotted the estimate of cline center temperature on graphs indicating the span of temperatures observed among each transect's six primary sites allowing visualization of the cline center temperatures along each transect and qualitative comparison of cline center temperature among the transects.

Qualitative Comparison of Morphological Cline Expanses and Ecotone Slopes: As one prediction of exogenous selection is an inverse relationship between hybrid zone expanse and ecotone slope, I evaluated this relationship with respect to the morphological clines across the five hybrid zones. I determined the elevational extent of the hybrid zone using estimates generated in the CFIT-7 analyses by scaling the phenotypic scores from 0

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to 1 and determining the elevational extent spanning values 0.2 and 0.8. (Numerous authors have employed this convention as a proxy for hybrid zone extent, e.g., May et al. 1975, Endler 1977, Hafner 1982.) For the ecotone slope I divided the change in mean low temperature across the hybrid zone by the associated elevational extent. As the temperature estimates are tied to primary sites, I determined the temperature difference between the two primary sites bracketing the hybrid zone determined above (one instance [WB] necessitated employing a primary site immediately above the lower boundary of the hybrid zone). This value was divided by the elevational extent between the primary sites (not the elevational extent of the hybrid zone). The estimated hybrid zone extents were plotted against ecotone slopes. The small number of observations (5) precluded an accurate evaluation of significance via linear regression analysis, however, visual inspection of the pattern of these two variables does permit discernment of whether the data generally fit, or grossly violate, the expectations of exogenous selection. These estimates of hybrid zone extent were also used to approximate ground-length distance using GOOGLE EARTH (http://earth.google.com/).

RESULTS

I amassed bi-hourly temperature readings from June 14th 2009 through October 9th 2010 (with a five-day gap in mid-October, 2009). Data clearly influenced by direct sunlight were omitted. Despite missing data due to sunlight influence and data logger failure/loss every sampling period was captured by at least one data logger. The temperature data revealed complex temperature variation along transects, with all transects non-monotonic for both high and low temperatures (i.e., possessing at least one primary site warmer than another primary site of lower elevation; Fig. 2.4). Nevertheless, the lowest primary site was consistently warmer than the highest primary site, and most Spearman's rho estimates were between -0.75 and -1 (Table 2.2), indicating a general trend of higher elevations exhibiting lower temperatures. The activity-hour calculations (which utilized the entirety of the temperature data) revealed a mid-elevation salamander activity hump within each transect (Fig. 2.5). Qualitatively, the centers of these activity-hour humps appear to coincide elevationally (~1030m) with

the exception of the one north-aspect transect (SM) where the activity-hour hump appears considerably lower in elevation.

I scored 481 specimens for the phenotypic characters. The genetic markers did not consistently PCR amplify or exhibit sufficient variation along every transect. However I managed to accrue sufficient sequence data for four to seven markers for each transect (Table 2.3). The preliminary model-fitting analyses revealed the minimum-parameter model sufficiently characterized all of the morphological data sets and three of the five molecular data sets, indicating the frequencies of the characters across the hybrid zones can be modeled with a simple sigmoid curve. For the two remaining molecular data sets, the best-fitting model included two parameters describing a right tail (Table 2.4); this reveals that for those two transects (SI & WB), as the distribution of *Plethodon shermani* is approached, the frequencies of alleles associated with *Plethodon teyahalee* diminish slower than would be predicted by a simple sigmoidal model. All of these clines are illustrated in Fig. 2.6 (data sets for which additional models were estimated to have $\Delta AIC_c < 2$ are presented as composite clines describing all equivocal models).

The analyses evaluating character cline accordance within transects identified the best model as morphological and molecular clines share the same center in four of the five transects (Table 2.4). For these four transects, this finding indicates that neither drift nor selection have disassociated the centers of the two character clines. Among these four transects, two (SI, TR) were found to have different extents for their respective character clines, with the molecular cline being much wider than the morphological cline (Fig. 2.6; Table 2.4). As the morphological characters are exposed to exogenous selection while the molecular markers are not, narrower clines for morphological clines relative to molecular clines is evidence for exogenous selection. In the analyses evaluating the accordance of anonymous and non-anonymous marker clines, the best model for all five transects had the two character-type clines sharing both the same center and extent; this indicates the two categories of molecular markers are not experiencing divergent selection regimes.

In most cases, analyses of accordance of the five hybrid zones identified the model allowing each transect to have a transect-specific center and slope to be the best model

(Table 2.5). In the analyses incorporating all five transects the unconstrained model was found to be the best fit regardless of data set or method of model-fit evaluation. In the analyses restricting the number of included transects to two or four (in each case, omitting the FC transect), the unconstrained model was also found to be the best fit save for the analyses of the morphology-only data sets; in these analyses, the coincident models were found to be the best fit either by LRT and AIC, or LRT alone (Table 2.5). In summary, these analyses find the different hybrid zones have different elevational centers and extents (analysis of the morphology-only dataset with one outlier hybrid zone omitted being the sole exception). However, morphological clines along different transects sharing the same center is evidence for exogenous selection.

The interpolated temperatures for the estimated cline centers indicate a broad range of cline center temperatures (both high and low) and do not appear to be coincident with a common temperature (Fig. 2.7). Visual inspection of the placement of the cline centers with respect to the activity hour estimates shows that the cline centers are below or at the lower-elevation limit of the mid-elevation humps (Fig. 2.5).

Inspection of the relationship between hybrid zone extents (Table 2.2) and ecotone slope reveals an apparently inverse relationship (Fig. 2.8). However, a linear regression analysis found the relationship to be nonsignificant ($P = 0.09$).

DISCUSSION

Hybrid zones are a common phenomenon in nature. Theory suggests that both exogenous and endogenous selection can maintain species limits for hybridizing taxa. Exogenous selection is occurs when the two species occupy different environmental conditions but come together and hybridize at an ecotone; animals of mixed ancestry may be successful at the ecotone, but traits that originate in one species are maladaptive in the range of the other. Endogenous selection results from the breakdown of advantageous suites of genes that work well together, but confer a selective disadvantage when combined with alleles from heterospecific individuals (the breakdown of coadapted gene complexes). Identifying whether stable hybrid zones are maintained by endogenous or exogenous selection is difficult, as the two forces can leave similar patterns. Situations

where species form multiple, independent hybrid zones with each other offer unique opportunities to identify which force is operating.

These findings provide the first robust assessment of the nature of hybrid zones formed between the salamanders *Plethodon shermani* and *Plethodon teyahalee*. I attribute the persistence of the hybrid zones to divergent exogenous selection operating at either end of the hybrid zones, while an intermediate selective regime supports populations of salamanders of mixed ancestry at the center of the ecotones. I find that where clines for molecular and morphological data are not concordant within a hybrid zone, the molecular data cline is of greater extent; I suspect this pattern is more common among hybrid zones than currently appreciated. Conversely, I find that within hybrid zones character clines are generally coincident, a prediction of secondary contact. I also find the various hybrid zones to not be coincident with a common elevation; this is neither surprising nor evidence against exogenous selection, given the diversity of aspects and habitat features observed among the hybrid zones. Finally, I recover mid-elevational humps in activity opportunity, the presence of which supports the hypothesis of divergent selective regimes across the hybrid zones and provide further evidence against endogenous selection being a significant factor in this system.

THE CASE FOR EXOGENOUS SELECTION

Other work indicates the hybrid zones formed between *P. teyahalee* and *P. shermani* are stable, and assortative mating is not sufficient to be the sole factor maintaining species limits in this system (Chapters 1 and 3, respectively), and therefore endogenous and/or exogenous selection are likely at play. The hybrid zones formed between *Plethodon shermani* and *Plethodon teyahalee* in the Nantahala and Unicoi mountains largely fit the expectations of hybrid zones maintained by exogenous selection. Specifically, five lines of evidence revealed by my studies implicate exogenous selection. First, in all three instances where the morphological and molecular clines were of different extents the molecular clines were greater. Under a scenario of endogenous selection all characters are expected to exhibit similar extents, as selection is operating on the genome as a whole; only in an exogenously maintained hybrid zone are characters

susceptible to exogenous selection limited in extent, while those that are neutral or that have a selective advantage can spread freely across the hybrid zone (Barton & Hewitt 1985, Stankowski 2013).

Second, character clines were very broad with respect to dispersal distance estimates in the literature. Despite being sufficiently narrow to rule out neutral diffusion (Chapter 1), these clines are also much broader than expected for endogenous selection. Under endogenous selection, hybrid zone extent is a balance of dispersal and selection, with greater dispersal ability resulting in wider hybrid zones. Even with weak selection against hybrids, endogenous selection predicts very narrow hybrid zones for organisms with weak dispersal abilities (Kruuk et al. 1999). Mark-recapture studies investigating *Plethodon* movement have found salamanders rarely move more than twenty meters in a single bout (Liebgold et al. 2011, Connette 2014), and one study found the average total distance moved over the course of a year was about 28 m (Wells & Wells 1976). My ground-length estimates of the morphological cline extents are between 0.79 km (TR) and 6.14 km (WB), seemingly much greater in extent than published dispersal events scaled up to lifetime dispersal estimates.

Third, the centers of the morphological clines consistently fall on the lower margin of identified areas of maximum foraging opportunity (mid-elevation activity-hour humps). With endogenous selection, hybrid zone centers are expected to find and remain on density troughs, which often occur in habitats of marginal quality for both parental species. (Hewitt 1975). While below the identified areas of maximum foraging opportunity, the cline centers were not associated with areas of minimal foraging opportunity, which were detected toward either or both ends of the transects. While abiotic factors also contribute to habitat suitability for *Plethodon* (e.g., the abundance and diversity of prey and predators), I feel that the increased opportunity for surface activity along with the general abundance of these salamanders at mid-elevations (pers. obs.) is sufficient to reject the hypothesis that these mid-elevations are a significant density trough (i.e., sink habitat) for these salamanders. This is supported by Gifford and Kozak (2012), who find that for *P. glutinosus*-group salamanders, population density and foraging opportunity are correlated.

Fourth, these five transects show a trend toward an inverse relationship between ecotone slope and morphological cline extent. If this apparent pattern is real, in addition to implicating exogenous selection (Gifford 2008, Stankowski 2013), it fits the expectations of the bounded-superiority hypothesis, where the distribution of intermediate populations follows the distribution of the ecotone (Moore 1977, Moore & Buchanan 1985).

Fifth, when only morphological data are considered and one outlier transect (FC) is omitted, the more conservative criterion of model selection (LRT) finds the best model has the centers constrained to the same elevational position (the coincident model). Assuming an ecotone/elevation association this suggests that the morphological characters investigated here are to some degree constrained to a common ecotone. The failure to find coincidence when molecular data are included in the analysis suggests the nuclear markers are not associated with ecological factors that are constraining the morphological characters. Contrary to the findings of the between-transect evaluations of accordance, the evaluations of molecular and morphological cline accordance within these four transects supported cline coincidence (indicating some level of spatial association that is likely a residual effect of secondary contact); the failure to find coincidence in molecular clines between these transects (both including and excluding morphological data) I interpret as an emergent property that further illustrates the ecological disassociation and random placement of the molecular cline centers.

I acknowledge that endogenous and exogenous selection are not necessarily mutually exclusive. It has been suggested that many hybrid zones may experience both kinds of selection (Dasmahapatra et al. 2002). Szymura & Barton (1986, 1991) showed that in a hybrid zone between *Bombina* frog species, there existed both environment-independent selection against hybrids, as well as character differences between the hybridizing species that are advantageous in the two species' respective distributions. Furthermore, Hewitt (1988) suggested the distinction between hybrid zones maintained by end- and exogenous selection, "may be illusory," as specific environmental conditions that species experience in allopatry may often drive the formation of coadaptive gene complexes. However, I feel that preponderance of evidence points to the actions of exogenous selection in this

system.

PHENOTYPIC CLINES MORE RESTRICTED THAN MOLECULAR CLINES

Three of the five transects included in this study exhibited different morphological and molecular cline extents, and in all three instances it was the molecular cline that had a greater extent. This supports the hypothesis that selection is acting to maintain narrow clines for phenotypic characters, while non-phenotypic characters are free to spread much further from the hybrid zone center. In a study of a hybrid zone formed between two species of manakin (Pipridae: *Manacus*), Brumfield et al. (2001) found variable cline widths for protein and restriction fragment length polymorphism data, and explained this as variation in purifying selection acting on the various characters. These findings were supported in a later study of the same system that incorporated microsatellite data (Yuri et al. 2009). I chose to collect nuclear SNP data as I felt this character type would best characterize the hybrid zones as they should be free of diversifying selection, and should be variable at this shallow divergence level on account of a lack of purifying selection (including synonymous sites in protein-coding genes). However, it appears alleles of these markers have spread clear across some transects (though divergent allele frequencies on either side of the hybrid zone still persist), and are not representative of the fundamental hybrid zone extents exemplified by allozymic (Weisrock et al. 2005) and phenotypic characters. Fortunately, clinal variation in nuclear markers across the transects still exists, and they still serve to help characterize the centers of the hybrid zones.

CHARACTER CLINES ACROSS TRANSECTS GENERALLY COINCIDENT

The morphological and combined-molecular data sets formed coincident clines across four of the five transects (SM, SI, TR, WB), and in all five cases the anonymous and non-anonymous data sets formed coincident clines. Though character cline coincidence is not an expectation of exogenous selection, and indeed exogenous selection can lead to non-coincidence of clines in some circumstances (Hewitt 1988), character cline coincidence is an expectation of secondary contact (Hewitt 1993). This finding supports the assertion of

Weisrock et al. (2005), who suggested hybrid zones formed between these two species are the result of secondary contact as a result of Pleistocene glacial cycles. Geographic sampling of *P. teyahalee* was meager in Weisrock et al. (2005) as it was not the focal taxon of the study; however, the results of this study appear to show phylogenetic diversity within *P. teyahalee* is greatest to the southeast of the current distribution of *P. shermani* (NW South Carolina), indicating the possibility of northwestern range expansion of *P. teyahalee* prior to secondary contact. Further research into the phylogeography of this species is needed.

Along the fifth transect (FC) the morphological cline has a center below that of the molecular cline. Non-coincident character clines have been seen in other hybrid zones. In their study of a manakin hybrid zone, Brumfield et al. (2001) found the center of the cline for secondary sexual characters (male plumage) to be offset from a center shared by molecular and morphometric characters. They suggested sexual selection is responsible for the cline center offset seen between these two groups of characters. They also proposed a mechanism for the positioning of the center of characters not influenced by sexual selection. As no ecotone occurs across the manakin hybrid zone and the cline center for these non-sexual characters rests on a "dispersal bottleneck" (a severe narrowing of habitable land), they suggested this hybrid zone is a tension zone. Intriguingly, I also found support for sexual selection along the FC transect (and this transect alone), with *P. shermani* males successfully mating with *P. teyahalee* females significantly more than the reciprocal pairing (Chapter 3).

COMPARING CLINES OF DIFFERENT TRANSECTS

I performed a large series of analyses aimed at shedding light on patterns of hybrid zone structure common to the different zones of contact. Under no circumstances were independent hybrid zones found to be in full accordance. Only under a limited set of circumstances (solely morphological data, one outlier transect omitted) was coincidence between different hybrid zones not rejected. The lack of overwhelming evidence for elevational coincidence among transects is unsurprising given the confounding effects of latitude, aspect, and moisture variation (culminating in non-monotonic temperatures

across these transects). Nevertheless, broad-scale patterns of an inverse relationship between temperature and elevation do exist (indicated by generally high Spearman's rho estimates) and may account for the limited evidence in support of coincidence which tellingly is only for the morphological data set, the data set expected to be coincident under exogenous selection. Further, the morphological cline centers are consistently associated with the terminus of an elevation band wherein animals are afforded increased foraging opportunity and therefore at a site of selection regime turnover (i.e., ecotone).

ABIOTIC CHARACTERIZATION OF *PLETHODON* HYBRID ZONES

I interpret the deviations from monotony inferred across all transects as evidence that at the scale of these transects, variables other than elevation have a significant impact on temperature. This is reflected in the broad elevational overlap between the forest types occurring along these transects (Hairston 1949, Day et al. 1988, Schafale & Weakley 1990). As the elevational extents of the transects are modest, the effects of local landscape features are able to overshadow the effects of elevation with respect to temperature. In particular, proximity to nearest stream and dominant woody plant species seem to strongly influence microclimates in this region. In a study of ground level temperatures in the Great Smoky Mountains, Fridley (2009) found that stream proximity had a strong, significant positive effect on temperature. Furthermore, a manipulation study conducted in the vicinity of the SI transect found *Rhododendron*-understory presence resulted in lower temperatures generally, and lower summer maximum and mean air temperatures specifically (Clinton 2003). Nevertheless, all transects exhibited a trend of cooler temperatures at higher elevations, and the bottoms of the transects were consistently warmer than the tops of the transects, indicating the potential for disparate ecological conditions bracketing the hybrid zones.

The activity-hour estimates across the transects suggest the mid-elevations may offer maximal activity opportunity for generalized *Plethodon glutinosus* group salamanders. I interpret these findings as indicating activity at higher elevations is limited by an excess of cold temperatures and activity at lower elevations is limited by an excess of hot temperatures. Different species (Spotila 1972) and conspecific populations (Bogert 1952,

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Elwood 2003) of *Plethodon* have different preferred temperatures, and it is safe to assume that *P. teyahalee* has a somewhat higher or broader temperature preference than *P. shermani*. As such, these mid-elevations may represent a suitability overlap, whereas the areas outside the hybrid zone are more suitable for one of the species. *P. teyahalee* achieves higher elevations outside the distribution of *P. shermani* than it does within (e.g., Highlands Plateau; Highton & Peabody 2000, pers. obs.). Further, a study of *P. teyahalee* in the Great Smoky Mountains showed the species has a broad thermal tolerance making it suitable for most of the elevational extents investigated in this study (Gifford & Kozak 2012). Therefore, *P. teyahalee* likely has a wider rather than a higher thermal preference with respect to *P. shermani*. This hypothesis is further supported by *P. teyahalee* being more broadly distributed than *P. shermani*, and other studies have shown that distributional extent is positively correlated with thermal tolerance breadth (Calosi et al. 2010, T. Markle unpub.). One salient conclusion from the observation of a mid-elevation activity-hour peak within the hybrid zones is its incongruence with a density trough, another expectation of a hybrid zone maintained by endogenous selection.

EVIDENCE SUPPORTING IMPORTANT BIOTIC INTERACTIONS

At the scale of these hybrid zones, biotic interactions should also be important. Others have suggested that at the scale of tens of kilometers, species distribution should be supremely influenced by biotic interactions (Pearson & Dawson 2003, Wisz et al. 2013), and I speculate this extends to the size and position of hybrid zones as well. In particular, the color pattern clines across these hybrid zones, which exhibit patterns consistent with selection (discussed above), may be under the influence of such biotic interactions. As aposematic signal consistency is maintained by predators (Kapan 2001, Noonan & Comeault 2009, Crothers & Cummings 2013), predator distributions may be in part dictating the fitness landscapes for these respective salamander species.

The context of the inferred ecological selection on phenotype may well be aposematism. The unpalatability of *P. shermani* has been demonstrated in studies using sympatric predators (Hensel and Brodie 1976, Brodie et al. 1979), as well as indirectly through laboratory studies involving this species and its Batesian mimic, *Desmognathis*

ocoee (Labanick & Brandon 1981). Though different colored legs (relative to body) of *P. shermani* and other plethodontids may serve a disruptive function under some circumstances (Stebbins 1949), the demonstrated unpalatability of *P. shermani* and survival conferred to red-legged (but otherwise palatable salamanders, see Labanick & Brandon 1981) serves to make a strong case for an aposematic function. A case can also be made for an aposematic function for the high-contrast pattern of *P. teyahalee*, which is achieved through eumelanin-blackened skin being overlain with clusters of white iridophores. The palatability of this species has not been evaluated, however, the amount of sticky substance exuded from dorsal granular glands is comparable to that of other large *Plethodon* (pers. obs.), and a similarly pigmented species that replaces *P. teyahalee* to the northwest (*Plethodon glutinosus*) has been shown to be even less palatable than *P. shermani* with respect to mammalian predators (*Blarina*; Brodie et al. 1979). Highton (1995) speculated that the white-spots-on-black motif seen in many *Plethodon* and *Aneides* species (including *P. teyahalee*), may serve as an aposematic signal to potential predators, but to date no studies have been performed to test whether predators trained on salamanders with this pattern will transfer this aversion to similarly patterned but otherwise palatable salamanders. However, black-and-white coloration is a common aposematic motif (Mappes 2005).

The question then is, if both lineages have successful aposematic strategies that do not appear to be mutually exclusive (one on legs, the other on torso), why would selection limit the spread of each aposematic syndrome into the distribution of the other? I hypothesize the success of the respective syndromes may be context dependent (Gamberale-Stille & Tullberg 2001). Though the species distributions of most potential predators span the distributions of both *P. teyahalee* and *P. shermani* (*Cryptotis parva* may be restricted to the distribution of the former and *Sorex cinereus* to that of the latter [Ford et al. 1994]), local populations of these species may have either evolved or learned to avoid large eastern *Plethodon* of one aposematic syndrome and are naive to the other, affording the native syndrome more protection than the novel one. Evidence has recently been put forth to explain regional variation among populations of *Plethodon cinereus* (Kraemer et al. 2016). Other sources of selection that could be at play include

conspecifics and the abiotic environment. However, there is no evidence that color hue or pattern affect courtship in *Plethodon* (and evidence against this has been found in the plethodontid genus *Desmognathus*; Labanick 1988), and it is difficult to envision an abiotic explanation for variation in color pattern.

CONCLUSIONS

Despite evidence for extensive introgression of nuclear markers, character clines across hybrid zones formed between *Plethodon shermani* and *P. teyahalee* are generally coincident within transects, and to a more limited degree coincident between transects. As these various clines appear coincident with an ecotone joining areas of high and low activity opportunity but not a density trough, a strong case can be made for exogenous selection in maintaining these hybrid zones. This case is strengthened by the fact that when morphological and molecular clines across a given transect are significantly different in extent, it is the morphological cline that is restricted in extent (suggesting selection acting on phenotype), and despite being narrower that molecular clines the morphological clines are still of a greater extent that the dispersal abilities of these salamanders. I suspect both biotic and abiotic variables may be contributing to the observed hybrid zone structure. I suggest further research into the contact zones formed between these two species, and recommend the use of genomic data which may reveal patterns of genetic introgression obscured in my SNP data set. Further, laboratory based studies of behavior and physiology could reveal differential adaptation with regard to important abiotic factors (e.g., temperature, humidity).
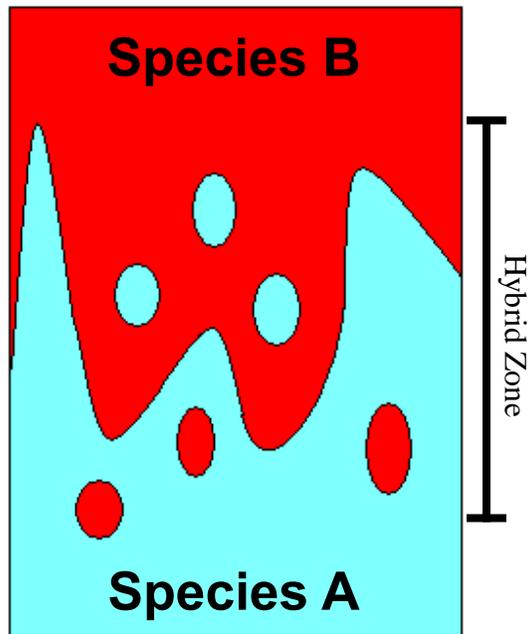
Figure 2.1. Schematic depicting a hypothetical mosaic hybrid zone. Note the hybrid zone portion of the figure consists of discrete populations of the two contacting species distributed in a patchwork fashion.
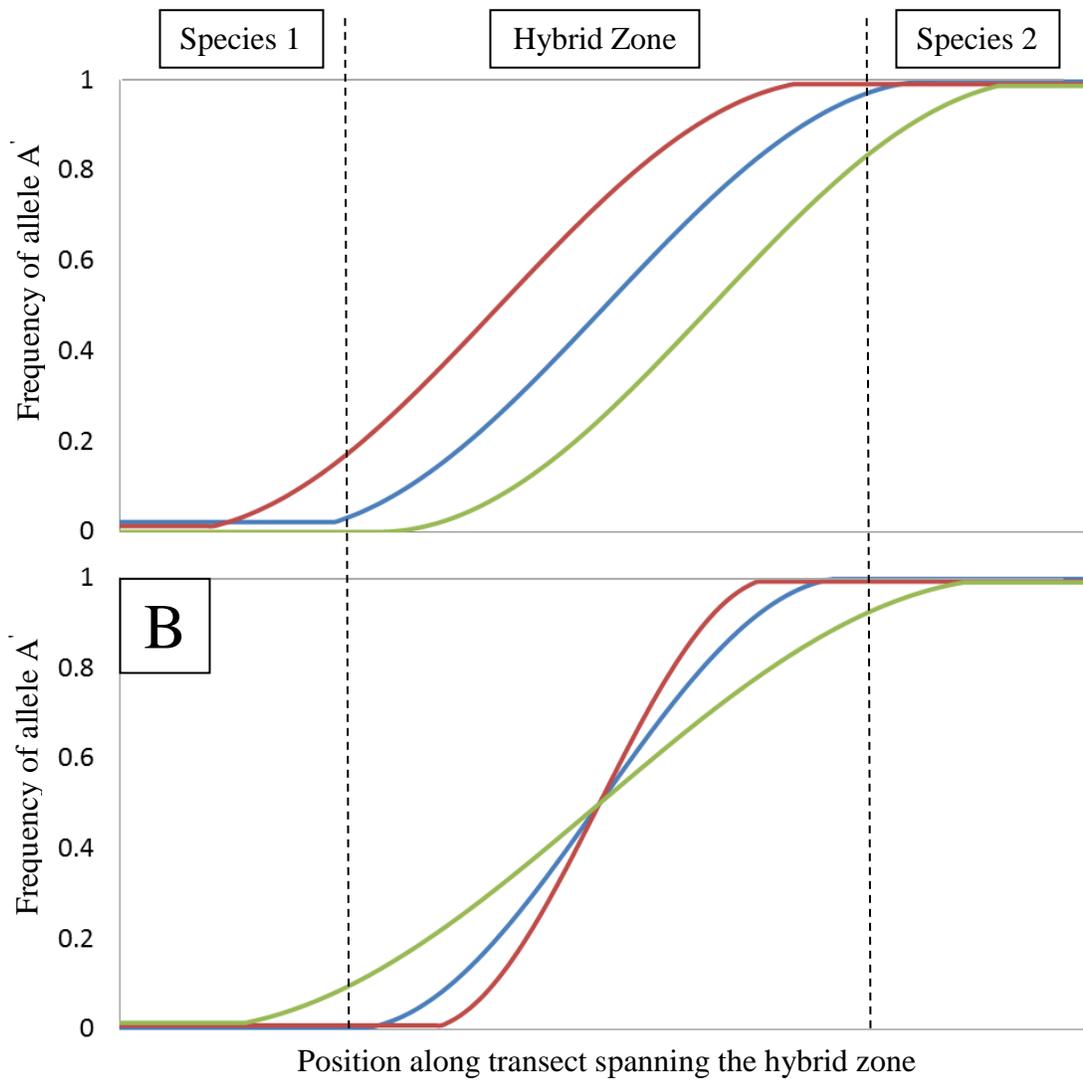
Figure 2.2. Schematic illustrating types of character cline accordance. **(A)**, concordance, where cline extents are equal, regardless of position of centers. **(B)**, coincidence, where clines share a common center, regardless of cline extent.
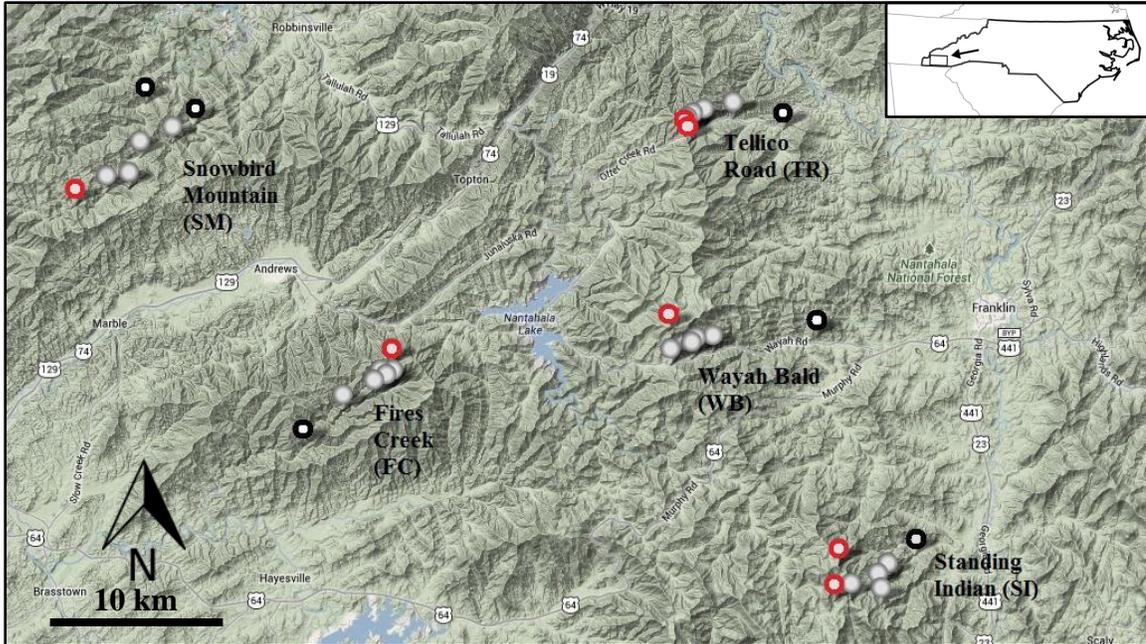
Figure 2.3. Hybrid zone transects. Circles with light grey margines indicate intra-transect sites inhabited by salamanders of mixed ancestry. Boldly outlined circles indicate parental sites, with black-margined circles indicating *P. teyahalee* parental sites and red-margined circles indicate *P. shermani* parental sites. Background topographic map courtesy of Google Maps[TM]. Inset map of North Carolina indicates extent of topographic map (rectangle indicated with arrow).
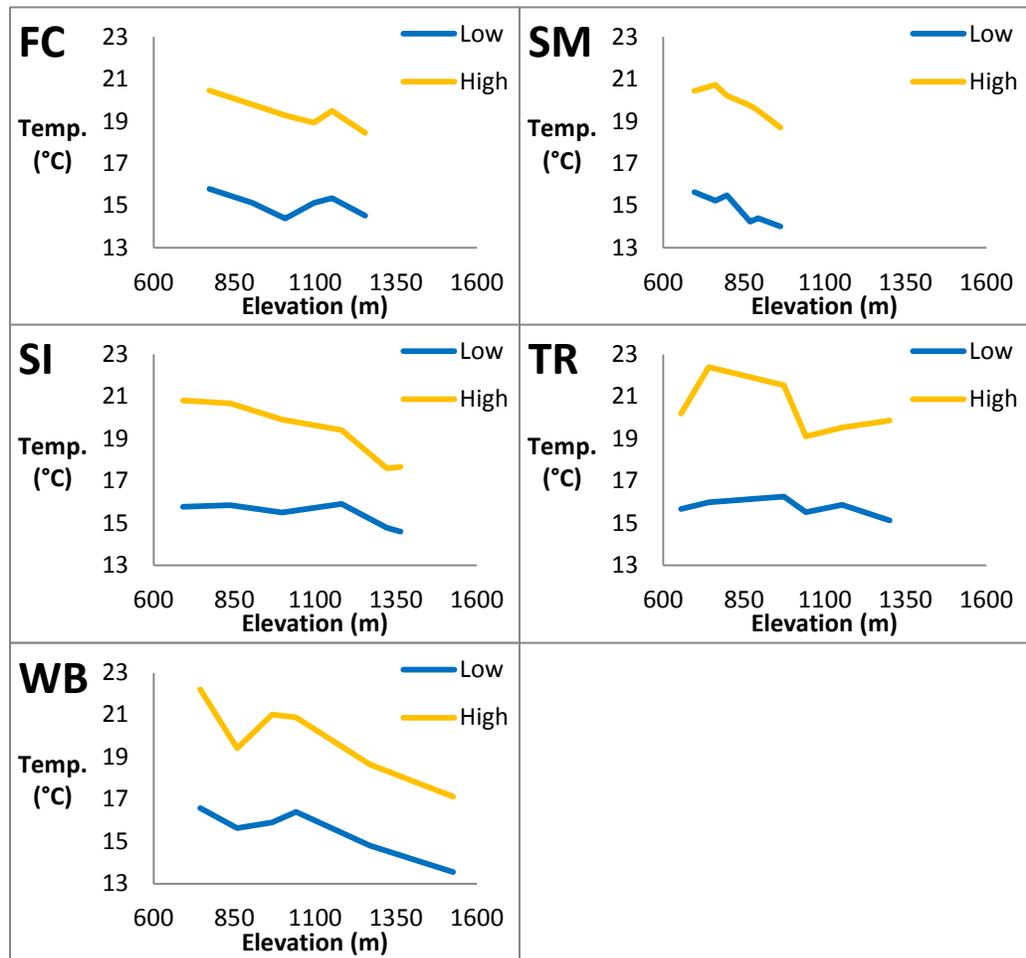
Figure 2.4. Temperature estimates across transects.
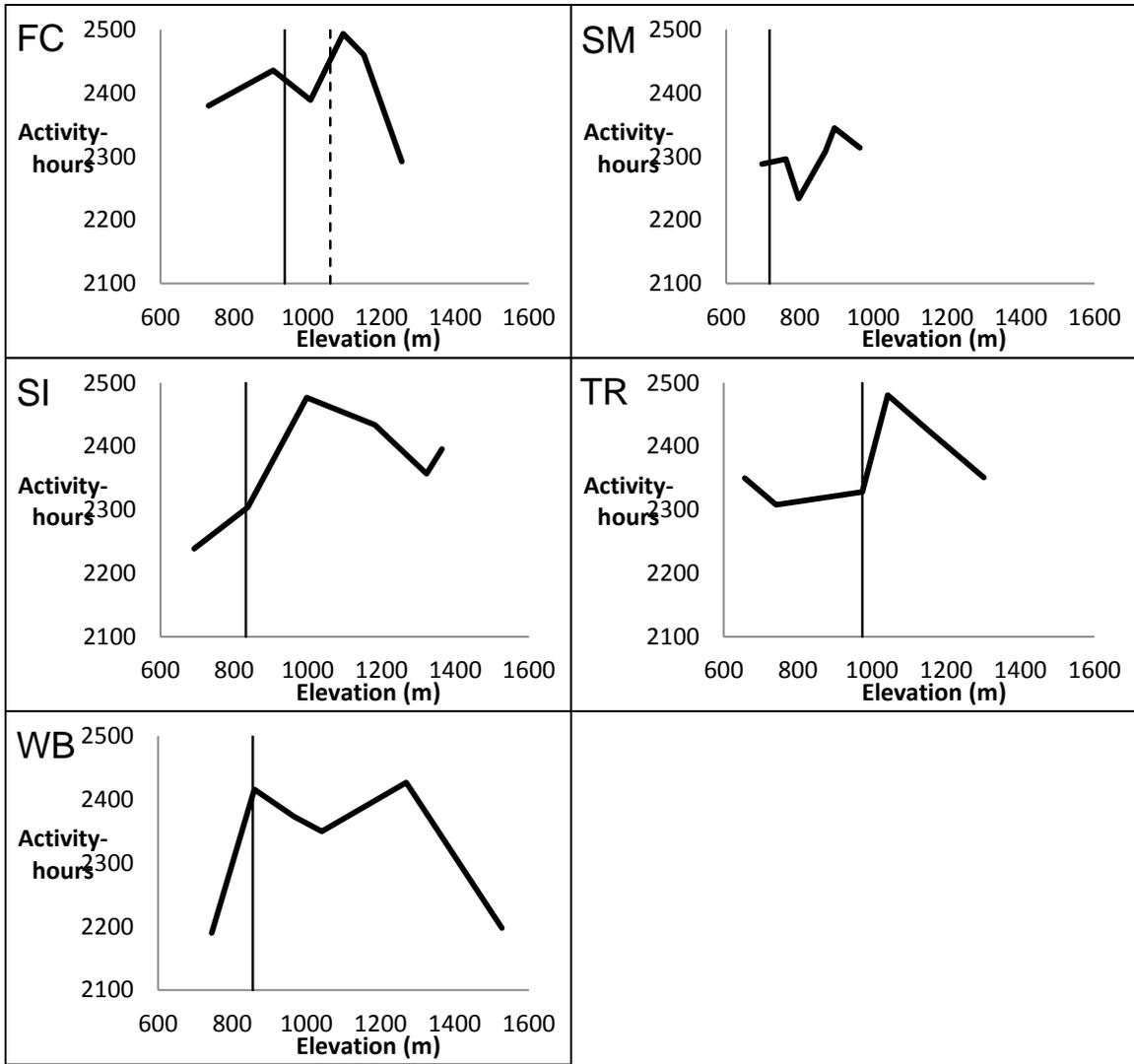
Figure 2.5. Activity-hours. Vertical lines indicate cline centers. For FC, the solid line indicates the morphological cline center and the dashed line indicates the molecular cline center. See Table 2.2 for explanation of transect abbreviations.
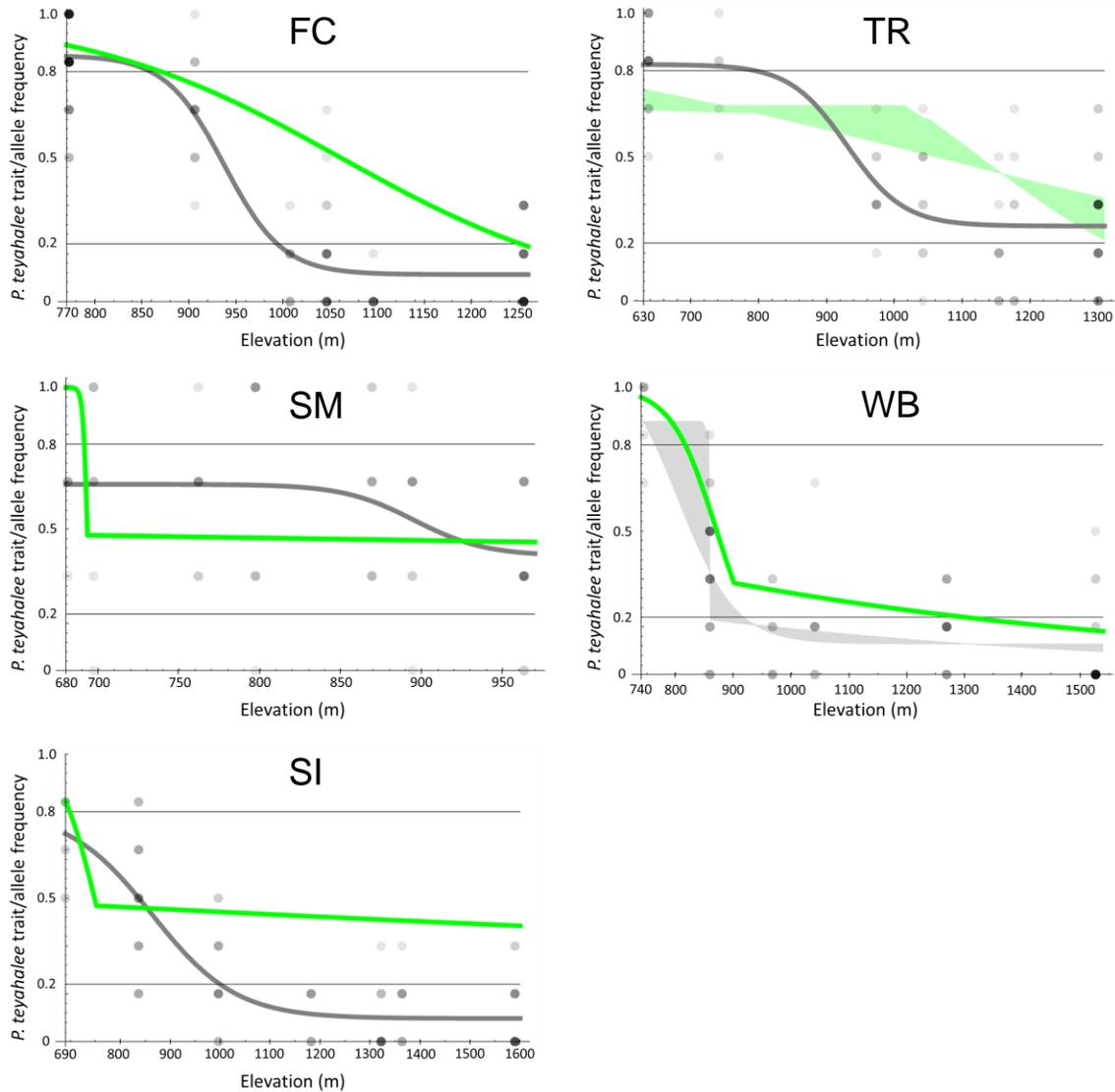
Figure 2.6. Clines across transects. Horizontal axis depicts elevation (meters). Vertical axis depicts *Plethodon teyahalee* trait value/allele frequency as determined by CFit-7. Grey lines/polygons indicate morphological clines. Green lines/polygons indicate molecular clines. Polygons are composite clines describing all models with $\Delta AIC_c < 2$ with respect to the best fit model. Circles indicate morphological observations, with circle darkness proportional to number of observations. Horizontal lines indicate frequency values of 0.2 and 0.8. See Table 2.2 for explanation of transect abbreviations.
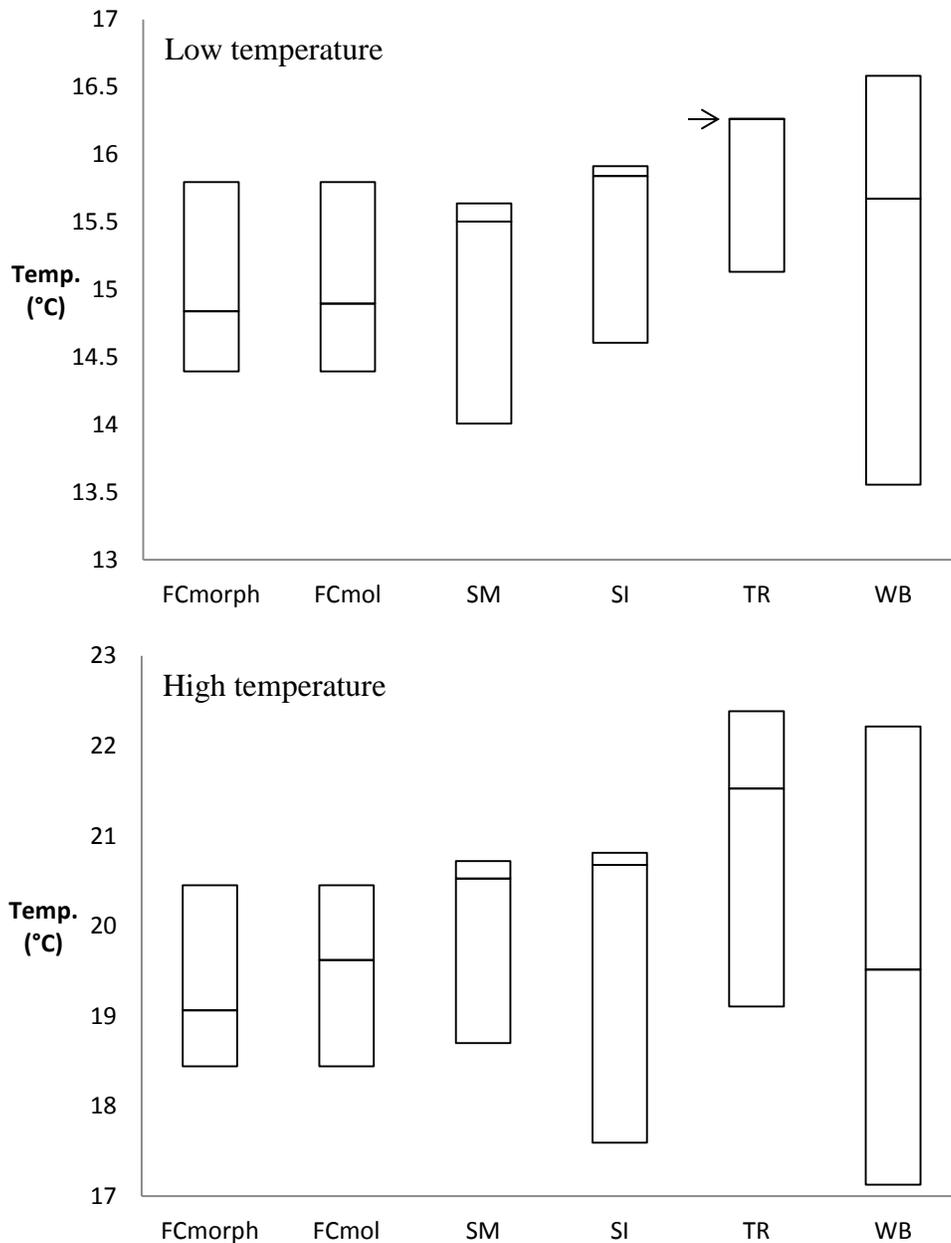
Figure 2.7. Mean temperature spans recorded on transects with interpolated temperature of cline center. Cline center interpolated mean temperature indicated with horizontal line (arrow adjacent to TR transect box plot indicates cline center interpolated mean low temperature). FCmorph and FCmol represent interpolated

mean temperatures for Fires Creek morphological and molecular clines, respectively. See Table 2.2 for additional explanation of transect abbreviations.
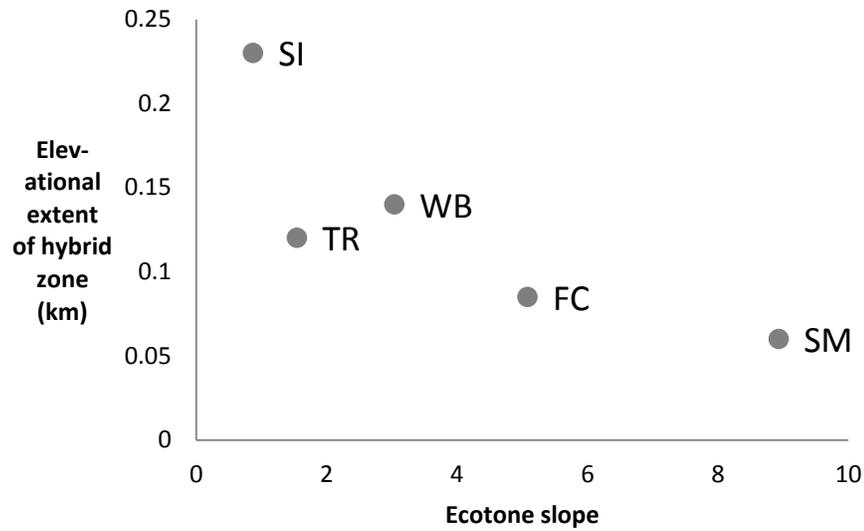
Figure 2.8. Morphological cline expanse plotted against ecotone slope. Ecotone slope is the change in mean low temperature divided by the change in elevation. See Table 2.2 for explanation of transect abbreviations.

Attribute	Exogenous	Endogenous
Relative extent of clines for neutral (= molecular) and selection-experiencing (= morphological) character clines across same transect	Morphological cline extents less than that for molecular	No expected relationship between morphological and molecular clines
Cline extent	Cline extent large with respect to dispersal ability	Cline extent of same magnitude as dispersal ability
Population density at hybrid zone center	Not predicted to differ from regions below and above center	Likely low; not high
Centers of the different hybrid zones	Coincident with a common center	Not predicted to be coincident with a common center

Table 2.1. Predictions for attributes under the two primary hypotheses.

Transect	Code	Isolate	Low elev.	High elev.	Aspect	HZ extent	Spearman's rho high	Spearman's rho low
Fires Creek	FC	Tusquitee	773	1255	SW	85	-0.828	-0.828
Snowbird Mountain	SM	Unicoi	697	963	NE	60	-0.942	-0.885
Standing Indian	SI	Standing Indian	691	1364	E	230	-0.942	-0.6
Tellico Road	TR	Wayah Bald	656	1301	E	120	-0.6	-0.485
Wayah Bald	WB	Wayah Bald	744	1527	S	140	-0.828	-0.771

Table 2.2. Transect characteristics. Elevations and hybrid zone (HZ) extent based on phenotypic data; in meters. Spearman's rho (i.e., Spearman's rank correlation coefficient) shown for high and low temperature estimates.

Marker	Locus category	Coding/non-coding	Transects*	Source
C3	Anonymous	Non-coding	FC, SM, SI, TR, WB	Novel locus
Pglut9	Anonymous	Non-coding	FC, SI, WB	Novel locus
Pglut10	Anonymous	Non-coding	SI, TR	Novel locus
Pglut19	Anonymous	Non-coding	FC, SI, WB	Novel locus
CXCR4	Non-anonymous	Coding	SM, SI, TR	Novel locus†
MC00	Non-anonymous	Non-coding (intron)	FC, SM	Chatfield et al. 2010
POMC	Non-anonymous	Coding	SM, SI, WB	Vieites et al. 2007
SLC8A3	Non-anonymous	Coding	SM, SI, TR	Rovito 2010

Table 2.3. Molecular markers.

*See Table 2.1 for explanation of transect abbreviations.

†Internal primers designed from locus of Roelants et al. 2007.

Transect*	Morphological Model	Molecular Model	Morphological vs. molecular clines	Anonymous vs. non-anonymous
FC	C & S	C & S	Non-accordant	Full accordance
SM	C & S	C & S	Full accordance	Full accordance
SI	C & S	R	Coincident	Full accordance
TR	C & S	C & S, L, Mir	Coincident	Full accordance
WB	C & S, R, Mir	R	Full accordance	Full accordance

Table 2.4. Cline models and accordance determinations from CFɪᴛ-7. All models with $\Delta AIC_c < 2$ presented. "C & S" indicates center and slope parameters. "R" indicates center, slope, position of right tail, and slope of right tail parameters. "L" indicates center, slope, position of left tail, and slope of left tail parameters. "Mir" indicates center, slope, position of tails, slope of tails parameters.

*See Table 2.1 for explanation of transect abbreviations.

Analysis block	Data set	Model	lnL	df	AIC	LRT
All Transects	Morph + Mol	Same	-1300.419113	31	2662.838226	
		Coin	-1256.419289	35	2582.838578	
		Diff	-1213.623713	39	**2505.247425**	x
	Morphological Only	Same	-729.5177774	28	1515.035555	
		Coin	-724.5130439	32	1513.026088	
		Diff	-713.6935534	36	**1499.387107**	x
	Molecular Only	Same	-515.5543783	6	1043.108757	
		Coin	-489.3607644	10	998.7215288	
		Diff	-459.4967578	14	**946.9935156**	x
No FC	Morph + Mol	Same	-1001.913402	26	2055.826805	
		Coin	-970.6684408	29	1999.336882	
		Diff	-950.5028178	32	**1965.005636**	x
	Morphological Only	Same	-532.5430896	22	1109.086179	
		Coin	-526.0037949	25	**1102.00759**	x
		Diff	-524.2402749	28	1104.48055	
	Molecular Only	Same	-452.7618464	6	917.5236928	
		Coin	-420.8400571	9	859.6801141	
		Diff	-413.529	12	**851.0580001**	x
TR & WB	Morph + Mol	Same	-431.9794949	14	891.9589897	
		Coin	-418.3817501	15	866.7635001	
		Diff	-413.2797619	16	**858.5595238**	x
	Morphological Only	Same	-282.3037947	12	588.6075894	
		Coin	-280.5588292	13	587.1176584	x
		Diff	-279.2528234	14	**586.5056469**	
	Molecular Only	Same	-134.1778933	4	276.3557865	
		Coin	-125.132898	5	260.2657961	
		Diff	-122.6506972	6	**257.3013944**	x

Table 2.5. Results from hypothesis tests. The "All Transects" analysis block includes data from all five transects. The "No FC" analysis block includes data from four transects, omitting the Fires Creek transect. The "TR & WB" analysis block includes

only the Tellico Road and Wayah Bald transects. "Morph + Mol" data sets employ both morphological and molecular data. The "Same" model constrains the center and slope of the transects to be the same (= full accordance). The "Coin" model constrains only the center of the transects to be the same (= coincidence). The "Diff" model allow both the centers and slopes to vary among the transects (= no constraint). "lnL" = log likelihood of the models given the data set. "df" = degrees of freedom. "AIC" = Akaike information criterion; the values for best model determined by AIC calculations are indicated with bold font. "LRT" = likelihood ratio test; an "**x**" in the LRT column indicates best model as determined in a likelihood ratio test (using a χ^2 table and a significance threshold of $P = 0.05$).

CHAPTER 3

TESTING FOR VARIATION IN FREQUENCY OF INTERSPECIFIC HYBRIDIZATION AMONG ISOLATES

ABSTRACT

Hybrid zones represent model systems for studying the causes and consequences of gene exchange between closely related evolutionary lineages. The salamanders *Plethodon shermani* and *Plethodon teyahalee* replace each other elevationally across several mountain ranges, but hybridize where they come in contact. Genetic, geographic, and life-history data, however, suggest that these two species are not in the process of merging into a single lineage. Potential factors preventing merger include genetic incompatibility, natural selection imposed by divergent ecological conditions, and behavioral isolation. To test the hypothesis that behavioral isolation maintains species limits in these salamanders, I performed courtship trials with individuals collected from opposite ends of five geographically disparate contact zones. I subjected male salamanders to overnight, "no-choice" trials with con- or heterospecific female salamanders, and evaluated whether courtship/mating took place the following morning. From these data, I estimated reproductive isolation (I_{PSI}) and compared patterns of isolation among the different contact zones with patterns of genetic divergence. In total, I conducted 1512 courtship trials. I found considerable variation in the degree of reproductive isolation among contact zones, and the degree of reproductive isolation was related to degree of genetic differentiation. My findings support the hypothesis that courtship/mating behavior facilitates reproductive isolation in this system and may be responsible for maintaining species limits.

INTRODUCTION

Positive assortative mating (Hogben 1946), a form of premating isolation wherein individuals preferentially mate with individuals more similar to themselves than the average available and compatible individual (hereafter 'assortative mating'), has received much attention from evolutionary biologists. Assortative mating has been shown to be a

powerful barrier to gene flow, particularly if there is a selective advantage to mating with similar individuals (e.g., conspecifics; Kirkpatrick & Ravigné 2002). There is great interest in assortative mating evolving to prevent maladaptive mating (i.e., reinforcement; Dieckmann & Doebeli 1999; Bolnick & Fitzpatrick 2007; Sætre et al. 1997, 1999; Barluenga et al. 2006). Assortative mating can also evolve in allopatry prior to secondary contact (Blair 1974, Nevo & Capranica 1985, Tregenza & Wedell 2000). Theoretical work suggests that such preexisting assortative mating can be a strong barrier to gene flow at secondary contact, particularly when interspecific matings result in the production of offspring with reduced fitness (Kondrashov & Shpak 1998). Additionally, this type of assortative mating may be very common (Tregenza & Wedell 2000). In contrast to the phenomenon of reinforcement, assortative mating serving to prevent the merger of species that hybridize at secondary contact has not been studied extensively in natural systems.   Situations where two species meet at multiple, independent zones of contact provide a good opportunity for determining whether assortative mating is maintaining species limits. Specifically, if assortative mating is preventing the merger of lineages and there is variation in the degree to which contacting populations mate assortatively, gene flow between the parental populations should be low where assortative mating is strong. Alternatively, if the species are maintaining integrity through other means (e.g., ecological selection, outbreeding depression), assortative mating should be random with respect to gene flow.

In this study, I assess assortative mating between two hybridizing species of salamanders of the genus *Plethodon* in the southern Appalachian Mountains using courtship trials. One of these species, *Plethodon shermani*, occurs in four isolated high-elevation metapopulations (hereafter, isolates); the other, *Plethodon teyahalee*, occurs at lower elevations. The two species form hybrid zones of variable extent where they meet at mid elevations, within which individuals exhibit phenotypic characteristics of both parental species. Specifically, these salamanders of hybrid ancestry possess some degree of red on their legs and white spotting on their flanks, characteristics attributable to *P. shermani* and *P. teyahalee*, respectively. Though phylogenetically close (belonging to the *Plethodon glutinosus* group; Highton & Larson 1979), *P. shermani* is not the sister

taxon of *P. teyahalee* (Fisher-Reid & Wiens 2011; Kozak et al 2009). Previous work demonstrated that *P. shermani* and *P. teyahalee* from allopatric and geographically disparate (~45 km) populations exhibit assortative mating, yet no assortative mating was detected between that *P. shermani* population and a geographically proximate (~9 km) population determined though morphology and genetics to be the product of *P. shermani*-*P. teyahalee* hybridization (Reagan 1992).

To establish that these species mate assortatively and to determine how assortative mating varies among different contact zones, I conducted inter- and intraspecific mating trials using animals from populations above (*P. shermani*) and below (*P. teyahalee*) five hybrid zones formed between these species. The four *P. shermani* isolates are represented among these five contact zones, as assortative mating is most likely to exhibit variation between isolates. To determine if elevated levels of assortative mating were associated with high interspecific genetic divergence, I generated pairwise estimates of genetic differentiation for the populations included in the courtship trials. If assortative mating is shown to vary among contact zones, and shown to be positively correlated with genetic differentiation (indicating reduced gene flow), I interpret this to be evidence for assortative mating contributing to the maintenance of species limits between these two salamander species.

METHODS
FIELD METHODS & EXPERIMENTAL DESIGN

Plethodon salamanders were field collected for use in courtship trials and for genetic characterization of the hybrid zone. Salamanders were collected from above and below five areas of hybridization ('transects'; Fig. 3.1; Table 3.1; hereafter I refer to salamanders from these ten localities as the 'courtship trial populations'). For nine of these ten sites, the salamanders corresponded to non-introgressed *P. shermani* or *P. teyahalee* (i.e., parental phenotype; based on morphology and previous molecular work [Peabody 1978]); these populations will be referred to hereafter as 'parental populations.' However, phenotypically parental *P. teyahalee* at one transect (WB) were insufficiently abundant for courtship trials and salamanders were instead collected from the next lowest

site along the transect (hereafter, the 'hybrid ancestry *P.teyahalee* population');
salamanders at this population largely correspond to *P. teyahalee* but do show some
influence from *P. shermani*. Salamanders were maintained in captivity in individual
plastic tubs in a growth chamber at 14.5 °C under light regime of 12 h light : 12 h dark.
They were acclimated for two weeks during which their tubs were cleaned and they were
fed crickets twice a week; subsequent to trial initiation, salamanders were fed and cages
were cleaned every fourth day.

To measure sexual isolation, I employed an experimental design and analytical
methodology that reduces the number of animals needed by including animals in multiple
trials while accounting for the non-independence of trials (Richmond & Jockusch 2007).
Animals were assigned to 'trial series' specific to the transect from which they were
collected. Each 'trial set' (all the salamanders assigned to a single trial series) contained
n males and *n* females of both species for a total of 4*n* animals per series. Within each
trial series, each male was afforded one opportunity to court each female in the trial set,
resulting in Xn, heterotypic and Xn homotypic encounters. In these "no-choice" (Coyne
1992) 'courtship trials,' the male and female were placed in a semi-translucent, $25 \times 14 \times 7$ cm courtship box containing ventilation holes and a moist towel pressed flat across the
bottom and left overnight at 14.5 °C in the environmental chamber. In the morning, the
cloacal region of the female was investigated for the presence of a spermatophore cap
(Fig. 3.2a), both salamanders were returned to their individual containers and provided
crickets, and the courtship box was investigated for evidence of courtship behavior (i.e.,
entire spermatophore, spermatophore base, disassociated cap; Arnold et al. 1993, Kozak
2003; Fig. 3.2). Salamanders were allowed three nights of rest between courtship trials.
In scheduling the courtship trials for a courtship set, the males and females were ordered
randomly. If the first courtship series did not result in sufficient courtship success to
evaluate assortative mating, additional salamanders were collected from the field and a
second trial series was conducted.

COURTSHIP TRIAL DATA ANALYSIS

I considered the presence of a spermatophore cap in the female's cloaca as evidence of

successful courtship. Using these data, I calculated the index of pair sexual isolation (I_{PSI}; Rolán-Alvarez & Caballero 2000) for each of the five transects as a measure of mating preference. I_{PSI} values span the range of -1 to 1, with negative, zero, and positive values denoting disassortative, random, and assortative mating, respectively. The software JMATING (v. 1.0.8; Carvajal-Rodriguez & Rolán-Alvarez 2006) was used to generate I_{PSI} values and ascertain statistical significance. Furthermore, this software identifies statistically significant sexual selection, where one sex of one species is preferred by all members of the opposite sex, irrespective of species, and asymmetrical hybridization, where one species shows a stronger preference for conspecifics than does the other. Because 'courtship failure,' spermatophore deposition concomitant with failed insemination, could reveal insights into assortative mating by indicating a "choosy sex," I also tabulated the number of failed courtship events. These were identified by the presence of a spermatophore base without the presence of a spermatophore cap in the female's cloaca. Potential failed courtship scenarios include an undisturbed spermatophore, a spermatophore cap separated from its base and lying on the towel (presumably trampled), and a capless spermatophore base and no evidence of the cap (presumably consumed; Marvin & Hutchison 1996, Picard 2005). For courtship series with greater than 20 failed courtship events (Dixon & Massey 1969), I performed chi-square tests to determine whether the distribution of courtship failures deviated significantly from the null hypothesis of random distribution. If significant, I subsequently examined the distribution of courtship failures to identify the pairings that resulted in an under- or overabundance of failures.

HYBRID ZONE CHARACTERIZATION

With regard to hybridizing taxa, if assortative mating is serving to restrict interspecific gene flow, parental populations should be most genetically distinct where assortative mating is strongest. This is because introgressed alleles increase the genetic similarity of the two parental populations. Alternatively, if assortative mating is present and varies in strength among independent contact zones but is not the force maintaining species limits, levels of assortative mating should vary randomly with respect to genetic divergence. To

this end, I estimated genetic differentiation between each pair of courtship trial populations. I used sequence data generated for the cline fitting exercise in Chapter 2 (see Chapter 2 for DNA extraction, PCR amplification, and sequencing methods). This data set comprises sequence data for eight nuclear markers. I used the program INDELLIGENT (v. 1.2; Dmitriev & Rakitov 2008) to resolve sequences heterozygous for length ('indels') for one locus (Pglut9). For other markers exhibiting length heterozygotes I instead truncated the sequences prior to the indel. I then used the software PHASE (v. 2.1.1; Stephens et al. 2001, Stephens & Donnelly 2003, Stephens & Scheet 2005) and SEQPHASE (Flot 2010) to render constituent haplotypes from the unphased diploid sequences. I used the program JMODELTEST (v. 2.1.4; Guindon & Gascuel 2003, Posada 2008, Darriba et al. 2012) to determine the best model of evolution for each marker. Subsequently I extracted and aligned the haplotypes belonging to the ten courtship trial populations and calculated Φ_{ST} statistics in ARLEQUIN (v. 3.5.1.3; Excoffier et al. 2005) for the five population pairs to quantify genetic differentiation across each transect. These analyses utilized the optimal substitution model when possible; however, where this was unavailable I employed the most similar available model. For the transect that necessitated using a hybrid ancestry *P. teyahalee* population in courtship trials (WB; Table 3.1) I performed two separate analyses: one using sequences from the hybrid ancestry population and one using sequences from a legitimately parental population of *P. teyahalee* from the same transect. In addition to presenting locus-specific Φ_{ST} estimates, I also calculated mean and weighted mean Φ_{ST} estimates. For weighting, I multiplied the individual marker Φ_{ST} estimates by the corresponding transect-specific sequence sample size, summed those products, and divided that sum by the total number of sequences for that transect.

The Φ_{ST} estimate for the WB populations was inexplicably low, therefore I utilized temperature data collected for another study (Chapter 2) to determine if, despite a considerable elevational and morphological difference between the courtship populations, temperature data revealed the salamander populations experienced similar ecological conditions, explaining their underlying genetic affinity. Two data loggers were placed at six sites along the transect (corresponding to sites of genetic sampling; Chapter 2). I

recorded temperature readings every two hours from June 14th through October 27th, and used these data to estimate mean daily high and low temperatures for these six sites, which include all parental and hybrid ancestry populations. Despite my best attempts to place the data loggers on the north side of trees and in unexposed positions, the high temperatures collected by some data loggers may have been compromised by direct sunlight. To remedy this I visually inspected the data and used the data from the data logger that appeared least affected by this phenomenon (if neither appeared compromised, I chose randomly). Furthermore, the daily low temperatures are not subject to this problem.

RESULTS

I performed a total of 1512 trials. Reproductive isolation was significant at the 0.05 level across two of the five transects (FC, SI; Table 3.2; Fig. 3.3). For two additional transects (SM, TR), I recovered moderate I_{PSI} values that were not statistically significant. Much lower I_{PSI} was estimated for the trial series that used the hybrid ancestry population.

Significant sexual selection was found in one of the courtship series (FC; $P = 0.0259$). This was manifest as a preference for *P. teyahalee* females by males of both species. Asymmetrical hybridization was found across one transect, also FC ($P = 0.0492$), wherein female *P. teyahalee* × male *P. shermani* pairings were significantly more successful than the reciprocal hybrid cross. The only courtship series with a quantity of courtship failures sufficient for statistical analysis was also FC ($n = 22$). The distribution of courtship failures deviated significantly from random (two-tailed $P = 0.0424$), with significantly fewer courtship failures in female *P. shermani* × male *P. teyahalee* trials than expected.

The nucleotide substitution model limitations of the program ARLEQUIN required selecting alternate models for four of the seven markers. However, for two markers where no single alternate model seemed most appropriate I compared the results from analyses performed using two different models (Table 3.3), and the Φ_{ST} estimates were effectively identical. The Φ_{ST} estimates showed considerable variation among the five

transects (Table 3.2; Fig. 3.3) and among the various markers (Table 3.4). For two transects, the courtship trial populations were found to be significantly differentiated with respect to multiple markers (FC and SM, 2 of 4 and 3 of 6, respectively). Courtship trial populations at a third transect (SI) were significantly differentiated with respect to one marker and differentiation was nearly significant with respect to an additional three markers (of a total of seven).

For the remaining two transects, courtship trial populations were not significantly differentiated at any locus. However, mean and weighted mean estimates of Φ_{ST} for WB using parental phenotype *P. teyahalee* were much higher than those estimated using the hybrid-ancestry *P. teyahalee*, indicating the WB I_{PSI} estimates generated in this study are potentially not reflective of I_{PSI} values for parental populations. The pairwise Φ_{ST} estimates for the WB *P. teyahalee* and the hybrid ancestry population were substantial (weighted mean Φ_{ST}: 0.203) given the morphological similarity and elevational proximity (115 m) of these two populations. In line with this finding, the temperature data revealed drastically divergent temperature regimes for these populations (Fig. 3.4).

The mean Φ_{ST} estimates corroborated the individual-marker estimates of significance, with estimates for FC, SI and SM courtship trial populations ranging from ~0.18 to ~0.34 and TR and WB estimates ranging from ~0.03 to ~0.07. The two transects exhibiting significant assortative mating (FC, SI) were among those for which a high Φ_{ST} was estimated. However, when all five courtship population pairs are evaluated together in a linear regression, I_{PSI} was not significantly correlated with either set of Φ_{ST} estimates.

DISCUSSION

ASSORTATIVE MATING IS PRESENT, CONTRIBUTING TO MAINTENANCE OF SPECIES LIMITS

Assortative mating can impede gene flow across zones of secondary contact, and prevent the merger of genetically compatible lineages, thereby maintaining species limits. Here, I present evidence for assortative mating across hybrid zones formed between *Plethodon shermani* and *Plethodon teyahalee*. Further, the different transects show variation in degree of assortative mating, and an apparent positive relationship between

degrees of assortative mating and genetic differentiation supports the hypothesis that where assortative mating is strong, it is serving to prevent admixture of parental populations.

For two of these transects, the courtship populations showed large, statistically significant assortative mating (FC, SI). For two additional pairs of courtship trial populations (SM, TR), assortative mating was estimated to be substantial, though not statistically significant. With the exception of the WB transect, the I_{PSI} values recovered (0.30–0.73) are within the range of biologically significant values found in other no-choice trial studies (e.g., Elmer et al. 2009, Dillon et al. 2011, Kim et al. 2012). Absent other factors I doubt these levels of isolation would suffice to maintain species limits (but see discussion of trial series SI below). However, in conjunction with outbreeding depression on account of genetic divergence accrued in allopatry and/or ecological selection, the degree of assortative mating seen across these transects would contribute to maintaining species integrity.

Assortative mating in concert with ecological gradients serving to maintain species limits between genetically compatible species is a well-documented phenomenon, having been demonstrated in marine gastropods (Hull 1998), gymnotiform fish (Cooke et al. 2014), and songbirds (Kirschel et al. 2011). The parental populations of *P. shermani* and *P. teyahalee* occur on either side of an ecotone, and the presence of an activity-hour peak in the center of all five transects (see Chapter 2) does suggest the areas occupied by parental populations present a more challenging foraging setting for generalized *Plethodon glutinosus* group salamanders. As foraging limitations constitute a form of selection pressure (McLaughlin 1989), this may be a source of divergent exogenous selection (Moore 1977).

TR AND WB TRANSECTS

When Φ_{ST} is calculated for the WB transect with a parental *P. teyahalee* population substituted for the hybrid ancestry population, a substantial Φ_{ST} value is returned (~0.3), indicating the only definitive Φ_{ST} outlier is the TR population. This level of genetic similarity is surprising, as salamanders from the parental populations associated with the

TR transect are highly divergent morphologically and separated by a relatively large elevational difference (664 m). Unfortunately this transect has not been studied previously, precluding corroborating my finding of genetic similarity with other markers (e.g., mtDNA, allozyme).

The pairwise Φ_{ST} values for the WB courtship trial populations (one of which was the hybrid-ancestry *P. teyahalee* population) were very small when compared with those estimated for the two parental WB populations (weighted mean Φ_{ST}: 0.038 and 0.318, respectively). Judging from the disparity of estimates it is clear that the hybrid-ancestry *P. teyahalee* were not an appropriate courtship series substitute for parental-phenotype *P. teyahalee*. Presumably if two parental populations were employed in this trial series the estimated I_{PSI} would have been substantially higher. A lack of assortative mating between a *P. shermani* population and a population of hybrid ancestry was also seen by Reagan (1992) in her study of assortative mating between various *Plethodon glutinosus*-group populations. These observations fit the expectations of the hypothesis that where assortative mating is weak, gene flow is sufficiently pervasive to result in an erosion of the genetic distinctiveness of the contacting species.

Interestingly, the pairwise Φ_{ST} for the two WB *P. teyahalee* populations (parental and hybrid ancestry) was relatively high (weighted mean Φ_{ST}: 0.203). This could imply that gene flow has been asymmetrical, with a greater preponderance of genes traveling down the mountain than up. Additionally, this may be attributable to the local conditions of the sites. Though the animals collected from this hybrid ancestry population overwhelmingly conform to proximal populations of parental-phenotype *P. teyahalee* (despite a minor reduction in size and occasional traces of red on the legs), estimates of temperature across this transect reveal this locality to be much cooler than one would expect given its low elevation and south aspect, and the temperatures estimated for adjacent sites (Figure 3.3). This discrepancy can be explained by a close-proximity creek and a particularly dense *Rhododendron* understory. (A manipulation study conducted at the SI transect found *Rhododendron*-understory presence resulted in lower temperatures generally, and lower summer maximum and mean air temperatures specifically; Clinton 2003.)

Considering that TR and WB *P. shermani* belong to the same isolate (Wayah), it is

surprising that the pairwise Φ_{ST} estimates for the parental populations are as different as they are. This finding indicates hybrid zone dynamics can be transect specific and may vary between different transects involving the same metapopulations (in this case, isolates). One possible explanation for this observation is the *P. teyahalee* populations are divergent, leading to divergent interspecific genetic compatibilities; this is unlikely given the short distance and continuous habitat connecting these *P. teyahalee* populations. A more likely explanation is the abiotic differences between the two transects. The WB transect has a south aspect, and the parental *P. teyahalee* population occurs on a relatively dry, exposed ridge. The TR transect has an east aspect and is situated within a mesic canyon. These abiotic differences could result in TR having a relatively salubrious climate fostering interspecific contact and a wide hybrid zone, and WB having a steeper environmental gradient which has functioned to maintain some genetic distinctiveness.

SI TRANSECT

The highest degree of assortative mating was observed in the SI transect ($I_{PSI} = 0.727$). This is an exceptionally high index of sexual isolation value for a no-choice trial (no-choice trials generally find lower I_{PSI} values than observed in the various choice-trial designs; Coyne et al. 2005), and indicates other barriers to gene flow may be at play. For instance, Dillon et al. (2011) observed similar I_{PSI} values (0.37 to 0.71) in a no-choice-trial experiment between a freshwater snail species (*Physa gyrina*) and congeners, and no offspring were observed to have resulted from these pairings suggesting postzygotic isolation existed as well. The SI transect is the best studied zone of contact between these species, and it has been used to make conclusions about hybridization between these species generally (e.g., Hairston et al. 1992, Walls 2009). As I estimated an I_{PSI} value 50% higher than the next highest transect investigated (FC), the SI transect is likely not characteristic of *P. shermani-P. teyahalee* hybrid zones generally. One factor that may be influencing the SI transect is gene flow from *Plethodon chattahoochee*. The SI isolate is the only *P. shermani* isolate to contact this species, and at that contact zone they form a hybrid zone (Highton & Peabody 2000). This allospecific gene flow involving

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one species but not the other could exacerbate the fitness reduction associated with hybridization by introducing *P. chattahoochee* genes filtered for compatibility with the *P. shermani* genome but potentially incompatible with that of *P. teyahalee*. This gene flow could also be contributing to the high Φ_{ST} estimate for this transect, however, pairwise Φ_{ST} estimates for the parental WB populations are comparable with those of SI without the benefit of gene flow from a third species. Furthermore, inferences from a study of mtDNA indicated that cytoplasmic gene flow was from *P. shermani* to *P. chattahoochee*, not the other way around (Weisrock et al. 2005).

FC TRANSECT

Analysis of the FC trial series found high I_{PSI}. Additionally, both sexual selection and asymmetrical hybridization exist between the respective populations, centered on female *P. teyahalee*. Specifically, female *P. teyahalee* were preferred over female *P. shermani*, and successful courtship was more likely to occur in trials consisting of female *P. teyahalee* and male *P. shermani* than in the reciprocal interspecific pairing. If this pattern was real, I should expect to see maternally inherited mtDNA to have introgressed from *P. teyahalee* into *P. shermani* but not in the other direction, and a previous study indicates this is the case (Weisrock et al. 2005).

Analysis of the failed courtship data revealed the relative lack of success of female *P. shermani*/male *P. teyahalee* pairings was not due to female choice, as no courtship failures were observed in these trials. Instead, pairings of female *P. teyahalee* and male *P. shermani* resulted in disproportionately more courtship successes and failures, indicating male interest was behind this pattern. The success of this pairing is particularly fascinating, as it represents, on average, the greatest size differential of the four sex × species combinations: in all *P. glutinosus* group salamander species males are smaller than females, and *P. shermani* is smaller than *P. teyahalee*. On average, female *P. teyahalee* weigh more than twice as much as male *P. shermani* (Lowe, BT, unpublished data). The disproportionate number of successful courtships in this pairing suggests size differential is not contributing to reproductive isolation. This is counterintuitive considering the elaborate courtship maneuvers required for successful

insemination. Courtship in all plethodontid salamanders involves the male engaging the female in a tail-straddle walk where the male guides the female to a position where her cloaca is immediately above the spermatophore, and knowledge of the female's body length seems important for success. Further, a study of skink lineages that lack post-zygotic barriers to gene flow showed that minute differences in body length prevent successful courtship (Richmond & Jockusch 2007). Body size has been demonstrated to not directly affect courtship success in the plethodontid genus *Desmognathus* (though size had an indirect effect via male-male competition; Houck 1988). Unlike in Houck's study (1988), the female *P. teyahalee*/male *P. shermani* pairing resulted in an increased number of courtship failures (successful courtship but insemination failure in Houck [1988]) which may be a consequence of pair size differential.

The sexual selection and asymmetrical hybridization detected in the FC courtship series possibly indicate a mechanism for gene flow despite elevated I_{PSI}. *Plethodon* females are larger than males, and male mate choice and preference for larger females has been demonstrated in various *Plethodon* species (Marco et al. 1998) including *P. shermani* (Eddy et al. 2016). Perhaps male preference for larger females, adaptive when a species is not sympatric with a larger congener, is overriding male preference for conspecifics and leading them to engage in maladaptive courtship with these larger heterospecific females at secondary contact. A similar scenario has recently been discovered where divergent lineages of Spotted Salamander (*Ambystoma maculatum*) have come into contact; in this female-choice system, it seems females of both lineages prefer the larger males of one lineage (Johnson et al. 2015).

SM TRANSECT

Modest, statistically insignificant assortative mating was observed in the SM courtship series. This is not surprising given certain characteristics of this transect. Morphological differentiation is limited along this transect, with the *P. shermani* population frequently exhibiting white lateral spotting. This is in addition to lacking red legs, a characteristic of *P. shermani* of the Unicoi isolate. This population has been hypothesized to be a *P. shermani* population that been swamped by gene flow from *P. teyahalee* (Highton &

Henry 1970), and this proposition has been supported by an analysis of mtDNA haplotypes (Weisrock et al. 2005). All this is in stark contrast to the pairwise Φ_{ST} data based on nuclear markers, which indicate substantial differentiation across this transect. Interestingly, this pattern of nuclear genome distinctiveness with respect to *P. teyahalee* corroborates earlier analyses of allozyme data from these populations (Peabody 1978, Highton & Peabody 2000; discussed in Weisrock et al. 2005). Weisrock et al. (2005) propose the mtDNA introgression into Unicoi isolate *P. shermani* may be due to mitochondrial capture that took place during past range expansions followed by long periods of isolation. Under this scenario, and in light of the findings of this study, the current interspecific contact along the SM transect is of recent origin.

CONCLUSION

With the discovery of assortative mating between parental *Plethodon* populations occurring at opposite ends of hybrid zones, a mechanism serving to maintain species limits for these hybridizing salamanders has been identified. Future research should focus on the interactions between parental populations and adjacent populations of hybrid ancestry. My findings, based on one transect (WB), suggest assortative mating does not exist between such populations, however, this transect may not be characteristic of other hybrid zones. Furthermore, studies into the fitness of hybrid salamanders, both F1 hybrids and hybrid swarm individuals from within the hybrid zones, will shed more light on maintenance of these hybrid zones. Unfortunately, creating F1 *P. shermani-P. teyahalee* hybrids in the laboratory is not without its difficulties, not the least of which is the exceptional sperm storage abilities of these salamanders (Eddy 2012). That said, methods for inducing *P. glutinosus* group salamanders to oviposit fertilized eggs in the laboratory exist (Eddy 2012), and though growing these F1 offspring to reproductive age is not feasible, hatching success could be used as an estimate of fitness. Ideally, these experiments would be conducted at temperatures characteristic of respective parental populations and mid-transect populations; this would serve to discriminate between the scenarios of general hybrid depression (endogenous selection) and bounded hybrid superiority (exogenous selection).
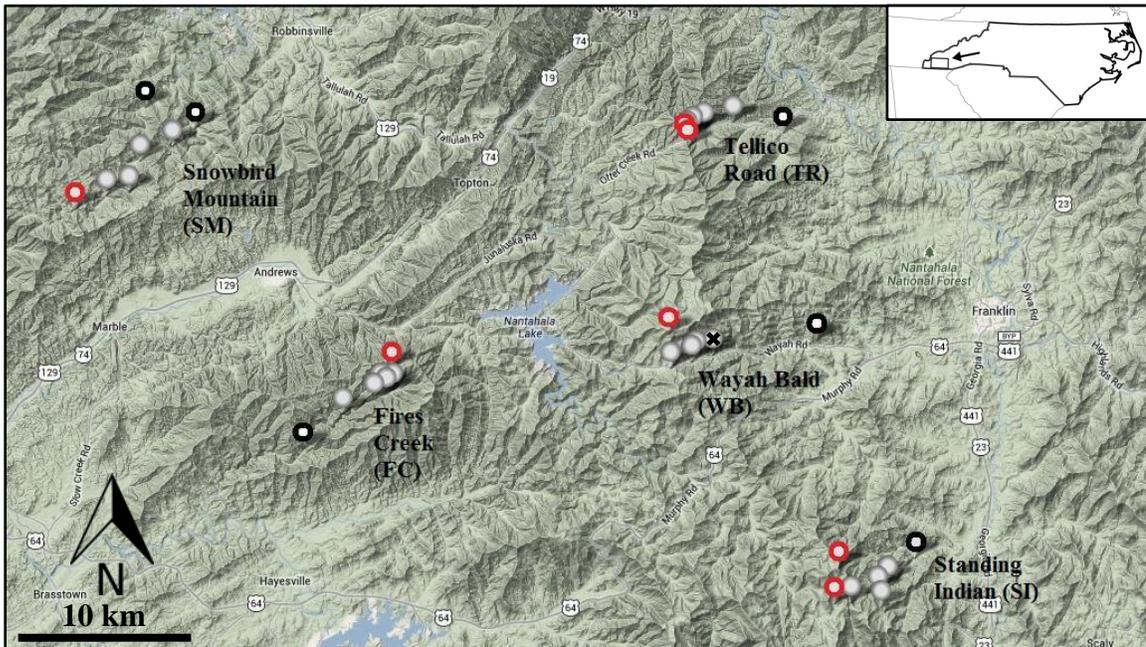
Figure 3.1. Transects investigated using courtship trials. Black-margined circles indicate parental *P. teyahalee* populations. Red-margined circles indicate parental *P. shermani* populations. Circle with black "×" along WB transect indicates *P. teyahalee* population of hybrid ancestry. Only southern SI parental *P. shermani* population represented in courtship trials. Background topographic map courtesy of Google Maps™. Inset map of North Carolina indicates extent of topographic map (rectangle indicated with arrow).
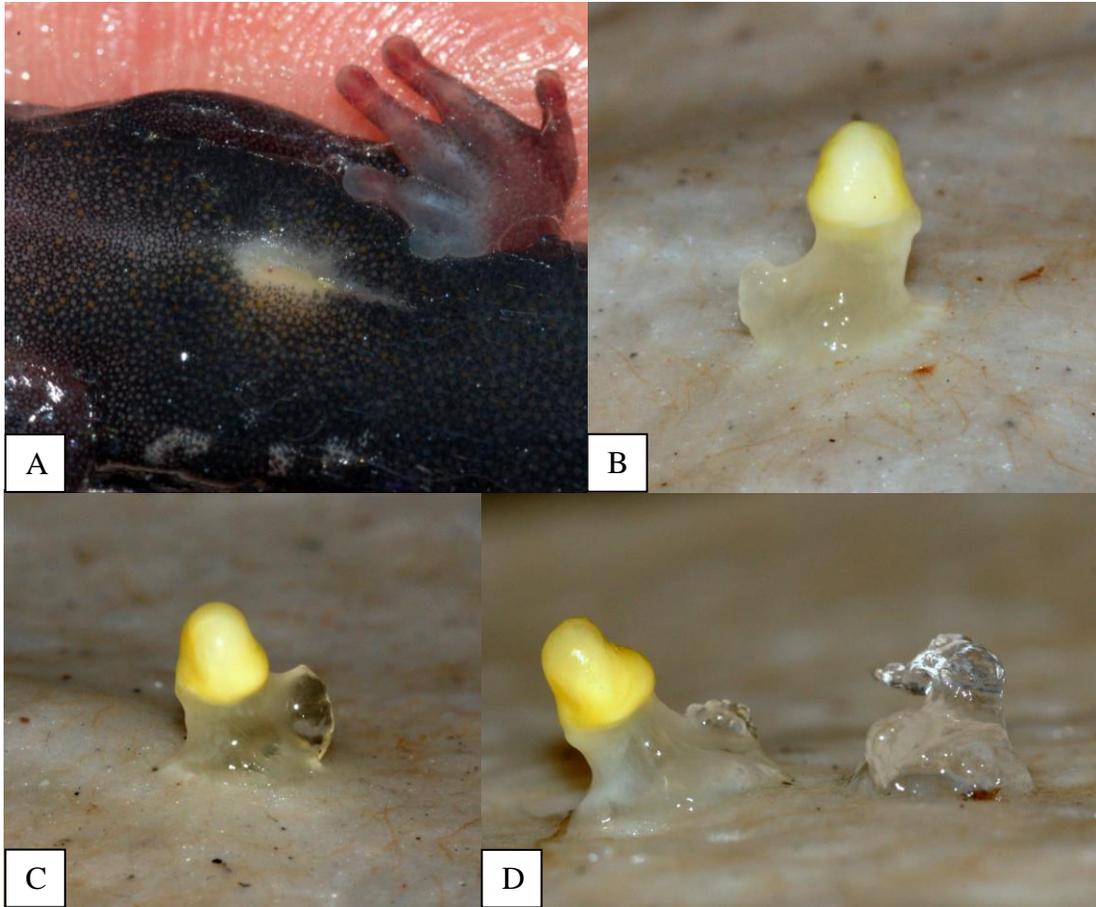
Figure 3.2. Spermatophores. A: female *Plethodon* with a spermatophore cap in cloaca. B: *Plethodon shermani* spermatophore. C: *Plethodon teyahalee* spermatophore. D: Intact spermatophore (left) and spermatophore base with missing spermatophore. These were deposited as shown on the same night by the same male.
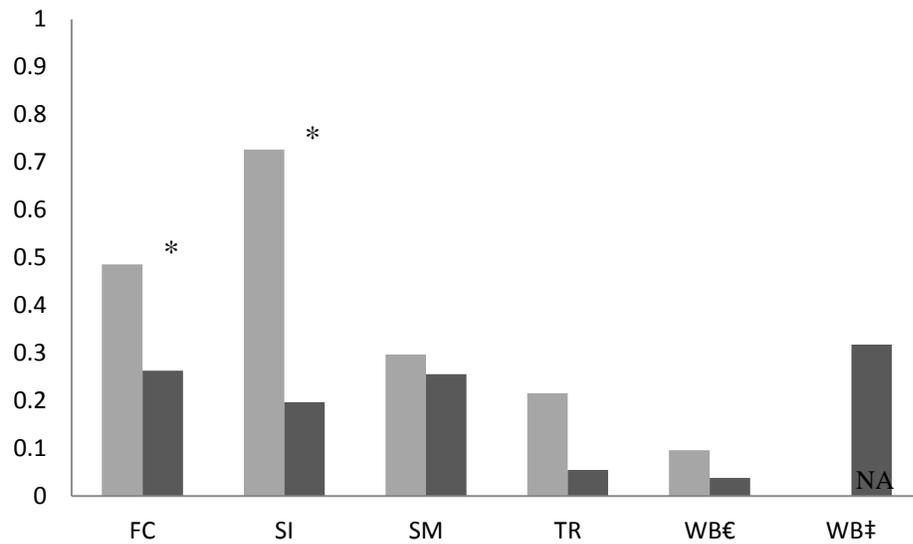
Figure 3.3. Estimates of assortative mating and genetic differentiation between transect population pairs. Light grey bars indicate I_{PSI} values. Dark grey bars indicate weighted mean Φ_{ST} values. * indicate statistically significant I_{PSI} values. € indicates WB population pair with hybrid ancestry *Plethodon teyahalee*. ‡ indicates WB population pair with parental *P. teyahalee*.
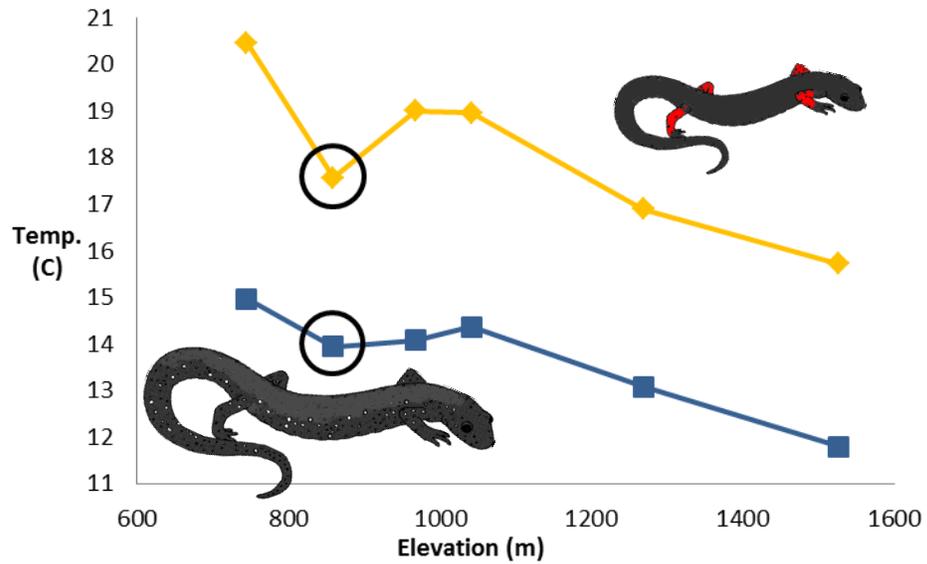
**Figure 3.4. Temperature across WB transect.** Presented to indicate disparate temperature regimes of first two transect sites (the first being the parental *P. teyahalee* population and the second being the hybrid ancestry population). Yellow line/symbols indicate mean daily high temperatures. Blue line/symbols indicate mean daily low temperatures. Black circles indicate the hybrid ancestry population. Salamander figures indicate the low (*P. teyahalee*) and high (*P. shermani*) ends of the transect (left and right, respectively).

Transect	*P. shermani*	*P. teyahalee*	Isolate
Fires Creek (FC)	35.15316, -83.75257	35.11242, -83.80682 35.12535, -83.79409	Tusquittee
Standing Indian (SI)	35.03437, -83.48063	35.05750, -83.43106	Standing Indian
Snowbird Mountain (SM)	35.23318, -83.94790	35.28431, -83.90480 35.27370, -83.87426	Unicoi
Tellico Road (TR)	35.26789, -83.57244 35.26451, -83.57056	35.27160, -83.51180 35.27409, -83.52036 35.26479, -83.51287 35.26258, -83.51169	Wayah
Wayah Bald (WB)	35.17068, -83.58260	35.15972, -83.55490*	Wayah

Table 3.1. Location of populations utilized in courtship trials. * indicates WB population of hybrid ancestry.

Transect	I_{PSI}	Mean Φ_{ST}	Weighted mean Φ_{ST}
FC	0.486*	0.179	0.263
SI	0.727*	0.215	0.197
SM	0.297	0.352	0.256
TR	0.216	0.083	0.055
WB €	0.096	0.038	0.038
WB ‡	NA	0.294	0.318

Table 3.2. Data for population pairs. I_{PSI} = index of pair sexual isolation. * indicates significant I_{PSI} value. € indicates WB population pair with hybrid ancestry *Plethodon teyahalee*. ‡ indicates WB population pair with parental phenotype *P. teyahalee*.

Marker	Best Model	Model Used in Φ_{ST} Estimation
C3	K81uf + I	HKY85, TrN*
Pglut10	TIM3 + I	TrN
Pglut19	TrNef + I	TrN
CXCR4	TPM2 + I	K80, TrN*
MC00	F81	F81
POMC	F81	F81
SLC8A3	JC	JC

Table 3.3. Marker-specific models of nucleotide substitution. Best models were identified with JMODELTEST. Φ_{ST} estimation models differ on account of limited models available in ARLEQUIN. * indicates model applied for determining effect of model selection but results of analyses not reported.

Transect	Anonymous Markers			Intron Markers			
	C3	Pglut10	Pglut19	CXCR4	MC00	POMC	SLC8A3
FC	0.293*		0.129		0.498*		-0.203
SI	-0.084	0.268*	0.205†	-0.265	0.283†	1†	0.096
SM	0.209*		0.894	0.196*	0.049	0.275	0.488*
TR	0.031		0.060	-0.081	0.219	-0.114	0.385
WB €	0.052		0.220†	0.090	0.111	-0.166	-0.081
WB ‡	0.663*		0.602†	-0.091	0.269	0.400	-0.081

Table 3.4. Marker-specific Φ_{ST} estimates between *Plethodon shermani* and *P. teyahalee* populations. € indicates WB population-pair including hybrid ancestry *P. teyahalee* population. ‡ indicates WB population-pair including parental *P. teyahale* population. * indicates statistically significant value ($P < 0.05$). † indicates near-significant value ($0.05 < P < 0.07$).

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