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Hybridization in the absence of an ecotone favors hybrid success in woodrats (*Neotoma* spp.)

Dylan M. Klure[1,*], **Robert Greenhalgh**[1], **Thomas L. Parchman**[2], **Marjorie D. Matocq**[3], **Lanie M. Galland**[2], **Michael D. Shapiro**[1], **M. Denise Dearing**[1]

[1]School of Biological Sciences, University of Utah, 257 S 1400 E rm 201, Salt Lake City, UT 84112.

[2]Department of Biology, University of Nevada Reno, 1664 N. Virginia Street, Reno, NV 89557.

[3]Department of Natural Resources and Environmental Science; Program in Ecology, Evolution and Conservation Biology, University of Nevada Reno, 1664 N. Virginia Street, Reno, NV 89557.

Abstract

Hybridization is a common process that has broadly impacted the evolution of multicellular eukaryotes; however, how ecological factors influence this process remains poorly understood. Here, we report the findings of a 3-year recapture study of the Bryant's woodrat (*Neotoma bryanti*) and desert woodrat (*N. lepida*), two species that hybridize within a creosote bush (*Larrea tridentata*) shrubland in Whitewater, CA, USA. We used a genotype-by-sequencing approach to characterize the ancestry distribution of individuals across this hybrid zone coupled with Cormack-Jolly-Seber modeling to describe demography. We identified a high frequency of hybridization at this site with ~40% of individuals possessing admixed ancestry, which is the result of multigenerational backcrossing and advanced hybrid-hybrid crossing. F_1, F_2 and advanced generation hybrids had apparent survival rates similar to parental *N. bryanti*, while parental and backcross *N. lepida* had lower apparent survival rates and were far less abundant. Compared to bimodal hybrid zones where hybrids are often rare and selected against, we find that hybrids at Whitewater are common and have comparable survival to the dominant parental species, *N. bryanti*. The frequency of hybridization at Whitewater is therefore likely limited by the abundance of the less common parental species, *N. lepida*, rather than selection against hybrids.

Keywords

Ancestry; apparent survival; genotyping-by-sequencing; hybridization; mark and release; *Neotoma*

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[*]=corresponding author (dylan.m.klure@gmail.com).

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Introduction

Hybridization is a consequential process that impacts evolution in a diversity of ways. For example, hybridization can introduce reticulated patterns of evolution (Larsen et al. 2010), and lead to a variety of detrimental effects such as outbreeding depression (Beauclerc et al. 2013), genetic swamping (Colella et al. 2018), and the accumulation of deleterious variation (Pfennig 2021). However, introgressive hybridization can also introduce novel genetic material for parental species, increasing genetic variation and potentially contributing to adaptive evolution (Hamilton and Miller 2016; Jones et al. 2018). Although hybridization is understood to be a common process that has shaped the diversification of plants (Whitney et al. 2010), a historical perception of its rarity has led to an underappreciation of its role in animal evolution (Mayr 1963). However, with the advancement of next-generation sequencing technologies, hybridization has been increasingly documented across Animalia (Lancaster et al. 2007; Gligor et al. 2009; Rheindt and Edwards 2011; Pereira et al. 2013; Cahill et al. 2015; Jones et al. 2018). The study of natural animal hybrid zones therefore presents a valuable opportunity to identify the evolutionary mechanisms underlying reproductive isolation, speciation and the acquisition of adaptive variation (Abbott et al. 2016). Additionally, hybrid zones often occur across intermediate environments (*i.e.*, ecotones) and variation in introgression can reflect the genetic architecture of postzygotic isolation at these sites (Gompert et al. 2017).

Despite the apparent influence of hybridization on evolution, we understand little about the factors that regulate this process. In animals, hybridization is limited by several forms of reproductive isolation, including intrinsic barriers such as prezygotic hybrid inviability or postzygotic hybrid sterility (Coughlan and Matute 2020), and extrinsic barriers such as the selection against fertile hybrids mediated by local ecological conditions (Vamosi et al. 2000). Research on intrinsic barriers to interspecific gene flow has improved our understanding of the mechanisms by which reproductive isolation evolves, such as through genomic imprinting (Brekke et al. 2016), interchromosomal conflicts (Davis et al. 2015), and elevated rates of gene regulatory evolution (Fitzpatrick 2004). However, as most research on hybridization has focused on intrinsic barriers to interspecific gene flow (Vamosi et al. 2000; Moran et al. 2021), our understanding of how extrinsic ecological barriers influence variation in hybrid ancestry remains relatively poor. To address this deficiency, we performed a survey of a woodrat (*Neotoma* spp.) hybrid zone to identify the role of ecological selection in regulating interspecific hybridization in this system.

Several *Neotoma* species hybridize along narrow secondary contact zones across multiple habitat types (Patton et al. 2007; Shurtliff et al. 2014; Hunter et al. 2017). This makes woodrats valuable for identifying patterns of ancestry variation associated with local environmental conditions. Two species in particular have been the focus of several studies on interspecific hybridization, the Bryant's woodrat (*N. bryanti* Merriam) and the desert woodrat (*N. lepida* Thomas; Shurtliff et al. 2013, 2014; Jahner et al. 2021; Nielsen and Matocq 2021). One of the hybrid zones is along a secondary contact zone in Kern County, CA, USA (lat/long: 35° 42' N; 118° 25' W), hereafter referred to as "Whitney Well". Whitney Well occurs across a strong ecotone where the Sierra Nevada foothills descend to the valley floor, creating an ecological transition from hilly-mesic to desert scrub habitat.

The ecotone at this site has been hypothesized to act as a barrier to interspecific geneflow as it facilitates strong niche partitioning in both habitat use and diet between parental individuals (Shurtliff et al. 2014; Nielsen and Matocq 2021), limits interspecific mating opportunities and selects against juvenile hybrids due to a lack of transitionary habitat (Shurtliff et al. 2014). Thus, the strong environmental gradient present at Whitney Well may create a strong selective gradient that influences the rate of interspecific hybridization. However, it is also possible that underlying genetic incompatibilities are influencing hybridization rates (Shurtliff et al. 2014; Jahner et al. 2021), and disentangling the role of these potential extrinsic and intrinsic barriers to interspecific gene flow is difficult when both may be at play. For this reason, we studied a second hybrid zone between *N. bryanti* and *N. lepida*, hereafter referred to as "Whitewater", which lacks an obvious ecological gradient, to ask whether environmental selectivity or genomic incompatibility is the main barrier to interspecific hybridization in these two species of woodrats.

Whitewater (Riverside County, CA, USA, lat/long: 33° 55' N; 116° 38' W), consists of hundreds of square kilometers of desert shrublands dominated by the highly toxic shrub creosote bush (*Larrea tridentata*), and lacks an obvious ecological gradient (*i.e.*, there is no elevation or vegetation gradient) in the areas where *N. bryanti* and *N. lepida* hybridize (Figure 1). Both woodrat species at this site have similar diets (Weinstein et al. 2021) consisting of largely creosote bush and rhatany (*Krameria* spp.). Based on the apparent overlap of habitat use and foraging behavior of *N. bryanti* and *N. lepida* at Whitewater, it is unclear what factors limit interspecific gene flow at this site and whether there is selection against hybrids in this relatively homogenous habitat.

Here, we report findings from a 3-year mark and recapture study that addresses the potential for ecological barriers and genomic incompatibilities to regulate the frequency of interspecific gene flow between *N. bryanti* and *N. lepida*. We used genome-wide analyses based on reduced representation sequencing to estimate woodrat ancestry and characterize population structure. In addition, we used demographic modeling to estimate apparent survival rates and recapture probabilities for woodrats of different ancestries. Specifically, we aimed to: i) characterize the spatial distribution of various ancestry classes, ii) determine the frequency and temporal variation of hybridization, iii) identify apparent survival patterns for woodrats of different ancestry classes, and iv) contrast the hybridization dynamics of the Whitewater and Whitney Well hybrid zones to further identify how intrinsic and extrinsic barriers might regulate interspecific hybridization between these two species.

Methods and Materials

Woodrat sampling

Woodrat sampling took place over three years within the creosote bush shrublands of the Whitewater hybrid zone located in the San Gorgonio Pass, Riverside County, CA, USA (latitude/longitude: 33° 55' N; 116° 38'), with a total of nine sampling events during the following months: January 2019, March 2019, May 2019, July 2019, November 2019, March 2020, July 2020, November 2020, and November 2021. Sherman live-traps (H. B. Sherman Traps, Inc., Tallahassee, FL) were baited with oats, opened at dusk near woodrat stick nests concentrated within the thickets of creosote bush, and checked at dawn. Locations where woodrats were captured were recorded by GPS (eTrex 10; Garmin, Olathe, KS). Woodrats were weighed, sexed and a small 2–3 mm piece of ear tissue was collected. Tissue samples were stored in a liquid nitrogen dewar while in the field and stored at −80°C in the lab prior to processing. Each woodrat was given an ear tag with a unique identifier and was subsequently released at their capture location so that individuals could be followed over time. The same transects and trapping effort were repeated for each trap event (four trap nights per trap site; 332 trap nights per event) for a total of 2,988 trap nights. In total, we sampled 203 unique individuals across 437 total captures. The ancestry coefficient and capture location of each woodrat across all captures is shown in Figure 2A. Woodrat trapping and handling was permitted by the California Department of Fish and Wildlife Scientific Collection Permits SC-008123 and SC-1379S, and University of Utah Institutional Animal Use and Care Committee protocol 19–01005. All animal handling protocols followed the guidelines set by the American Society of Mammologists (Sikes 2016).

Reduced-representation genomic library preparation and sequencing

To generate reduced representation sequencing libraries, genomic DNA from ear tissue was extracted from 176 individual Whitewater woodrats of unknown ancestry using the Qiagen DNeasy Blood and Tissue Kit (Qiagen; Germantown, MD). We estimated the concentration of extracted DNA using a Qubit 4 Fluorometer (Invitrogen; Waltham, MA) with the dsDNA High Sensitivity Assay Kit. We generated genotyping-by-sequencing libraries using two restriction enzymes (essentially a ddRADseq approach; Peterson et al. 2012) as described in Parchman et al. (2012). In brief, the restriction enzymes *EcoR1* and *Mse1* (NEB; Ipswich, MA) were used to digest genomic DNA and modified Illumina adapters were ligated to the *EcoR1* and *Mse1* cut sites with the addition of a 10 bp DNA barcode unique to each individual on the *EcoR1* end of the DNA fragments. We then pooled the barcoded restriction-ligation products from all individuals and conducted PCR amplification with Illumina primers and a high-fidelity proofreading polymerase (Iproof, BioRad; Hercules, CA). Thermocycler conditions consisted of 30 PCR cycles (84°C for 20s, 60°C for 30s, 72°C for 2 min) followed by a final cycle of 98°C for 3 min, 60°C for 2 min, and 72°C for 10 min. We size selected PCR products for DNA fragments between 350–425 bp in length using a Pippin Prep unit (Sage Science, Beverly, MA), and performed quality screening with a BioAnalyzer device (Agilent; Santa Clara, CA). The library pool was then sequenced on a single lane of an Illumina NovaSeq 6000 with S2 chemistry to produce 100 bp single-end reads at the University of Texas Genomic Sequencing and Analysis Facility.

Read processing and variant calling

We removed known contaminants from the raw read data such as PhiX and *Escherichia coli*, as well as sequences matching Illumina oligos using `bowtie2_db` v.2.3.0 (Langmead & Salzberg, 2012). Custom `Perl` scripts were used to parse reads to individual FASTQ files (scripts are available at https://datadryad.org/stash/dataset/doi:10.5061/dryad.wstqjq2kd). Reads were aligned to the chromosome-level *N. bryanti* reference assembly (Greenhalgh et al. 2022) using the default settings of the *mem* algorithm of `bwa` v.0.7.17 (Li and Durbin 2009) to generate sequence alignment map files, which were then converted to binary alignment format with `SAMtools` v.1.15 (Li et al. 2009). Reference-guided read mapping did not include the mitochondrial genome. We called single nucleotide polymorphisms (SNPs) and estimated genotype likelihoods using `SAMtools` and `BCFtools` v.1.15 (Li and Barrett 2011), requiring a minimum base site quality score of 20, a mapping quality score of 20, and a genotype quality score of 10 or greater. We used `VCFtools` v.0.1.16 (Danecek et al. 2011) to filter SNP calls prior to downstream analyses. We filtered out loci that were not covered by reads in at least 70% of individuals, then removed individuals with more than 50% missing data (as an abundance of missing data indicates a low-quality library). To generate the final genotype matrix, we retained only autosomal and bi-allelic SNPs with reads present in at least 90% of individuals and with a minor allele frequency (maf) > 0.05, and removed any sites with depth greater than two times or less than half the mean depth for all sites.

Ancestry estimation

We used genotype likelihoods to estimate ancestry coefficients (q) for each individual using the hierarchical Bayesian modeling software `entropy` v.2.0 (Gompert et al. 2014; Shastry et al. 2021). `entropy`, which assumes that individual genomes consist of loci with ancestry from k ancestral clusters, is particularly appropriate for reduced representation sequencing datasets as it incorporates genotype uncertainty into estimates of genotype probabilities and ancestry coefficients (Gompert et al. 2014). To aid Markov Chain Monte Carlo (MCMC) convergence, we initialized individual ancestry coefficients in chains using the cluster membership assignments from k-means clustering of principal components using genotype likelihoods in a linear discriminant analysis with the package `MASS` v.7.3–55 (Venables and Ripley 2002) running in the `R` v.4.1.2 (R Core Team 2021) environment. This approach aids in MCMC convergence but does not inhibit posterior sampling. Input files were prepared for entropy using `vcfR` v.12.0 (Knaus and Grünwald 2017). As we sampled individuals representing parentals and hybrids from a single site, we ran entropy (q model) for $K = 2$ for 50,000 MCMC steps following a burn-in period of 10,000 steps and sampled every tenth step. We assessed chain convergence by plotting the inferred parameter estimate over the runtime of each chain and deemed convergence to be satisfactory if the chain demonstrated good mixing and reached a stationary distribution. Trace plots for the `entropy` q model are available in Figure S1. To further classify hybrid individuals, we used the ancestry complement model in `entropy` to estimate interspecific ancestry coefficients (Q_{12}). This information is valuable for delineating between F_1 and F_2 individuals as well as hybrids produced through additional generations of hybrid crosses (Gompert and Buerkle 2016; Shastry et al. 2021). Parental individuals have a Q_{12} of 0 as they lack loci heterozygous for alleles from an interspecific source, F_1 individuals have a Q_{12} of 1 as each of their parents contributed different ancestries, and F_2 individuals have a Q_{12} of ~0.5, as their genomes consist of approximately equal parts intra- and interspecific ancestry due to recombination (Shastry et al. 2021). Backcross individuals have intermediate Q_{12} values between parental and F_1 individuals, and can be further distinguished from other hybrid groups as they have q values < 0.5 or > 0.5 (Shastry et al. 2021). Estimates of q and Q_{12} were used to characterize the ancestry classes and ancestry distribution of woodrats at Whitewater. We executed `entropy` (complement model) for 50,000 MCMC steps following a burn-in period of 10,000 steps and sampled every tenth step.

Estimating the genetic differentiation between the Whitewater and Whitney Well hybrid zones

To assess population structure between parental individuals from each hybrid zone, we aligned the entirety of the Whitney Well (n = 383; https://doi.org/10.5061/dryad.bnzs7h4bd; Jahner et al. 2021) and Whitewater sequence datasets (n = 154) in tandem to generate a database containing shared SNPs. Read mapping, variant calling and variant filtering was performed as previously described. Genetic differentiation between parental *N. bryanti* and *N. lepida* within and across sites was estimated by calculating the mean pairwise fixation index (F_{ST}) with `VCFtools` v.0.1.16 (Danecek et al. 2011) using the Weir and Cockerham method (Weir and Cockerham 1984). We used the maximum likelihood framework in `ADMIXTURE` v. 1.3.0 (Alexander et al. 2009) to identify the most likely number of ancestral populations for the Whitewater, Whitney Well and combined datasets. We employed `ADMIXTURE` iteratively from K = 1 to K = 6 with 1,000 bootstrap replicates to obtain estimates of model precision and identified which K value had the highest model support using a 10-fold cross-validation approach.

Mark and recapture analyses

We constructed capture histories for each unique individual for each sampling trip by assigning a “0” to indicate an individual was not recaptured, a “1” to indicate the recapture of an individual, and “2” to indicate an individual was recaptured but died before release (this last event occurred only once). We used the capture histories of each individual and the open-population Cormack-Jolly-Seber (CJS) modeling approach (Lebreton et al. 1992) to estimate apparent survival (ϕ) and recapture probability (p) for *N. bryanti*, *N. lepida* and hybrids. Apparent survival differs from true survival in that it is fitted with the probability that an individual may have migrated out of the study site (Sandercock 2006). Using `marked` v.1.2.6 (Laake et al. 2013), we constructed 16 candidate time-dependent and open-population CJS models (including null models) to assess the relationship between ancestry (q) and sex on estimates of apparent survival and recapture probability (Table S1). We assessed candidate model support using Akaike Information Criterion model selection and proceeded with the model with the strongest support (Table S1). As this model did not include an interaction term between ancestry and sex, we did not estimate the apparent survival separately for males and females of each genotype.

We computed apparent survival and recapture probability over time with an open-population CJS model in a Bayesian MCMC framework with 10,000 burn-ins across 100,000 iterations to obtain estimates of model precision. Woodrats were binned by their genotype (determined from q) into one of three groupings: parental and backcross *N. lepida* ($q < 0.4$), parental and backcross *N. bryanti* ($q > 0.6$), and hybrids ($0.4 \leq q \leq 0.6$) for comparisons of apparent survival and capture probability. Parental and backcross individuals were binned for this analysis as backcross individuals in woodrats have previously been found to occupy similar habitat and display similar foraging behavior to the parental species with which they share a majority of their ancestry (Shurtliff et al. 2014; Matocq et al. 2020). Thus, we assumed the phenotype of backcross individuals likely mirrored their more genetically similar parental species. However, hybrids with various hybridization histories are certainly not homogenous in their ecology and the way in which they are grouped may influence model results. Therefore, we constructed an alternative model with animals grouped by q estimates from 0 to 1 with bin widths of 0.2 to assess for any influence of ancestry bins on model estimates. We corrected the models to reflect the actual temporal spacing between sampling events and did not include juvenile individuals or individuals lacking genotype assignments. We also did not include trapping effort as a covariate in the models as this was held constant across sampling trips. Additionally, we did not include capture histories from recaptured individuals in the CJS models from the sampling events in 2021 as newly caught individuals from the previous sampling event were not genotyped. In total, 141 adult individuals with known ancestry and sex were used in the CJS models. We determined differences in apparent survival and recapture probability across groups to be significant if the model estimates had non-overlapping 95% confidence intervals, as is routine practice for similar demographic models (Wood et al. 2018; Schleimer et al. 2019; Strinella et al. 2020).

Microhabitat use and dispersal analyses

We used the `R` package `Kriging` v.1.1 (Olmedo 2014) to estimate fine-scale habitat use of woodrats with different ancestries. Spatial interpolation modeling was used to predict the areas of occurrence of different ancestry coefficients (q) at our study site. We fitted a semivariogram model to our spatial data with the spherical algorithm of `gstat` v.2.0–9 (Pebesma and Wesseling 1998). An interpolated map of ancestry coefficients was then generated using the Ordinary Kriging algorithm to produce a prediction of responses (q) for each location using the weighted average of the nearest neighbors. To characterize within-site dispersal, we calculated the pairwise distance traveled in meters for each individual across sampling events using the latitude and longitude coordinates for each capture with the R package `geodist` v.0.07 (https://hypertidy.github.io/geodist/).

Results

DNA sequencing

The genotype-by-sequencing libraries generated 295,285,492 raw reads across 176 individuals. After quality filtering, we retained 59,721 bi-allelic SNPs with a mean site coverage per individual of 8.82 ± 4.71 (standard deviation) in our final genotype matrix. We retained 154 individuals after the removal of 22 individuals with a high degree of missing data. This genotype matrix was used for all downstream analyses.

Ancestry distribution

The estimated ancestry composition of Whitewater during the sampling periods covered in this study consisted of parental *N. bryanti* (n = 73; $q \geq 0.95$), parental *N. lepida* (n = 3; $q \leq 0.05$), backcross (BC)-*bryanti* (n = 41; q = 0.6–0.9), BC-*lepida* (n = 9; q = 0.1–0.4), F_1 hybrids (n = 3; $0.4 \leq q \leq 0.6$, $Q_{12} > 0.90$), and F_2 hybrids (n = 6; $0.4 \leq q \leq 0.6$, $0.4 \leq Q_{12} \leq 0.6$). The remaining individuals could not be confidently assigned to early generation hybrid classes and are likely the product of advanced hybrid crosses (n = 19). These results are consistent with the presence of multigenerational backcrossing and hybrid-hybrid mating (Figure 2: B, D), and stand in contrast to the Whitney Well hybrid zone which demonstrates little evidence for advance generation hybrids (Figure 2: C, E). We expected parental *N. bryanti* at Whitewater to be ~3× more common than parental *N. lepida* based on recent sampling efforts at this site (Dearing et al. 2022). However, we found parental *N. lepida* to be exceedingly rare, representing only 2% of the total individuals sampled (Figure 2B). We were initially concerned that the low number of parental *N. lepida* in the original dataset may have biased inference of ancestry coefficients, especially those of admixed individuals. For this reason, we repeated the `entropy` q modeling with the addition of 10 parental *N. lepida* from Whitney Well that had the highest shared read coverage with the Whitewater libraries (Jahner et al. 2021). We found that the inferred ancestry estimates between the two `entropy` q models were highly similar and that the inclusion of additional parental *N. lepida* did not meaningfully change the ancestry estimates for admixed individuals (Figure S2). In particular, individual estimates for q and Q_{12} only varied on average by 0.03 ± 0.03 (standard deviation) and 0.02 ± 0.03, respectively. Based on these results, we interpret the original results to reflect an accurate characterization of the ancestry classes present at Whitewater.

Population structure and differentiation between Whitewater and Whitney Well

Due to limited overlap in read coverage between the Whitewater and Whitney Well libraries, the combined and filtered dataset consisted of only 4,286 SNPs. Using this dataset, we performed a principal components analysis which identified evidence for population structure between the parental *N. bryanti* at Whitewater and Whitney Well, but little population structure between the parental *N. lepida* from each site (Figure S3). The imputed pairwise fixation indices (F_{st}) between parental individuals from within and between each hybrid zone are summarized in Table S2. We found little differentiation between Whitewater and Whitney Well *N. lepida* ($F_{st} = 0.060$) but elevated differentiation between parental *N. bryanti* from each site ($F_{st} = 0.249$). Parental species at Whitney Well ($F_{st} = 0.362$) were more differentiated than at Whitewater ($F_{st} = 0.291$). As anticipated, the most supported number of ancestral populations (K) for the Whitewater and Whitney Well populations when analyzed separately was 2, consistent with two species occurring in sympatry with interspecific hybridization (Figure S4). For the combined dataset, the most supported K was unclear with $K = 3$ to $K = 5$ demonstrating similar model support (Figure S4). The cross-validation estimates of the combined dataset are likely a factor of the substantial population structure between parental *N. bryanti* at each site. We used this SNP dataset with only the Whitney Well individuals to repeat the `entropy` q and complement models as previously described to summarize the ancestry distribution of this site as shown in Figure 2. These results were nearly identical to the original characterization of ancestry classes at Whitney Well (Jahner et al. 2021), despite the use of a substantially smaller SNP dataset.

Apparent survival rates, recapture probabilities, and body mass estimates

Parental and backcross *N. bryanti* ($q > 0.6$) and hybrids ($0.4 \leq q \leq 0.6$) had higher mean 60-day apparent survival estimates of $\phi = 0.80 \pm 0.07$ (standard error of the mean) and $\phi = 0.75 \pm 0.10$ respectively, compared to parental and backcross *N. lepida* ($q < 0.4$; at $\phi = 0.62 \pm 0.15$ (Figure 3A). The period with the lowest 60-day apparent survival rates across all ancestry groups was between July 2020 and November 2020, with mean apparent survival rates for each ancestry group as follows: parental and backcross *N. bryanti* $\phi = 0.67 \pm 0.05$, hybrids $\phi = 0.60 \pm 0.09$, and parental and backcross *N. lepida* $\phi = 0.43 \pm 0.12$ (Figure 3A). Mean 60-day apparent survival rates for each ancestry class were extrapolated into the following annual survival rates: ~25% for parental and backcross *N. bryanti*, ~20% for hybrids, and ~6% for parental and backcross *N. lepida*. Female woodrats had marginally lower but not significantly different recapture probabilities across all sampling dates compared to males (Figure S5). Apparent survival rates imputed from the model with alternative ancestry bins demonstrated similar results to the original model (Figure S6). Parental *N. lepida* individuals were more common during trapping events that occurred in the breeding season and constituted a higher proportion of the total animals surveyed for these trips: 12% for January 2019, 15% for March 2019, and 6% for March 2020, compared to less than 3% for any trapping event outside the breeding season (typically between January to April of each year).

Additionally, we evaluated the adult body mass of individuals with different ancestry classes, as a larger body mass in woodrats is often correlated with dominance in sympatric populations (Dial 1988). We used the maximum recorded body mass for each individual and excluded individuals for which only juvenile weights were recorded (< 90 g). After confirming homoscedasticity between comparison groups with a Levene's test, we used a two-way ANOVA to explore the effects of genotype and sex using the same ancestry groups as the apparent survival analyses and performed post hoc analysis following the Tukey Honest Significance Difference procedure. We found both a significant effect of sex ($F_{1:129} = 40.735$; $q < 0.001$) and a significant interaction effect between genotype and sex ($F_{2:129} = 4.617$; $q = 0.012$) on mean body mass (Figure 3B). We found that parental and backcross *N. bryanti* males ($n = 48$) were significantly larger than parental and backcross *N. lepida* males ($n = 7$) with mean body masses of 183g and 147g, respectively (Figure 3B). We also found evidence for sexual dimorphism in body size between parental and backcross *N. bryanti* males and females, but not for parental and backcross *N. lepida* (Figure 3B). This overlap in mean body mass between male and female parental and backcross *N. lepida* is likely a factor of the small sample size of these groups and due to the fact that a third of the measurements occurred in the breeding season, indicating some female individuals may have been pregnant.

Microhabitat use by ancestry class

We did not identify any strong signals of spatial autocorrelation between woodrats with similar ancestry coefficients at Whitewater (Figure 2A; Figure S7). The predicted occurrence of ancestry coefficients seems to be largely stochastic without the obvious spatial autocorrelation that is typical of bimodal hybrid zones (Figure S7). Additionally, any mild signals of spatial autocorrelation among parental *N. bryanti* are likely a factor of their overall higher abundance at Whitewater compared to parental *N. lepida*. The occurrence of hybrid individuals throughout the sampling site further suggests there is no spatial structure of ancestry classes across the hybrid zone. Within-site dispersal was low for woodrats of all ancestry classes and did not substantially differ based on sex. For parental *N. bryanti* ($q \geq 0.95$) that were recaptured at least once, 40.0% (14/35) of males and 46.7% (14/30) of females had moved from their previous capture locations and traveled a mean distance of 103 and 128 meters, respectively. For hybrids ($0.05 < q < 0.95$), 34.6% (18/50) of males and 40.5% (17/42) of females had moved from their previous capture locations and traveled on average 43 and 34 meters, respectively. We were unable to assess the within-site dispersal patterns of parental *N. lepida* due to a low sample size and recapture rate. The largest within-site dispersal recorded was by a female *N. bryanti* who traveled 733 meters between captures in November 2020 and November 2021. The greatest distance of within-site dispersal we could measure was ~1.05 km, based on the distance between the farthest pair of trapping locations used in our sampling efforts.

Discussion

The role of ecological selection and genomic incompatibility in regulating the frequency of interspecific hybridization among mammals is poorly understood. Most mammalian hybrid zones occur along ecotones, which are areas with sharp ecological transitions (Gava and de Freitas 2002; Gligor et al. 2009; Shurtliff et al. 2014; Haines et al. 2019). Ecotones are thought to facilitate the differential local adaptation of parental forms to distinct habitat types, restrict interspecific mating opportunities, and select against hybrid individuals in the absence of sufficient transitionary habitat (Anderson 1948; Shurtliff et al. 2014; Tarroso et al. 2014; Haines et al. 2019). However, assessing the relative roles of ecological selection and genetic incompatibilities is difficult when both may be present. In the absence of an ecotone at Whitewater, we found substantial evidence for interspecific hybridization between *N. bryanti* and *N. lepida*, including advanced-generation hybrids, multigenerational backcrossing, a stable hybrid population and no evidence for habitat segregation between ancestry classes. We discuss these findings in comparison to another woodrat hybrid zone, and their implications in greater detail below.

Interspecific hybridization is frequent at Whitewater

The frequency of hybridization at Whitewater is high, with ~40% of the individuals surveyed possessing admixed ancestry. This is ~2.5× greater than the frequency of hybridization documented in woodrats at the Whitney Well site (Shurtliff et al. 2014), and elsewhere (Coyner et al. 2015). Advanced hybrid generations at Whitewater with a substantial backcrossing bias towards *N. bryanti* were present; ~30% of individuals with admixed ancestry lacked at least one unadmixed parent, and instead were likely the result of advanced hybrid crosses (*e.g.*, $BC_n \times F_n$ hybrid, $BC_n \times BC_n$). This result is in contrast to the population at Whitney Well where most hybrid individuals were the offspring of early generational parent-parent or parent-hybrid crosses (Shurtliff et al. 2014; Jahner et al. 2021). In addition, we also identified several advanced generation hybrids including a handful of potential F_2s at Whitewater, indicating that hybridization at this site is largely regulated by ecological factors such as competition for nesting sites, mating opportunities and dietary resources, rather than intrinsic barriers (*e.g.*, underlying genomic incompatibilities). In particular, we find that there is complete overlap in habitat use and diet (Weinstein et al. 2021; Dearing et al. 2022) between parental *N. bryanti* and *N. lepida* at Whitewater; thus, interspecific competition may play a substantial role in regulating the frequency and outcomes of hybridization at this site. The lack of clear reproductive barriers between *N. bryanti* and *N. lepida* may be a consequence of their relatively recent divergence of ~1.5 mya (Patton et al. 2007). However, other possibilities include a lack of sufficient niche divergence to facilitate divergent selection (Nosil et al. 2009), or interspecific geneflow is sufficient between these species to preclude reproductive isolation.

Based on the limited number of genetic surveys of the woodrat population at Whitewater, it is possible that the frequency of hybridization may vary temporally. The first genetic survey at Whitewater occurred in 2004, and although limited in scope, found only *N. bryanti* with no evidence for the presence of hybrids or *N. lepida* (n = 6; Patton et al. 2007). Whitewater was next genetically sampled between 2017 to 2018 and the ancestry composition of the woodrat population during these periods consisted of 50% *N. bryanti*, 19% *N. lepida*, and 31% hybrid individuals (n = 36; Dearing et al. 2022). Based on these previous surveys and the results of this study, it is possible that the ancestry composition of Whitewater may fluctuate over time, and in particular, the abundance of parental *N. lepida*, which may influence interspecific hybridization outcomes. The long-term temporal dynamics of interspecific hybridization at this site remain unclear, including whether there are periods where hybridization is absent. For this reason, we are continuing annual genetic sampling of this site to identify any temporal trends in hybridization frequency and ancestry class composition.

Apparent survival varies by ancestry class without evidence for genotype-specific habitat segregation

We found evidence for reduced site persistence in individuals with ancestry coefficients biased towards *N. lepida*. Parental and backcross *N. lepida* had substantially lower apparent survival rates across all sampling periods with an estimated annual survival rate of ~6%, compared to hybrid individuals and parental and backcross *N. bryanti* of ~20 and ~25%, respectively. The annual survival rates of Whitewater parental and backcross *N. bryanti* and hybrids were similar to the annual survival rates of parental *N. bryanti* and *N. lepida* at Whitney Well (Shurtliff et al. 2014). The lower apparent survival of parental and backcross *N. lepida* suggest that there could be environmental selection against *N. lepida* genotypes at Whitewater or that *N. lepida* is being competitively excluded by *N. bryanti*. As *N. lepida* has a greater evolutionary experience with creosote bush shrublands and is better able to exploit creosote bush as a food resource than *N. bryanti* (Dearing et al. 2022), we believe that the latter hypothesis is more likely. Although hybrid individuals had marginally lower apparent survival rates than parental and backcross *N. bryanti*, their temporal survival is sufficient that a large proportion of the hybrid population is able to reach reproductive maturity, typically between 6 to 9 months of age (Matocq 2004), which may further explain the presence of advanced hybrid crosses and multigenerational backcrossing at Whitewater.

The high frequency of hybridization despite the rarity of parental *N. lepida* is surprising, and may be a consequence of the seasonality of breeding at this site. *Neotoma lepida* may be dispersing from surrounding areas into Whitewater, primarily during the breeding season (late January through April). They might successfully mate with *N. bryanti* and hybrid individuals, but otherwise not survive in appreciable numbers outside of this period. This hypothesis is supported by our findings that parental *N. lepida* constituted a higher proportion of the total animals captured during sampling events that occurred within the breeding season. This short breeding season at Whitewater likely restricts females to 1–2 litters per year and coincides with the arrival of ephemeral plants that may reduce interspecific competition for food, potentially supporting more parental *N. lepida*. It is also possible we had limited power to accurately census parental *N. lepida* due to their low apparent survival rates coupled with the 60–120 day spacing between sampling events.

We found no evidence for genotype-specific habitat segregation at Whitewater, indicating this population does not demonstrate a bimodality of habitat use between parental individuals. It appears that parental *N. bryanti* have marginally higher within-site dispersal than hybrids, regardless of sex; however, dispersal rates were low, and the vast majority of individuals were either recaptured at the same location or had moved less than 100 meters from their previous capture site. Similar to most small mammals, the frequency of dispersal in woodrats is highest in subadults as they leave their natal nesting sites to establish home territories (Sakai and Noon 1997). We only measured the dispersal characteristics of adults in this study, so we cannot rule out the possibility of genotype-specific dispersal patterns for juveniles and subadults. Nevertheless, the high nest site fidelity of adult hybrids suggests they are successfully defending their home territories and that ecological selection against hybrids may be reduced at Whitewater compared to Whitney Well. However, it is also worth noting that interspecific differences in dispersal behavior can influence apparent survival estimates. In this study, we found both high apparent survival and low within-site dispersal for *N. bryanti* and hybrids at Whitewater. In contrast, we were unable to characterize the dispersal characteristics of *N. lepida* due to an insufficient sample size and thus cannot rule out the possibility that *N. lepida* may have a higher capacity for dispersal. However, *N. lepida* surveyed in similar Mojave habitat demonstrated high site and nest fidelity across sampling years (Smith 1985), suggesting that it is unlikely that adult *N. lepida* demonstrate a high enough rate of between-site dispersal to fully explain their exceptionally low site persistence at Whitewater.

Neotoma bryanti **is the numerically dominant species despite less evolutionary experience with creosote bush**

Neotoma bryanti is the most abundant parental species at Whitewater and likely maintains some form of competitive advantage in this habitat. This finding is unexpected given *N. lepida*'s propensity to both co-occur with creosote bush across much of its range (Cameron and Rainey 1972), and its greater ability than *N. bryanti* to tolerate creosote bush resin (Dearing et al. 2022). However, male parental and backcross *N. bryanti* were ~15% larger in body mass compared to male parental and backcross *N. lepida*, and this size differential was also apparent at Whitney Well (Shurtliff et al. 2014). Furthermore, *N. bryanti* is more aggressive than *N. lepida* in both field and laboratory experiments (Shurtliff et al. 2013). Woodrats are territorial and compete against sympatric congeners for resources (Cameron 1971; Kinsey 1977), with the larger species predominately occupying higher quality nesting sites (Dial 1988). Therefore, the larger body size of male *N. bryanti* likely confers a competitive advantage that enables it to exclude *N. lepida* from prime nest sites and mates, potentially limiting *N. lepida*'s site persistence despite its ability to better utilize a common food resource, creosote bush.

The role of transitional habitat in regulating interspecific competition and hybridization

The Whitewater and Whitney Well hybrid zones emerged as a product of secondary contact between *N. bryanti* and *N. lepida* ~60 kya (Patton et al. 2007). Much of our prior understanding of the dynamics of woodrat hybrid zones came from Whitney Well, which has a clear ecotone. As Whitewater is non-ecotonal, characterizing the hybrid zone dynamics at this site is useful to identify the role of habitat composition in regulating interspecific hybridization. Though hybridization occurs at both secondary contact zones with and without ecotones, the composition of the hybrid population may differ based on the presence or absence of an ecological gradient. For example, we found at Whitewater the presence of both early generation and advanced generation hybrids compared to the hybrid population at Whitney Well, which consisted mainly of early generation hybrids (Shurtliff et al. 2014; Jahner et al. 2021). This indicates that ecotones may limit the frequency of hybrid-hybrid mating by selecting against early generation hybrids, potentially reinforcing species boundaries.

The substantial overlap between *N. bryanti* and hybrids at Whitewater facilitated by the lack of an ecotone indicates that there is great opportunity for ecological and genomic interactions between parental and recombinant genomes. The evolutionary consequences of such interactions are unclear, but may lead to a variety of potential outcomes such as lineage collapse (Taylor et al. 2006), hybrid speciation (Mallet 2007) and introgression (Jahner et al. 2021). Due to the high frequency of multigenerational backcrossing at Whitewater, introgression, especially in the direction of *N. bryanti*, may occur. Introgression of *N. lepida* haplotypes into *N. bryanti* may be adaptive if it confers beneficial traits related to creosote bush feeding, maladaptive if it leads to an accumulation of deleterious variation (Pfennig 2021), or largely neutral with respect to selection. Future studies documenting the pattern of introgression across hybrid zones will show how ecological selection influences the rates of interspecific introgression, and the fitness consequences such events entail.

Current implications, and the future of this hybrid zone

Collectively, these results indicate that the ecological context in which secondary contact occurs can have profound effects on hybridization. The comparison of the Whitewater and Whitney Well contact zones presents a unique opportunity to consider the influence of ecological barriers and genomic compatibilities on interspecific gene flow. In particular, these two hybrid zones indicate that the presence or absence of sharp ecological boundaries can influence interspecific mating opportunities, hybrid success, and the distribution of ancestry classes. In areas with sharp ecological transitions, interspecific mating opportunities are reduced due to strong parental habitat segregation (Patton 1973; Shurtliff et al. 2014), and hybrids may be selected against if they express intermediate phenotypes to those of the parents (Konuma et al. 2013) or express traits that are maladaptive under local conditions (Hudson et al. 2021). Therefore, ecotones may reinforce species boundaries and promote the formation of stable parapatric contact zones between hybridizing species. Conversely, in hybrid zones without strong ecological gradients, the frequency of hybridization may be more variable over time, and thus, repeated temporal monitoring is required to accurately characterize the outcomes of hybridization at such sites.

We conclude by highlighting potential concerns for the ongoing conservation of this hybrid zone in the face of environmental change. Whitewater is presently being threatened by substantial anthropogenic disturbances and the future of this site remains uncertain. The hybrid zone is presently divided by several roadways and highways — including the massive I-10 interstate — and habitat is actively being lost to urban sprawl. The impacts of these disturbances on resident woodrat populations are unknown, but they could dramatically alter the hybrid zone dynamics if habitat connectivity suffers and dispersal is limited. These disturbances are further compounded by ongoing changes in abiotic conditions as the American Southwest experiences increased aridification (Cook et al. 2015), which has already caused range contraction for some woodrat populations (Moritz et al. 2008). Woodrats are keystone species (Whitford and Steinberger 2010), and their potential loss in this community may significantly impact plant, arthropod, and predator abundances. Therefore, ongoing monitoring of Whitewater is vital for assessing temporal shifts in the population dynamics and structure of this hybrid zone, each of which can alter rates of interspecific gene flow and act as an indicator for the future success of these two species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability:

Sequence data generated as part of this study is available from the NCBI SRA under BioProject PRJNA767020. The code and metadata associated with this article are available from the Center for Open Science (https://doi.org/10.17605/OSF.IO/DNE8R).

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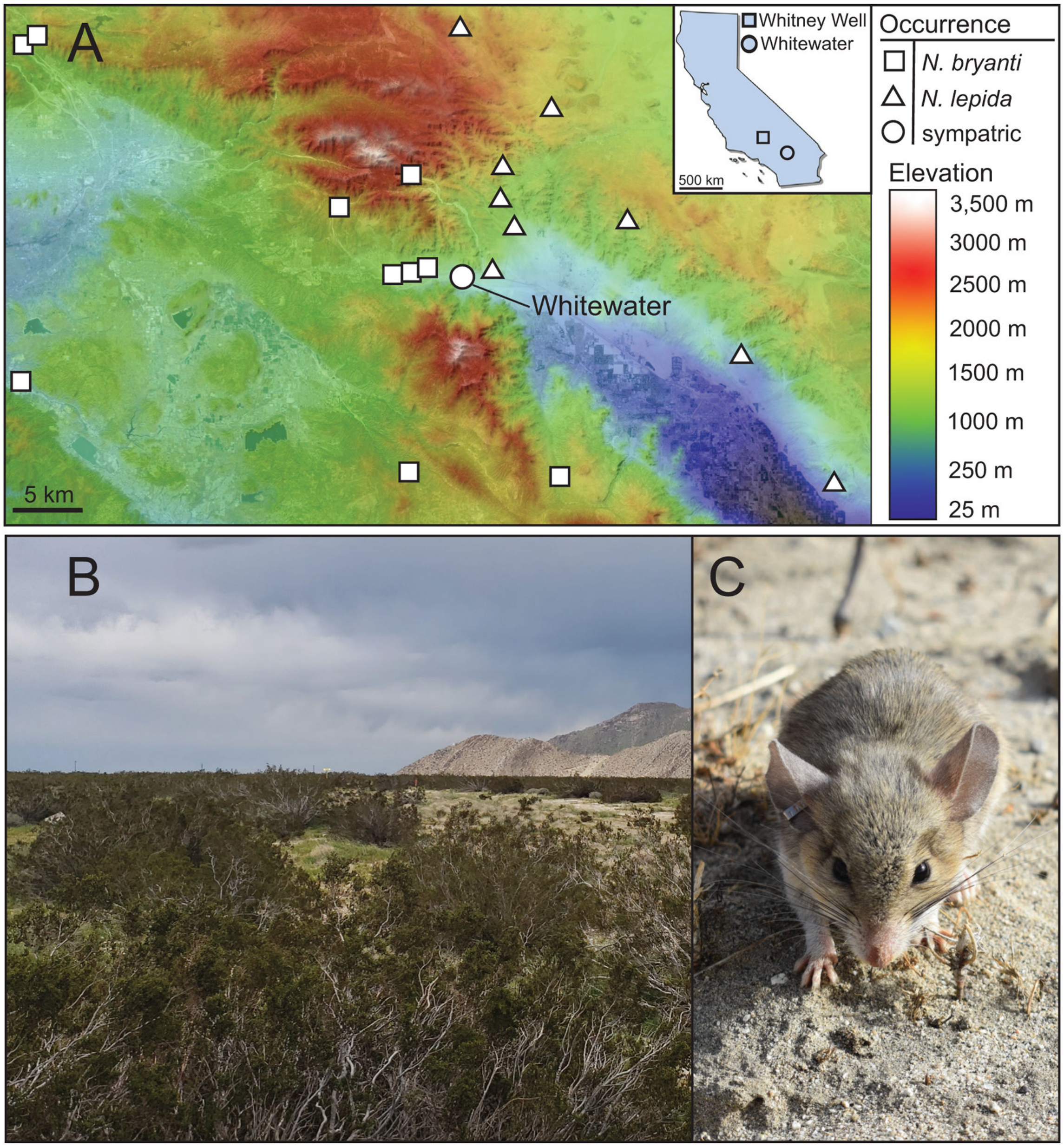

Figure 1.

A) Topographic map of the San Gorgonio Pass, Riverside County, CA, USA. Squares, triangles, and circles represent known areas of occurrence of *N. bryanti*, *N. lepida*, or hybridization zones respectively, based on genetic sampling of woodrats from Patton, et al. 2007 and Dearing, et al. 2022. Topographic data is from Yamazaki et al. 2017. B) Whitewater occurs within hundreds of square kilometers of creosote bush (*L. tridentata*) shrublands that span the San Gorgonio Pass in an east-west orientation (photograph by D.M. Klure). C) Photograph of a female subadult backcross *N. bryanti* from Whitewater (photograph by M.L. Doolin).

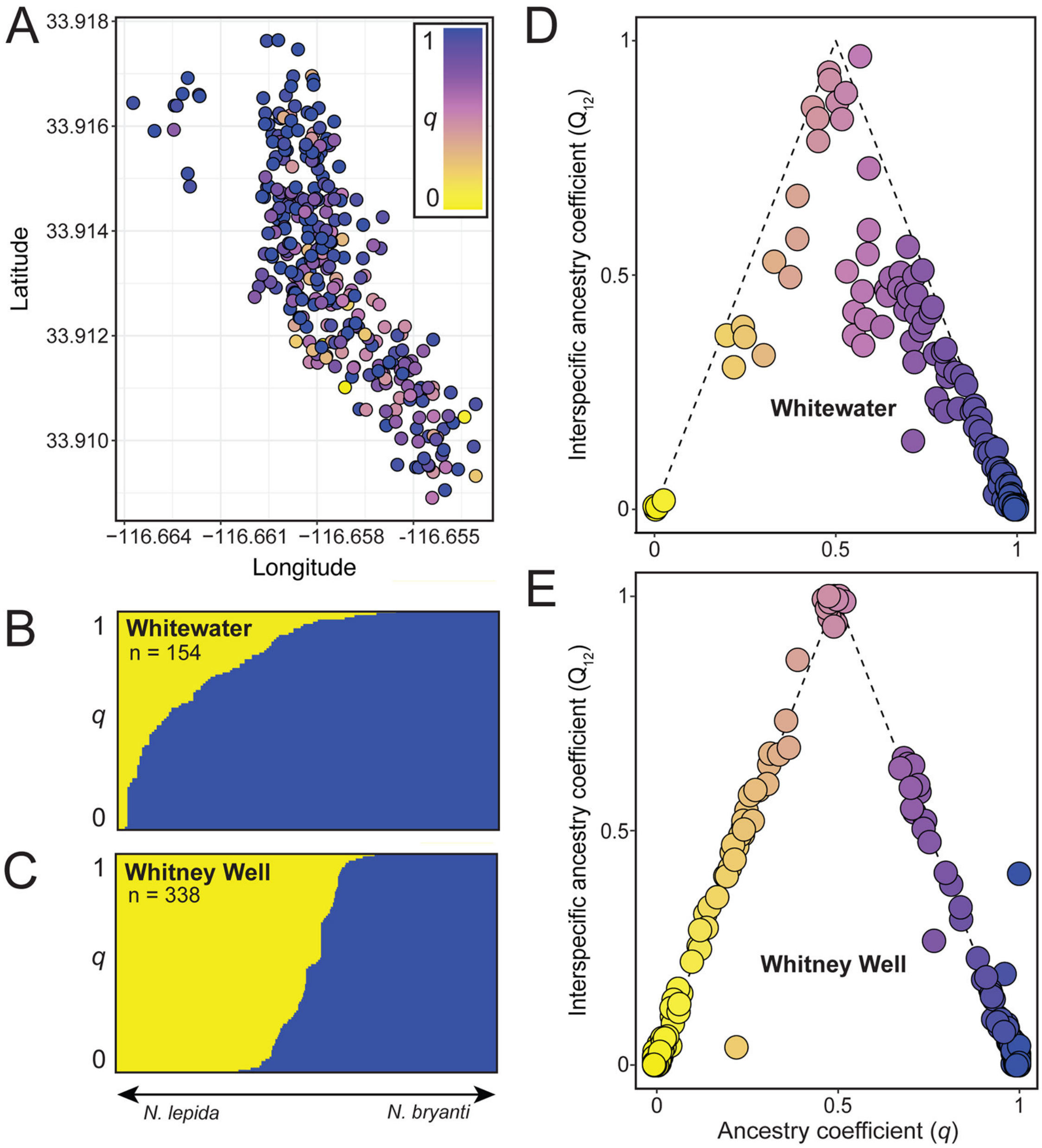

Figure 2.

A) Individual capture location for woodrats across all sampling periods colored by their inferred ancestry coefficient (*q*). Points were jittered (± 0.001) to avoid overplotting. The distribution of ancestry coefficients at B) Whitewater and C) Whitney Well. Individuals are ordered along the x-axis by decreasing values of *q*. Individual ancestry classes were assigned based on individual (*q*) and interspecific (Q_{12}) ancestry coefficients as estimated from the *q* and complement models in `entropy` for D) Whitewater and E) Whitney Well woodrats. Individuals that reside on the dashed line have at least one unadmixed parent compared to individuals towards the center of the triangle, which are the progeny of two admixed individuals. Points were jittered (± 0.01) to avoid overplotting.

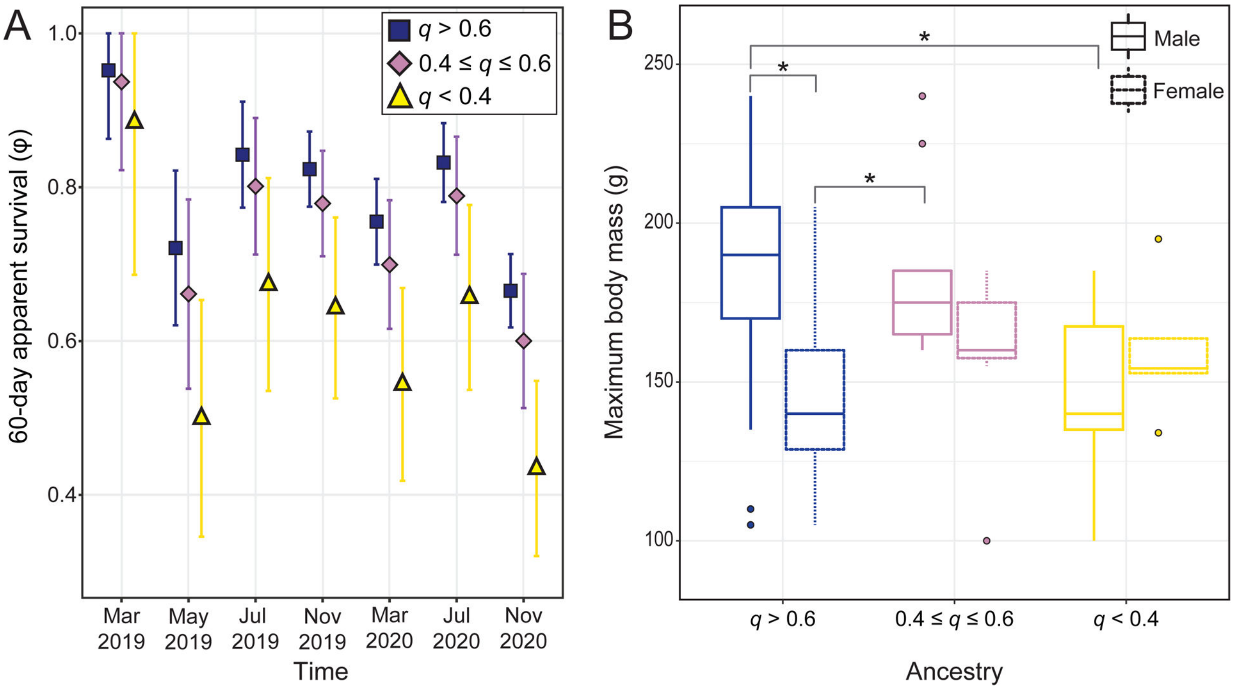

Figure 3.

A) Apparent survival estimates for woodrats grouped by ancestry classes as estimated from an open-population and time-dependent Cormack-Jolly-Seber model. B) Boxplots of the maximum recorded body mass for adult woodrats grouped by ancestry and sex. Individual points represent statistical outliers within each group and significant differences between groups are denoted by an asterisk (p-value < 0.05).