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Estimating red wolf ancestry in a population of canids on Galveston Island, Texas

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ESTIMATING RED WOLF ANCESTRY IN A POPULATION OF CANIDS ON
GALVESTON ISLAND, TEXAS

By

Tanner M. Barnes

A THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Forest Ecology and Management

MICHIGAN TECHNOLOGICAL UNIVERSITY

2021

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This thesis has been approved in partial fulfillment of the requirements for the Degree of
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Abstract

In the United States biologists aim to prevent species extinctions through different policies and laws, the most prominent of these, the Endangered Species Act of 1973 (ESA), sets the precedent for federal protection of endangered or threatened species. However, the ESA does not have a clear policy for groups of organisms that do not meet discrete species classifications, such as hybrids (Fitzpatrick et al. 2015). Hybrids are the offspring of two different species or varieties. When hybrids are the result of a mating between a common species and an endangered species, those hybrids are not necessarily protected under the ESA. However recently biologists are seeing the benefit of hybrids in species conservation because they can be source of genetic rescue.

Genes are distinct segments of nucleotides forming part of a chromosome that are passed down from parent to offspring and are how systematics contemporarily delineate species classifications (Davis & Nixon 1992). There are thousands of allelic variants present within all individuals of a species, but the loss of alleles in rare species decreases their ability to adapt to changing environmental conditions, which increase their risk of extinction. Hybrids of an endangered species can be a source of lost genetic variation and with new emerging technologies such as genome editing tools or de-introgression strategies alleles from rare species can be recovered from hybrid populations.

Here we aim to describe a newly discovered population of red wolf/coyote hybrids to determine the importance this population may have to the critically endangered red wolf species. Then we compare the two parental species in a systematic review to realize the hybridizing species niche overlap for future conservation actions.

1 Estimating red wolf ancestry in a population of canids on Galveston Island, Texas

1.1 Abstract

Hybridization can be a conservation concern if genomic introgression leads to the loss of an endangered species' unique genome, or when hybrid offspring are sterile or less fit than their parental species, but hybridization can also be an adaptive management tool if rare populations are severely inbred. One such species, the red wolf, is critically endangered in the United States, where all extant red wolves are descended from 14 founders which has contributed to an increase in inbreeding over time. Red wolves were considered extirpated from Texas in 1980, but before they disappeared, they hybridized with encroaching coyotes creating a hybrid population. In 2018, a genetic study identified individuals on Galveston Island, Texas with significant amounts of red wolf ancestry. We returned to Galveston for a more in-depth analysis of this hybrid population to identify the extent of red wolf ancestry in the population and mechanisms that support it on the landscape. We identified 23 individuals from Galveston Island and 8 from mainland Texas with greater than 10% red wolf ancestry. Two of those from mainland Texas had greater than 50% red wolf ancestry estimates. Additionally, this population had 6 private alleles that were absent in all North America reference canid populations, possibly representing lost red wolf genetic variation. We additionally identified several individuals on Galveston Island and the mainland of Texas that retained unique red wolf mitochondrial haplotype. Our results confirm the presence of substantial red wolf ancestry persisting on Galveston Island and mainland Texas. This population has the potential to benefit red wolf conservation through novel reproductive techniques and

ultimately through de-introgression strategies, with the goals of recovering extinct red wolf variation and reducing inbreeding within the species.

1.2 Introduction

Hybridization and the resulting introgression, or the movement of alleles from one species into another, occurs naturally in many organisms and is thought to be an important evolutionary strategy (Fredrickson & Hedrick 2006; Hufbauer et al. 2015; Hamilton and Miller 2016; Kronenberger et al. 2018; vonHoldt et al. 2018; Burgarella et al. 2019; Brzeski et al. 2021). However, contemporary anthropogenic activities, such as human translocations and habitat loss, are resulting in an increase in hybridization events because they facilitate the breakdown of reproductive barriers (Rhymer & Simberloff 1996; Crispo et al. 2011; McFarlane & Pemberton 2019). Hybridization and the resulting introgression can be harmful to populations if hybrid offspring are sterile or if outbreeding depression occurs (Rhymer & Simberloff 1996). Additionally, introgression can lead to the loss of genetic distinctiveness between species which can affect the persistence of rare species (Rhymer et al. 1994; Wolf et al. 2001; Allendorf & Luikart 2007; Placyk et al. 2012). For instance, hybridization with introduced mallard ducks (*Anas platyrhynchos*) has been a factor in population declines of the New Zealand grey duck (*Anas superciliosa*) (Rymer et al. 1994) and the endemic Hawaiian duck (*Anas wyvilliana*) (Shallenberger & Fefer 1989; Rhymer & Simberloff 1996).

Alternatively, hybridization can genetically rescue and restore fitness in critically endangered populations or species when crossed with more common species or subspecies (Pimm et al. 2006; Amador et al. 2014). For example, in 1967 the Florida panther (*Puma concolor coryi*) was federally listed as endangered, and by the 1990s

deleterious traits were becoming fixed through genetic drift and inbreeding depression from decades of low population size (Hedrick & Fredrickson 2010). In addition, a survey of microsatellite loci showed the Florida panther had much lower molecular variation than other North American populations (Culver et al. 2000; Driscoll et al. 2002). In 1995, a program was initiated to release Texas panthers to genetically restore fitness in the Florida panther. Studies after the release found a dramatic reduction in deleterious traits in animals with Texas ancestry (Mansfield and Land 2002; Land et al. 2002), and from 1995 to 2008 the population grew at a rate of 12% per year (Hedrick and Fredrickson 2010).

Today, recent advances in sequencing technology help identify hybrid populations. Developing technologies, such as genome editing, are also providing new means of genetic rescue. For instance, genome editing may prove useful in supplementing endangered taxa with lost genetic variation (ghost alleles) that may only be present in frozen cryo-banks or living hybrid individuals (Amador et al. 2014; Quinzin et al. 2019; Brzeski et al. 2021). For example, genes of the recently extinct Galapagos tortoise (*Chelonoidis elephantopus*) were detected within hybrid individuals of the Wolf Volcano Giant-Tortoise (*Chelonoidis becki*) (Garrick et al. 2012). These hybrid individuals are now being selectively bred to capture the largest genomic representation of the extinct Galapagos tortoise to hopefully recover the species (Quinzin et al. 2019).

Whether hybridization is a conservation concern, or an adaptive management tool depends on a variety of factors, such as the size of an endangered population, the current genetic variation present, and importantly the strengths and weaknesses of reproductive barriers operating in a system (Fredrickson & Hedrick 2006). For example, species will

hybridize on the edge of their ranges when mating with a closely related species or losing a mating opportunity are the only options (Pfennig 2021). As a result, multi-generational admixture can produce a diversity of phenotypes, making edge populations phenotypically and genetically distinct from those in the center (Excoffier et al. 2009; Gruber et al. 2017; Pfennig 2021). These hybrid zones can be reinforced with reproductive barriers such as assortative mating but its dynamics in natural populations are poorly understood (Schumer et al. 2017). Studying the ancestry and population structure of hybrid populations can help us understand reproductive barriers or assortative mating patterns that lead to stable hybrid zones or widespread introgression, which in the face of contemporary anthropogenic landscape change, will be critical for effective conservation planning.

The red wolf (*Canis rufus*) and the coyote (*Canis latrans*) historically hybridized on the edge of their historic ranges in the United States. Once widespread throughout the southeastern United States, the red wolf suffered severe population declines due to human persecution and habitat loss (Hinton et al. 2013). Their populations become more isolated in small, declining populations on the western edge of their historic range in states including Missouri, Arkansas, Texas, and Louisiana, where red wolves appeared phenotypically distinct from eastern conspecifics (Goldman 1937, 1944). The phenotypic differences of red wolves across their range could be due to variation in habitats or potentially infrequent historic hybridization with encroaching coyotes from the western United States. Eventually, red wolves were extirpated from every state but Texas and Louisiana, and in these states' reproductive barriers between red wolves and coyotes collapsed, increasing the frequency of hybridization (Hinton et al. 2013). Concerned with

a declining wolf population and fearful that continued hybridization with coyotes would lead to the genomic extinction of the red wolf, the United States Fish and Wildlife Service (USFWS) initiated a large trapping effort to create a captive breeding population (Phillips & Parker 1988; Allendorf et al. 2001; Phillips et al. 2003). This effort ended in 1980, where ultimately just 14 individuals from Texas and Louisiana became the founders of all extant red wolves today (Phillips & Parker 1988).

Following trapping efforts, red wolves were considered extinct from the wild in 1980 until they were reintroduced as a nonessential experimental population (NEP) in North Carolina in 1987 (McCarley & Carley 1979; USFWS 1990; Pritchard et al. 2000; Hinton et al. 2013). Yet, coyotes with large body sizes continued to be reported in Texas and Louisiana (Giordano & Pace 2000). This prompted two studies that independently discovered significant red wolf genetic ancestry in canids in both Texas and Louisiana (Heppenheimer et al. 2018a; Murphy et al. 2019). These discoveries suggest that red wolf introgression may be common along the Gulf Coast, and red wolf genetic diversity can persist at relatively high levels without human intervention. In North Carolina studies suggest that despite coyote presence, red wolves will seek out conspecific mates or mates with mixed ancestry rather than hybridize with coyotes (Bohling & Waits 2015; Hinton et al. 2018). Finding hybrid canids with significant amounts of red wolf ancestry along the Gulf Coast four decades after they were considered extinct suggest there are assortative mating patterns such as variation in prey selection, territorial aggression, or body size differences that limit introgression between red wolf hybrids and coyotes (Hinton et al. 2018).

Galveston Island, located in Texas, was one of the initial locations where canid populations with red wolf ancestry was discovered (Heppenheimer et al. 2018a). Galveston represents an ideal system to better understand mechanisms that promote the persistence of red wolf ancestry given its canid population likely experiences reduced gene flow with southeastern coyotes on the mainland and it is a relatively closed system. Additionally, Galveston Islands landscape spans between a rural and urban interface. This provides an opportunity to examine patterns of habitat selection as a function of canid ancestry. For example, larger carnivores will avoid areas with high human density and modify their behavior as human density increases (Dellinger et al. 2013). In North Carolina, red wolf x coyote hybrids are larger than coyotes (Hinton & Chamberlain 2014), therefore, canids with red wolf ancestry in Galveston may be selecting more undeveloped habitats thereby forcing coyotes into more developed areas.

To better understand the distribution of red wolf ancestry in this population and the mechanisms promoting it, our objectives included to 1) estimate red wolf ancestry and the distribution of ancestry on Galveston Island, 2) evaluate baseline genetic variation of Galveston Island canids and compare it to mainland Texas canids surrounding the island to measure for restricted gene flow and inbreeding and 3) estimate relatedness, genetic substructure, and abundance of canids on Galveston island to determine habitat characteristics that promote red wolf ancestry. We accomplished our objectives through systematic noninvasive fecal sampling across Galveston Island, opportunistic noninvasive fecal sampling from National Wildlife Refuges on mainland Texas surrounding Galveston, and opportunistically collecting roadkill tissue samples on and off Galveston Island. Reintroduced red wolves in North Carolina have recently suffered

recovery challenges and there are currently less than a dozen wild wolves today. This study provides a crucial step in understanding how endangered red wolf ancestry distributes itself on the landscape and mechanisms that reinforce the persistence of red wolf genes.

1.3 Methods

1.3.1 Study Area

Our study was located south of Houston, Texas on Galveston Island, in San Bernard and Anahuac National Wildlife Refuges. Galveston Island is a barrier Island situated approximately 80 km south of Houston on the Texas Gulf Coast. It is 160 square kilometers, 43.5 km long, and no more than 4.8 km at its widest point. It has a year-round population of 48,000 residents (Census 2010) and supports many tourists throughout the year. Galveston Island is completely disconnected from the mainland, and only has two access bridges, from highway 45 at the north end and San Luis Pass on the west end. The closest distance to the mainland is approximately 1 km at San Luis Pass.

San Bernard National Wildlife Refuge is ~65 km west of Galveston in east-central Texas. It is a ~185 km² wildlife refuge established in 1968 (Yao et al. 2020). The refuge is a mix of three main habitat types: salt marsh, coastal prairies, and bottomland hardwood forests (Yao et al. 2020). The Anahuac National Wildlife Refuge is ~ 40 km east of Galveston Island in eastern Texas. It is a part of the Texas Chenier Plain Refuge Complex and covers 140 km² (Lane 2017).

The climate is classified as humid subtropical, as prevailing winds from the south/southeast bring moisture from the Gulf of Mexico (Weather Stats 2008). Summer

temperatures can exceed 32°C and the constant humidity increases the heat index. Winter temperatures can vary but average around 10-16°C (The Weather Channel 2006). Annual rainfall averages over 1,000 mm with some areas receiving more than 1,300 mm (The Weather Channel 2006). Hurricanes are an ever-present threat during the summer and fall seasons as the low-lying coastal island can receive storm surge (Berger 2008).

1.3.2 Field Methods

We systematically sampled the island for feces to collect genetic samples for noninvasive genetics. We designed our study to account for degraded DNA that is collected from fecal samples by having repeated sampling. We collected fecal samples and recorded their GPS location along designated 25 transects distributed east to west (*Figure 1.1*) across the island during three field seasons, January 2020, July 2020, and January 2021. Each transect was routinely walked or driven over multiple days to retrieve fresh fecal samples. Based on a pilot study in August 2019 (*Appendix A*) designed to test fecal collection protocols, we collected fecal samples by swabbing them with a sterile cotton swab that was subsequently placed in a 2 mL tube of Longmire buffer.

Additionally, the whole fecal sample was collected in a labelled paper bag and dried for future diet analysis. We also opportunistically collected fecal samples and recorded their GPS location from mainland Texas, including National Wildlife Refuges surrounding Galveston to compare Galveston Island to nearby parts of Texas. Swabs were stored in a freezer at -20°C and whole fecal samples were stored in a clean dry area at Michigan Technological University (MTU), Houghton, USA. We additionally collected roadkill tissue samples from Galveston Island and mainland Texas. Canid tissue was placed on silica or ice and transported to MTU for long term storage at -20°C. All research was

approved by the MTU Institutional Animal Care and Use Committee (#1438689) and the Texas Parks and Wildlife Department (SPR-0220-020).

1.3.3 Genetic Assessment Methods

We extracted DNA from fecal samples using a modified QiAmp Fast DNA Stool protocol (Qiagen, Inc., Hilden, Germany), in a laboratory dedicated to low-quality DNA at MTU. We extracted DNA from tissue samples using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) following the manufacturers protocol in a laboratory dedicated to high-quality DNA samples. We amplified a portion of the cytochrome B region of the mitochondrial genome via polymerase chain reaction (PCR) to confirm DNA isolated from fecal samples was collected from wild canids and to gather information on matrilineages, which is useful in determining canid taxonomy (Adams & Waits 2007).

Extant red wolves are only represented by a single mitochondrial haplotype, so we compared the mitochondrial lineages of our generated sequences with previously published sequences to assess if they had the red wolf ancestry. For low quality DNA extracted from fecal samples we targeted a ~ 200 base-pair fragment using primers ScatSeqF (5` - CCATGCATATAAGCATGTACAT-3`) and ScatSeqR (5` - AGATGCCAGGTATAGTTCCA-3`). The PCR mix consisted of 10mM of each primer, 0.2mM dNTPs, 1x Buffer II, 2.5 mM MgCl₂ and 0.5 µL Amplitaq Gold (Applied Biosystems) in a 15 µL reaction with 1.5 µL of DNA extract. We used an Eppendorf Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany) using an initial denaturation step of 95°C for 10 minutes, 40 cycles (95°C for 30 s, 48°C for 45 s, 72°C for 60 s) and a final extension for 7 minutes at 72°C. For high quality DNA extracted

from tissue we targeted a ~ 420 base-pair fragment using primers Thr-L 15926 (5'-CAATTCCCCGGTC TTGTAAACC-3') and DL-H 16340 (5'-CCTGAAGTAGGAA CCAGATG-3') (Vila et al. 1999). The PCR mix consisted of 10mM of each primer, 0.2mM dNTPs, 1x Buffer II, 2.5 mM MgCl₂ and 0.5 µL Amplitaq Gold (Applied Biosystems) in a 10 µL reaction with 1 µL of DNA extract. The Eppendorf Mastercycler Gradient Thermal Cycler conditions were an initial 10-minute denaturation at 95°C, 40 cycles (95°C for 30 s, 55°C for 45 s, 72°C for 60 s) and a final extension for 7 minutes at 72°C. The Each PCR was run with a negative control from a DNA extraction to monitor for contamination. We visualized each PCR reaction on a 2% agarose gel run at 115 volts for one hour. Samples that contained nonspecific amplification were reamplified with an annealing temperature of 55°C. We enzymatically cleaned samples using the product ExoSapIT (Bell 2008) on an Eppendorf Mastercycler Gradient Thermal Cycler following manufacturers protocol and sent to GENEWIZ (New Jersey, U.S.A.) for Sanger Sequencing; mitochondrial sequences were viewed, trimmed, and aligned with Geneious software v2021.1.1 (Kearse et al. 2012).

We compared our generated sequences to all previously published sequences in the NCBI BLAST database using Geneious. The samples confirmed to be from the target sequence were aligned using a pairwise MUSCLE alignment in Geneious using 8 iterations. The shorter sequences that were amplified using the Scatseq primers were matched to a longer sequence from the Thr-L 15926 and DL-H 16340 primers for haplotype identification. We used a Muscle alignment in Geneious to compare our mtDNA haplotypes to all extant North American canid haplotypes from NCBI GenBank.

We genotyped nuclear DNA (nDNA) at 17 microsatellites at the Laboratory for Ecological, Evolutionary, and Conservation Genetics (University of Idaho, Moscow, U.S.A.) to identify individuals from fecal samples, estimate ancestry, and estimate relatedness. This multi-locus microsatellite panel has been used extensively in the past for identifying red wolf X coyote hybrids (Adams et al. 2003; Miller et al. 2003; Bohling & Waits 2011; Bohling et al. 2013; Murphy et al. 2019). We generated genotypes using two multiplexes (Bohling & Waits 2011; Bohling et al. 2013). The first multiplex contained 0.06 μ M of CXX.377, 0.07 μ M of CXX. 172, CXX.173, and CXX.250, 0.13 μ M of CXX.109, 0.16 μ M of CXX.200, 0.20 μ M of AHTq121, 0.60 μ M of AHT103, 0.71 μ M of CXX.20, 1X Qiagen Multiplex PCR Kit Master Mix, 0.5X Q solution, and 1 μ L of DNA extract in a 7 μ L reaction (Ostrander et al. 1993, 1995; Mellersh et al. 1997; Miller et al. 2003; Murphy et al. 2019). The second multiplex contained 0.06 μ M of FH2010, 0.07 μ M of FH2062 and FH2054, 0.10 μ M of FH2001, 0.16 μ M of FH2145, 0.24 μ M of FH2004, 0.36 μ M of CXX.225, 0.80 μ M of CXX.403, 1X Qiagen Multiplex PCR kit Master Mix, 0.5X Q solution, and 1 μ L of DNA extract in a 7 μ L reaction (Ostrander et al. 1993; Mellersh et al. 1997; Miller et al. 2003). We amplified tissue samples in duplicate and performed up to four and six replicate PCRs for the tissue and fecal samples respectively that consistently amplified. We visualized PCR products using a 3130xl DNA Sequencer and scored allele sizes using Genemapper 3.7 (Applied Biosystems, Inc., Foster City, U.S.A.). Assessment of sample quality and genotype screening methods followed those described by Adams and Waits (2007). We calculated Hardy-Weinberg Equilibrium using package Pegas in R v4.0.2 (Paradis 2010) and used a Bonferroni correction that corrects for multiple, simultaneous comparisons (Weissstein

2004). We calculated probability of identity (PID) and probability of identity for siblings (PID_{SIBS}) using nine loci in Multiplex 1 with GenAlEx v6.5 (Peakall & Smouse 2006) and performed a matching analysis in GenAlEx to determine how many individuals were detected in the fecal genotypes after Multiplex 1. Only five loci were necessary to differentiate between individuals, so only unique individuals were amplified with Multiplex 2.

1.3.4 Statistical Methods

Our first objective was to estimate red wolf ancestry proportions in each individual, by assessing the mitochondrial lineage and determining the distribution of red wolf ancestry in nDNA across the island and adjacent mainland Texas. To accomplish this, we estimated gene trees using Bayesian methods implemented in BEAST v.1.10.4 (Drummond et al. 2012), with a constant size coalescent tree prior, an uncorrelated lognormal relaxed molecular clock, and a random starting tree. We conducted Markov Chain Monte Carlo (MCMC) analyses with 20 million steps, sampling every 2000 steps, and combined tree estimates from each run with LogCombiner v.1.10.4 (Rambaut & Drummond 2015) with a 10% burn-in. We calculated the maximum clade credibility in TreeAnnotator v.1.10.4 (Helfrich et al. 2018) and uploaded the most likely tree in the Interactive Tree of Life v3.6.3 online webtool to visualize the gene tree and mtDNA lineages (Letunic et al. 2016).

To determine red wolf nDNA ancestry, we used a Bayesian assignment method (Bohling et al. 2013) implemented in program STRUCTURE v2.3.4 (Pritchard et al. 2000). Galveston Island and mainland samples were assigned ancestry proportions to our

Canis references. References included Mexican wolves (n=14), domestic dogs (n=38), gray wolves (n=38), red wolves (n=19), and southeast coyotes (n=107). For the STRUCTURE analysis we set the number of populations (K) a priori to five (e.g., Mexican wolf, domestic dog, gray wolf, red wolf, and coyote) and ran 10 independent runs of the admixture model with correlated allele frequencies with a burn-in of 20,000 Markov chain Monte Carlo iterations followed by 50,000 iterations to estimate a posterior probability of ancestry (q). To prevent bias that can arise from including related individuals among our samples, we used the PopFlag prior that uses a Boolean variable to indicate learning samples. Because the Popflag prior can be sensitive to admixed individuals in the reference populations, after an initial STRUCTURE run, we removed reference individuals with $\geq 25\%$ ancestry assignment to a difference reference group (i.e., gray wolf with 25% dog ancestry). This left us with Mexican wolves (n=14), domestic dogs (n=37), gray wolves (n=36), red wolves (n=18), and southeast coyotes (n=98). Individual summaries of ancestry proportions were visualized using pie charts plotted spatially using ArcGIS Pro v2.5.2 software. We used a T-Test to compare mean red wolf ancestry proportions between males and females. We further evaluated genetic clustering with a principal component analysis (PCA) using package Adegenet (Jombart 2008) and package Factoextra (Kassambara & Mundt 2017) in R v4.0.2. We conducted two PCAs, the first with all reference populations and second with a subset (gray wolf, red wolf, coyote, our samples) to better visualize clustering with only one outgroup.

Our second objective was to evaluate the baseline genetic variation of Galveston Island canids and compare it to Texas mainland canids surrounding the island to measure for restricted gene flow and inbreeding. We calculated standard measures of genetic

variation in several ways. First, we calculated observed and expected heterozygosity, which are measures of genetic diversity within a population and Fixation Index (F), which are measures of population differentiation using program GenALEx (Peakall & Smouse 2006). We calculated these metrics for all Galveston Island canids, Texas mainland samples that were close geographically proximity to the island, and on *Canis* reference populations to have a comparison of genetic variation to other canid populations (*Table 1.1*). Next, we assessed genetic differentiation of Galveston Island and our Texas mainland samples in comparison to reference populations by estimating pairwise F_{ST} (Takezaki & Nei 1996) and F_{IS} values per population using package Hierfstat in program R (Goudet, Jombart, & Goudet 2015). We additionally calculated allelic richness and private alleles using package PopGenReport in program R (Adamack et al. 2014).

Our third objective was to estimate relatedness, genetic substructure, and abundance of canids on the island and determine habitat characteristics that support red wolf ancestry. To identify possible family groups, we first estimated genetic relatedness of Galveston Island canids by calculating pairwise relatedness between all individuals on Galveston Island using the maximum likelihood approach implemented by the program ML-Relatedness (Kalinowski et al. 2006). We estimated average relatedness of all individuals on Galveston Island and evaluated differences in relatedness between the sexes. Next, we used STRUCTURE to evaluate the genetic substructure of canids on Galveston Island. We conducted 10 independent runs for each K value with the admixture model $K = 1-10$, using 50,000 repetitions after a burn-in of 20,000 Markov chain Monte Carlo iterations. The most likely number of genetic clusters represented by

the data was estimated by considering delta K (Evanno et al. 2005), calculated with STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt 2012). However, delta K cannot provide support for $K = 1$ or $K = 10$ as it is based on the rate of change in log-likelihood between successive K values, so we also used the log-likelihood (Ln Probability) values inferred from STRUCTURE (Pritchard et al. 2000). We used pie charts plotted spatially in ArcGIS Pro to visualize differences in genetic clusters ($K=2-7$). We considered individuals to have high assignment to a given inferred cluster if the ancestry proportion (q) was greater than or equal to 0.8. We separated individuals into family groups based on their assignment. We then separated the family groups by the habitat characteristics based on the GPS location they were collected within (developed or undeveloped), to test for differences in mean red wolf ancestry proportions between developed and undeveloped natural areas of Galveston Island. We used The National Oceanic and Atmospheric Administration (NOAA) geospatial classification system with a 30-meter resolution (NOAA 2010). We ran a F-test to test for unequal variances and then ran a one sample T-Test on the ancestry proportions of the individuals within each habitat characteristic.

Next, we used NOAA land cover class (NOAA 2010) in ArcGIS Pro and grouped all individuals into three habitat categories: open, low intensity developed, and high intensity developed based on where they were sampled. The open category included habitats with little to no human presence including Galveston Island State Park, East End Nature Preserve, Artist Boat Coastal Heritage Preserve, and Galveston Bay Foundation Conservation Preserve; the low intensity category included green spaces with some development including golf courses, airports, and RV parks; the high intensity category

included Galveston City. We conducted an ANOVA in Microsoft Excel to test for a difference in mean red wolf ancestry between the three habitat categories. To estimate abundance, we ran a Cormack-Jolly-Seber (CJS) model in program MARK v9.x (White & Burnham 1999) which provides estimates of survival and recapture probability between our three field seasons. We estimated N using the fecal samples collected along the 25 transects using the three field seasons as effort to meet model assumptions. We ran the most simplistic CJS model to obtain an estimate of survival and recapture probability between occasions.

1.4 Results

1.4.1 Field Results

We collected a total of 229 fecal samples and 32 tissue samples for genetic analysis, of which 168 fecal samples were collected along the 25 transects across Galveston Island, Texas. An additional 21 fecal samples were collected from Galveston Island during a pilot study in August 2019 and 21 tissues were collected opportunistically on Galveston Island throughout the duration of the study. An additional 40 fecal samples and 11 tissues were collected on mainland Texas. (*Figure 1.2*).

1.4.2 Genetic Assessment Results

We extracted DNA from 182 fecal samples and 32 tissue samples for genetic analysis. We successfully obtained a mitochondrial genotype at the cytochrome B control region for 94 fecal samples for a 51% success rate and 32 tissue samples. We removed 9 samples that were not wild canids: 8 domestic dogs and 1 otter. We identified four mitochondrial haplotypes on Galveston Island that matched previously published haplotypes (Accession #'s AY280924, FM209385, KU696410, and AY280913). Two

individuals from Galveston Island matched the red wolf haplotype (Accession #AY280913) that is the only haplotype in extant red wolves (Adams et al. 2003). One tissue sample from GI matched a haplotype that most associated with gray wolves (Accession #KU696410). The rest of the samples on GI were represented by haplotypes AY280924 and FM209385 that match coyotes. Of the mainland Texas samples we identified the same four haplotypes found on GI and an additional three Accession #'s AY280913, JN982579, and a new haplotype not previously published. An additional three samples from the mainland matched the red wolf haplotype and five matched the gray wolf haplotype (*Figure 1.3*).

We obtained a nDNA consensus genotypes from 61 fecal samples for a 34% success rate and 32 tissues from canids on Galveston Island and mainland Texas. We did not obtain a nDNA genotype from 33 fecal samples we sequenced a mtDNA haplotype from, nor did we obtain a nDNA genotype for either of the individuals with the red wolf mtDNA haplotype. The highest PID_{SIBS} for five loci was 0.0081, thus any combination of five loci would ensure our ability to distinguish between individuals and still be below the PID_{SIBS} threshold of 0.01 (1 out of 100 siblings are predicted to have matching genotypes). We performed a matching analysis in GenAlEx and identified a total of 51 individuals from GI and 17 individuals from mainland Texas. One sample from Galveston Island was later revealed to be a domestic dog and was removed from further analyses, leaving 50 individuals from Galveston Island (*Supplemental File A for genotypes*). We confirmed sex from 47 of the 50 samples from Galveston Island. The number of detections per individual ranged from 1 to 4. Three of the tissue samples from GI matched to a fecal sample. We removed 4 individuals collected from mainland Texas

from the rest of the analyses because they were from south Texas, not adjacent to Galveston Island, and thus not a genetically relevant group for assessing gene flow between Galveston Island and the adjacent mainland.

Two of the 17 loci significantly deviated from HWE after Bonferroni correction. Previous studies found that even when assumptions of equilibrium are ignored, STRUCTURE still performed well compared to distance-based methods (Hauser et al. 2006; Rodriguez-Ramilo et al. 2009; Bohling et al. 2013). We reran STRUCTURE after removing the two loci that deviate from HWE and the results do not qualitatively change, thus we kept in the loci for analyses (*Supplemental File B*).

1.4.3 Statistical Results

For objective one, we estimated ancestry of the 50 Galveston Island canids and the 13 Texas mainland canids. The main mtDNA haplotypes found on Galveston Island (Accession #FM209385; Hailer & Leonard 2008 and Accession #AY280924; Adams et al. 2003) both are coyote lineages. Two fecal samples matched the red wolf mtDNA haplotype (Accession #AY280913; Adams et al. 2003) that match the red wolf captive breeding lineage. The final haplotype found on Galveston Island (Accession #KU696410; Koblmuller et al. 2016) was found in a tissue sample from a wild canid but most closely resembled a gray wolf mtDNA lineage (*Figure 1.4*).

On Galveston Island red wolf nDNA ancestry averaged 11% and ranged from 1.5-32%, 23 out of 50 individuals had ancestry proportions greater than 10% (*Figure 1.5*). There was no difference in red wolf ancestry between the sexes (one sample t-test, p-value=0.66). Red wolf ancestry was spread across Galveston Island, where we did not

observe high ancestry clusters in one area of the island (*Figure 1.6*). Red wolf ancestry averaged 22% and ranged from 1.8-51%, in samples from mainland National Wildlife Refuges and other nearby parts of mainland Texas. Seven out of 13 of these individuals had ancestry proportions greater than 10%, and 6 of those were greater than 30%. PCA clusters were consistent with taxonomic classifications and previous analyses (Pollinger et al. 2011; Heppenheimer et al. 2018a), where Galveston Island and mainland Texas canids spanned PC1 between the red wolf and coyote taxonomic reference groups (*Figure 1.7*).

For objective two, we compared the genetic variation of Galveston Island and Texas mainland canids to all our reference North American canids. The smallest differentiation in pairwise F_{ST} estimates was between southeastern reference coyotes and Galveston Island canids ($F_{ST} = 0.0700$) followed by Texas mainland samples and reference coyotes ($F_{ST} = 0.0878$) (*Table 1.2*). Galveston Island and mainland Texas samples had similar heterozygosity estimates to our reference coyote groups. Galveston Island and mainland samples both had three private alleles compared to reference groups. Galveston Island canids showed little inbreeding compared to other groups ($F_{IS} = 0.041$).

For objective three, we calculated relatedness, genetic substructure, and abundance of canids on Galveston Island to determine habitat characteristics that promote red wolf ancestry. We calculated pairwise relatedness of every individual on Galveston Island. The variance in relatedness between individuals was (0, 0.78). There was no difference in relatedness between the sexes (p-value=0.61). Many individuals were related to another, 41 out of 50 individuals we genotyped on Galveston had at least one

parent-offspring or full sibling equivalent relationship assignment ($r \geq 0.4$), and 47 out of 50 had a half-sibling equivalent relationship assignment ($r \geq 0.25$). Only three out of the 50 individuals we genotyped did not have a relationship assignment to another individual. Two of these samples came from the middle of the island and one came from the western part. These were areas with lots of private land where we could only collect samples from the roads, so we are potentially missing additional family groups in these locations. We identified four main family groups spread across the island named based on geographic location: East End Lagoon Nature Preserve (10), Scholes International Airport (13), Middle Island (12), and Galveston Island State Park (7) (*Figure 1.8*). Forty-two out of the 50 individuals we sampled on Galveston Island had high assignment to one of the four family groups ($q \geq 0.8$) (*Figure 1.9*). The other 8 individuals did not have high assignment to one group, these may be individuals from family groups we missed during sampling or offspring of individuals from separate family groups. Of the family groups we identified, East End Lagoon Nature Preserve and the Galveston Island State Park groups ($n=17$) both represented undeveloped habitat and the groups from Scholes International Airport and the middle of the island ($n=25$) represented developed habitat. There was no difference in sample variances (f-test for unequal variances, $p=0.088$), but we did find a difference in ancestry between undeveloped and developed habitat types (one sample t-test, $p=0.023$).

However, when we grouped all individuals into three category types (open, low intensity developed, high intensity developed) there was no difference in mean red wolf ancestry between those habitat types (ANOVA, $p=0.54$). This may be confounded at the individual level by a lack of independence. The CJS mark recapture model converged at a

consensus of recapture probability of 0.31 with a large confidence interval (0.028, 0.87). Therefore, canid abundance was estimated at 106.8 (37.8, 1178.6) individuals on Galveston Island which is approximately 0.67 canids/km².

1.5 Discussion

We evaluated the ancestry, genetic variation, and genetic structure of a canid population on Galveston Island, Texas where red wolf ancestry was previously detected in two roadkill individuals (Heppenheimer et al. 2018a). We returned to Galveston Island for a more extensive sampling effort and our results confirmed the findings of Heppenheimer et al. (2018a), demonstrating there is a significant number of individuals within the population with red wolf ancestry. Many individuals on Galveston Island had greater than 10% red wolf ancestry with 8 individuals having ~25-30%. Notably, 6 out of the 13 individuals from mainland Texas we sampled had greater than 30% red wolf ancestry, and two of those had greater than 50%. Additionally, we detected the presence of the red wolf mtDNA haplotype both on and off the island. The only other location this haplotype was detected in wild individuals outside of the nonessential experimental population (NEP) in North Carolina, is southwestern Louisiana (Murphy et al. 2019), another location that was part of the last remaining stronghold of wild red wolves (Carley 1975; Nowak 2002).

Galveston Island canids had observed heterozygosity estimates similar to other reference coyote populations in the southeastern United States, and we did not detect significant amounts of inbreeding (F_{IS}) or population genetic differentiation (F_{ST}) even though many individuals were related, suggesting there is gene flow between Galveston

Island and mainland canids. A minimum of one migrant per generation is said to minimize the loss of polymorphism and heterozygosity within subpopulations (Mills & Allendorf 1996), and our data suggest this threshold is met. These results suggest reduced gene flow due to water between the mainland and the island is not the main mechanism promoting the persistence of red wolf ancestry on Galveston Island. Other mechanisms such as prey selection, territorial aggression, or body size assortative mating like seen in North Carolina (Bohling et al. 2015; Hinton et al. 2018) may be of more importance.

This geographical area was the last stronghold of wild red wolves before they were considered extinct from the wild. Our reference red wolf population is represented by 13 of the 14 genetic founders of the captive breeding population, three offspring of the fourteenth genetic founder, and three additional animals who never reproduced. This is a vast underrepresentation of the original genetic diversity of the species. Between the Galveston individuals and the mainland Texas individuals, we detected 6 private alleles that were not found in any of the reference populations, where some of the individuals with private alleles had high red wolf ancestry (*Table 1.3*). These alleles could represent unique red wolf genetic variation that was lost when the species experienced the bottleneck to just 14 individuals.

Additionally, in the mid-1900s, red wolf/coyote hybrids were documented using morphometrics in Texas (Paradiso & Nowak 1972a,b), Louisiana (Nowak 1967; Goertz et al. 1975), Arkansas (Gipson et al. 1975), Missouri (Elder & Hayden 1977), and Oklahoma (Freeman & Shaw 1979). These were the first states coyotes had to migrate through to colonize the southeastern United States. We only compared our samples from

Texas to coyotes east of these states. If red wolf/coyote hybrids originally colonized the southeastern states, we could be underrepresenting red wolf ancestry throughout the southeast because ancient red wolf genetic variation from these hybrids would appear as southeastern coyote genetic variation not red wolf.

We identified two main maternal lineages on Galveston Island based on the two main mtDNA haplotypes. Interestingly, these haplotypes were divided spatially with one lineage in the west and one in the east. From these, we identified four main family groups, where some individuals did not assign to any group. This may be due to sampling bias where we missed areas on the island. For instance, there was a 7 km distance on the west side of the island where we could only sample along the main road and the public beach because the rest was private land, which we did not have access to. Areas such as this created gaps in our analysis where we could be missing additional family groups. Additionally, two mtDNA haplotypes from fecal samples on the west end of Galveston Island matched to the red wolf haplotype but did not genotype. Therefore, we could be missing an important family group with high amounts of red wolf ancestry in this area. From the groups we did identify, the ones with the largest percentage of red wolf ancestry were found in The East End Lagoon Nature Preserve and the Galveston Island State Park (*Figure 1.8*). These are well managed natural areas of the island, yet our tests of whether more natural landscapes support red wolf ancestry were not conclusive. When we grouped families into developed and undeveloped areas of the island, we found that undeveloped areas held family groups with significantly higher red wolf ancestry than developed areas ($p\text{-value} = 0.023$). But this pattern was not supported when we grouped individuals by habitat characteristic between high-intensity developed, low-intensity

developed, and open habitats on Galveston Island (p-value=0.54). The inconsistency in results could be due to capture bias in sampling or a lack of independence between individuals because many individuals were related. We collected 26 canids in open habitats, 15 in low-intensity, and 9 in high-intensity developed habitats. It was more difficult to locate scats from wild canids in the City of Galveston than it was in open habitats like the State Park even though we had reports of canids in both locations.

Our abundance estimates indicate there may be around 106 individuals (0.67 canids/km²) on the island but with large confidence intervals. Coyote density can vary for a variety of reasons; in southeastern Colorado, coyote density averaged 0.29 coyotes/km² (Gese et al. 1989), but in the urban landscape of Nacogdoches, Texas coyote density was estimated at 1.38 coyotes/km² (Lombardi et al. 2017). Although our evidence supports there is gene flow between the island and the mainland, dispersal off the island is still likely challenging. An individual warrants significant risk because they must cross one of two highway bridges or swim across the strait, therefore, Galveston Island may hold a higher density of canids than similar habitats elsewhere.

The discovery of these hybrid canids has important implications for red wolves, which are critically endangered and in need of conservation action. First, hybrid populations can act as reservoirs of historic or lost genetic variation (Heppenheimer et al. 2018ba, 2020), or as a source for genetic rescue, something needed given contemporary red wolves have become inbred over time (Brzeski et al. 2014). Through genome editing tools or de-introgression strategies, admixed individuals could be bred over time to recover the extinct genotype (Amador et al. 2014). Second, the USFWS established that a

large habitat of 170,000 acres in size is needed to reintroduce red wolves in the wild (USFWS 1984). This requirement limits the available options for additional red wolf reintroductions. Conditions that maintain red wolf ancestry may be less restrictive than previously thought and areas that support hybrid populations may be able to help inform future red wolf reintroduction sites. Third, there is little known about red wolf ecology prior to historic population declines and the red wolf/coyote hybrids along the Gulf Coast represent a rare opportunity to learn about the ecological impacts and natural histories of a species thought to be regionally extinct.

One aspect that remains unevaluated is the selective benefit of red wolf/coyote hybridization and subsequent introgression. Hybridization may have provided an adaptive advantage to expanding coyotes by introducing genetic material already filtered by natural selection in southeastern environments (Heppenheimer et al. 2018b). This process has been documented in northeastern coyotes, where hybridization with eastern wolves (*Canis lupus lycaon*) has resulted in selection for larger body size and skeletal proportions (vonHoldt et al. 2016). Through adaptive introgression, individuals with red wolf ancestry may be adapted to the southeastern environment promoting persistence of red wolf genetic variation. Multi-generational admixture can produce a diversity of phenotypes and genotypes (Excoffier et al. 2009; Gruber et al. 2017; Pfennig 2021). We have determined that the Gulf Coast canid population does have a diversity of genotypes with some individuals having little to no red wolf ancestry and some having 50-60%. Important next steps are to evaluate the diversity of phenotypes within the population and identify regions of admixed genome that may provide an adaptive advantage from past red wolf introgression.

This map displays the coastline of Jamaica with 25 numbered sampling stations. The stations are distributed along the entire length of the island's coast, from the northern tip (station 1) to the southern tip (station 25). Key geographical features and locations labeled on the map include Hebechock, Bayou Vieja, Taki Island, Pelican Island, Port Antonio, Spanish Town, Jamaica Beach, and Port Antonio Bay. A compass rose in the top left corner indicates North (N), South (S), East (E), and West (W). A scale bar at the bottom center shows distances in miles, ranging from 0 to 8. The map is sourced from Esri, HERE, Garmin, FAO, NOAA, USGS, and OpenStreetMap contributors.

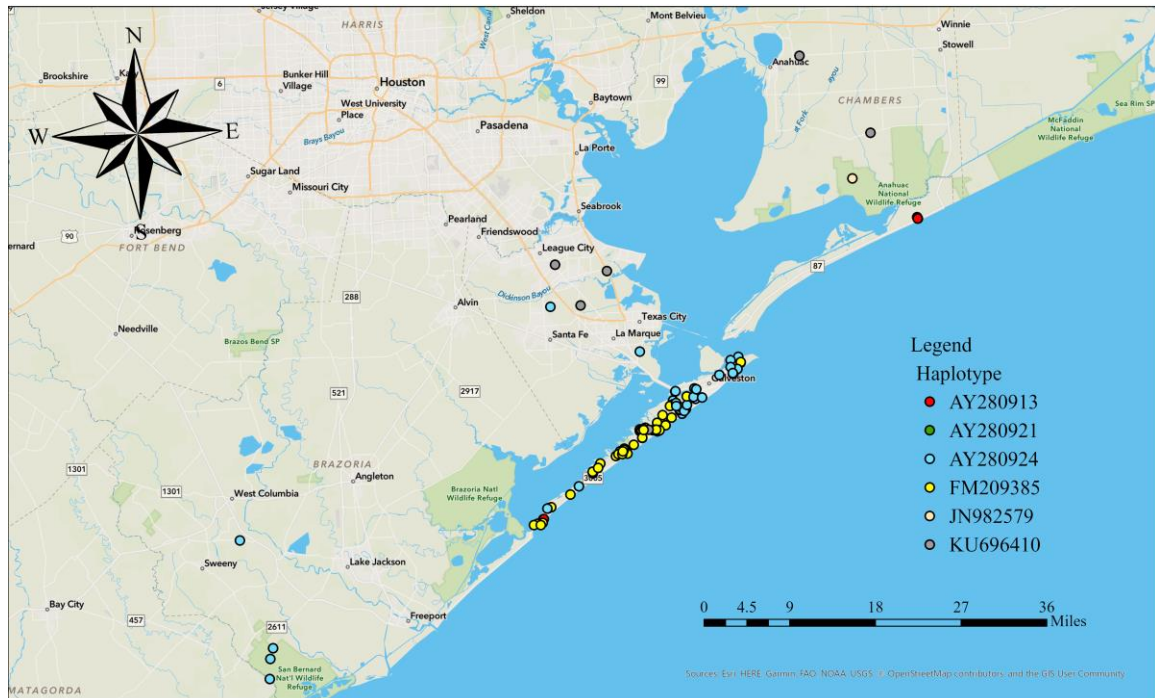


Figure 1.1.3. Mitochondrial haplotypes found on in southern Texas on and surrounding Galveston Island from coyote fecal and tissue samples. The haplotype accession names were appropriated from NCBI GenBank.

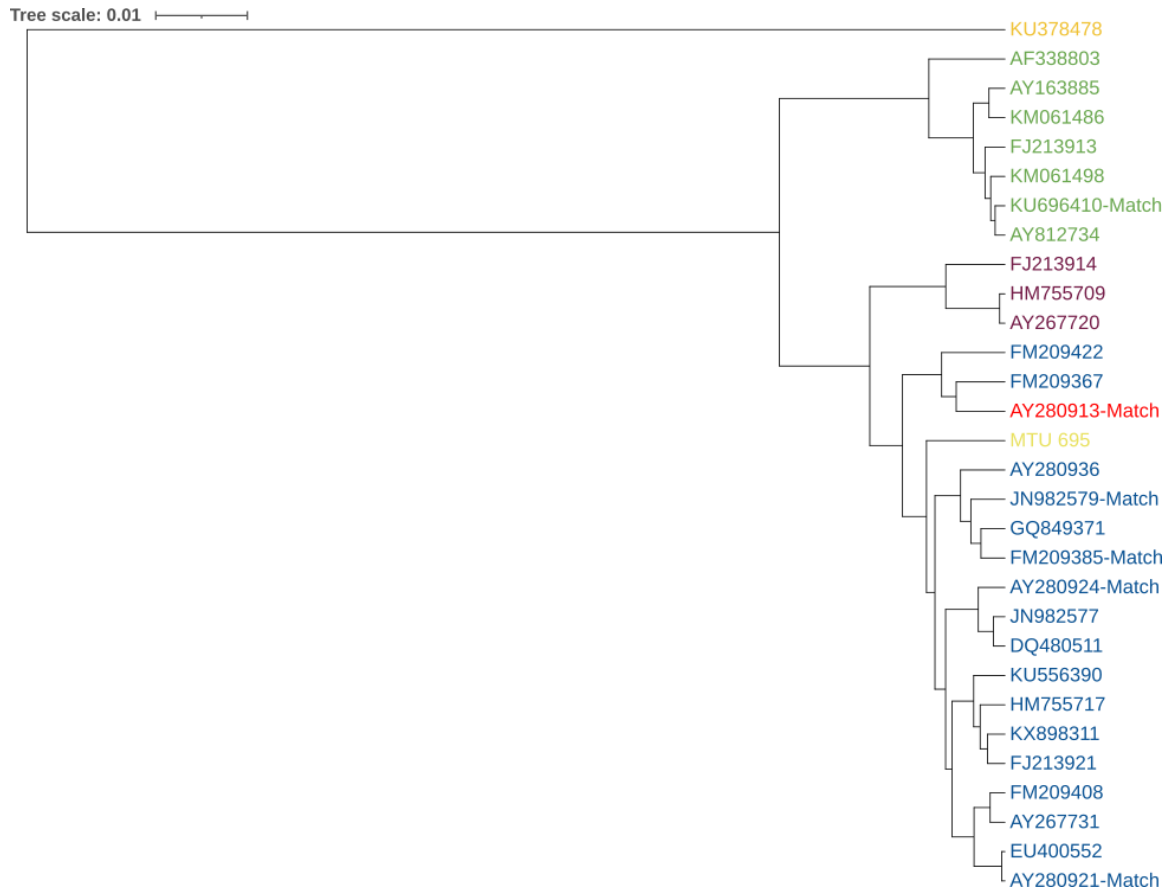


Figure 1.1.4. Mitochondrial Haplotype gene tree of the cytochrome B region of the mitochondrial control region. Accession numbers were appropriated from NCBI GenBank. The accession numbers that match our generated sequences are shown here. Dark yellow represents the outgroup red fox (*Vulpes vulpes*), green is gray wolf (*Canis lupus*), purple is eastern wolf (*Canis lupus lycaon*), blue is coyote (*Canis latrans*), red is red wolf (*Canis rufus*), and yellow is a new coyote haplotype we found.

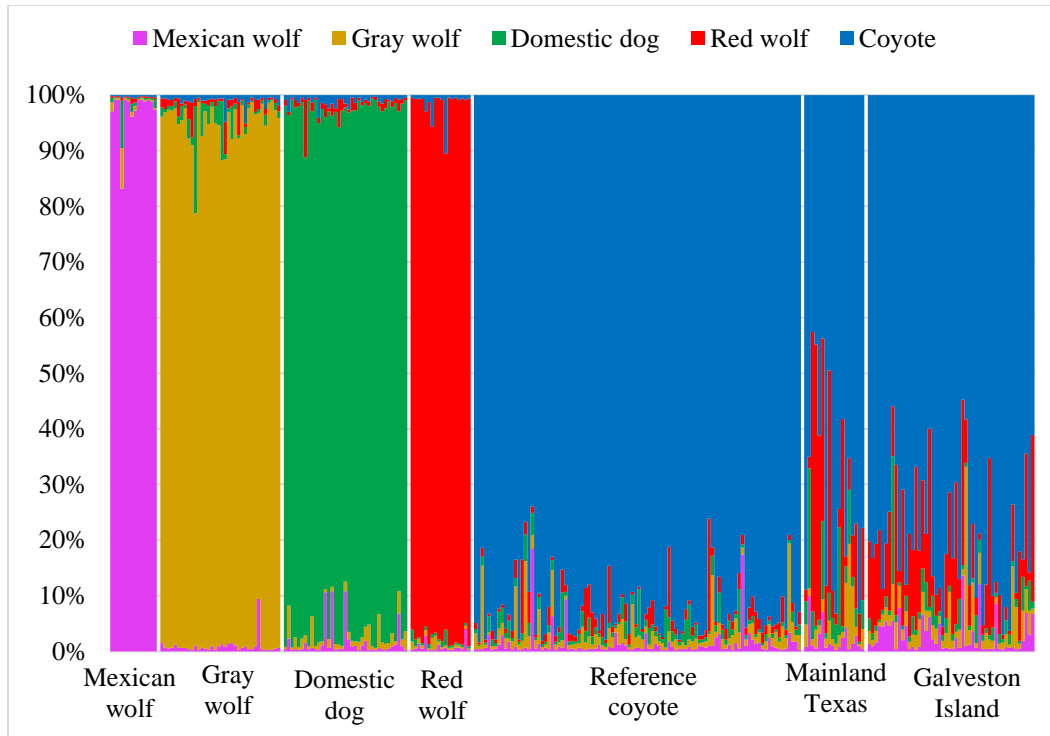


Figure 1.1.5. Bar plot of estimated *Canis* nDNA ancestry coefficients from the STRUCTURE model of five genetic clusters (K=5). Each bar represents ancestry coefficients for one individual. The populations correspond to domestic dogs (*C. lupus familiaris*), gray wolves (*C. lupus*), Mexican wolves (*C. lupus baileyi*), red wolves (*C. rufus*), and coyotes (*C. latrans*) from five southeastern states. Our samples from mainland Texas and Galveston Island are the last two populations.

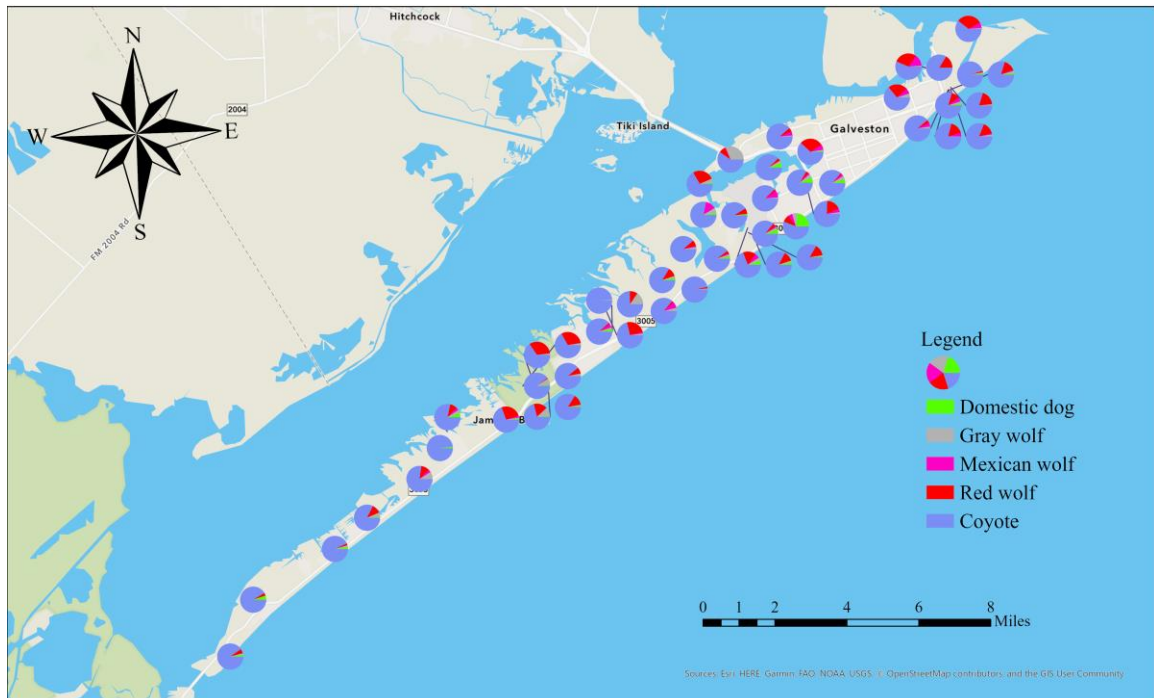


Figure 1.1.6. Map of how red wolf ancestry is distributed across Galveston Island, Texas. Ancestry estimates inferred using the program STRUCTURE. Each pie chart represents a single canid.

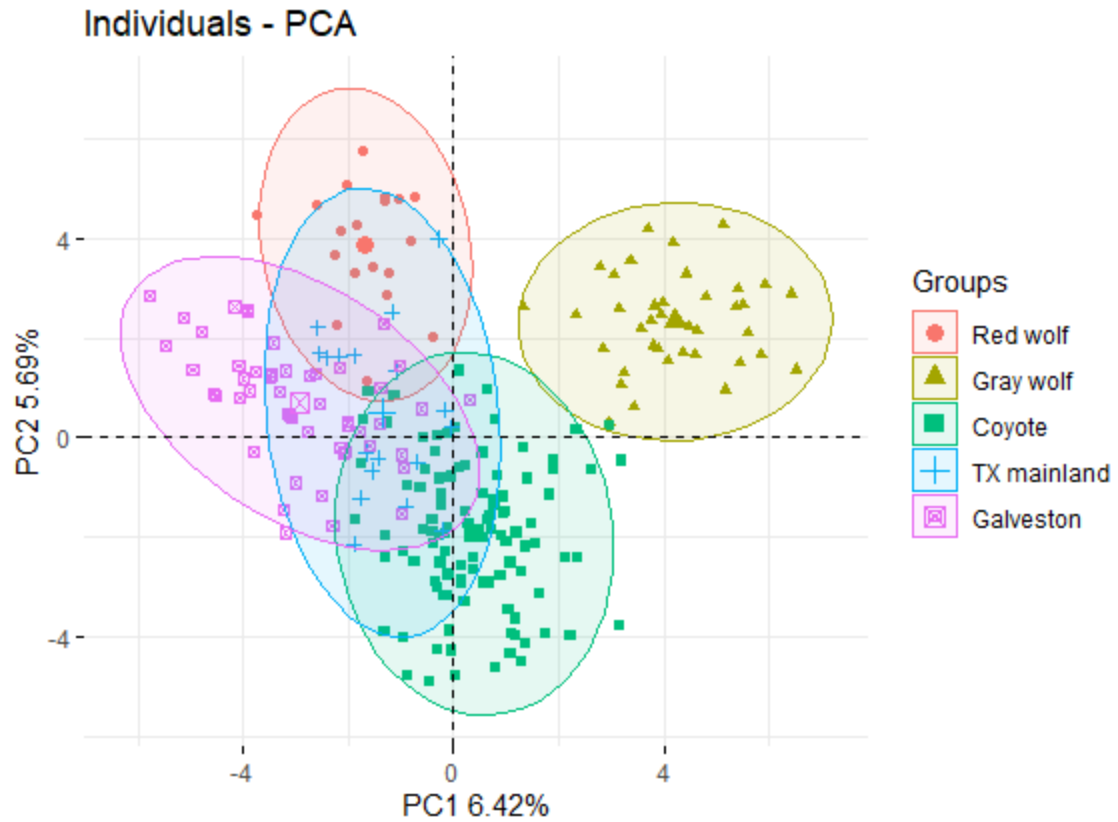


Figure 1.1.7. Results from the principal component analysis of sampled *Canis*. Red wolf (*Canis rufus*) reference group red circles, gray wolf (*Canis lupus*) yellow triangles, reference coyotes (*Canis latrans*) green squares, Texas mainland samples blue cross, Galveston Island, Texas samples purple shape.

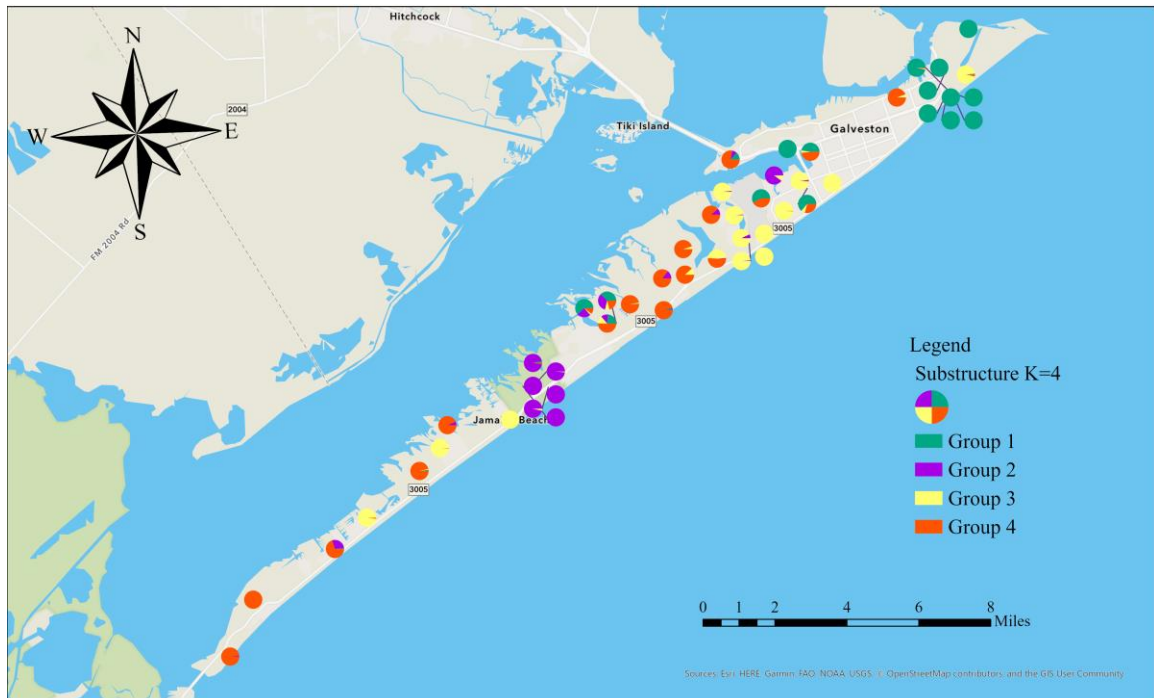


Figure 1.1.8. Population genetic substructure ($K=4$) from program STRUCTURE of individuals on Galveston Island, Texas.

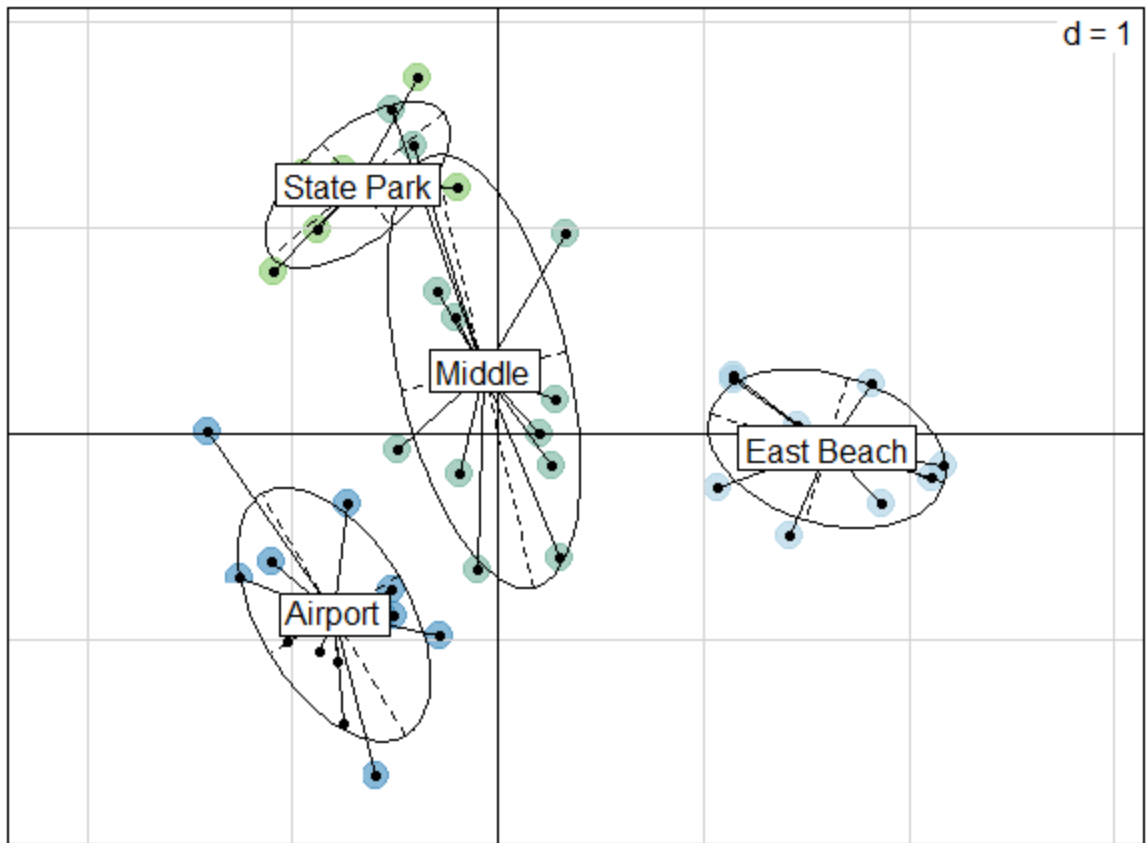


Figure 1.1.9. Principal component analysis showing deviation of the family groups on Galveston Island, Texas.

1.7 Tables

Table 1.1.1. Diversity statistics for each reference group of *Canis* in North America. Summary statistics derived from 17 microsatellites include: H_o : observed heterozygosity, H_e : expected heterozygosity, A_R : allelic richness, N_{PA} : number of private alleles, F : fixation index, F_{IS} : inbreeding coefficient.

Population	n	H_o	H_e	A_R	N_{PA}	F	F_{IS}
Red wolf	19	0.657 (0.03)	0.630 (0.026)	3.75	1	-0.050 (0.035)	-0.015
Gray wolf	38	0.634 (0.031)	0.720 (0.027)	4.41	0	0.121 (0.026)	0.1328
Domestic dog	38	0.583 (0.046)	0.686 (0.047)	4.45	5	0.142 (0.035)	0.1641
Mexican wolf	14	0.433 (0.068)	0.428 (0.060)	2.38	1	-1.85E^-4 (0.056)	0.0250
Reference coyote	107	0.717 (0.026)	0.805 (0.017)	5.57	22	0.110 (0.023)	0.1142
TX Mainland	7	0.730 (0.050)	0.723 (0.024)	4.67	3	-0.005 (0.059)	.0678
Galveston Island	50	0.719 (0.029)	0.741 (0.023)	4.65	3	0.027 (0.029)	0.041

Table 1.1.2. Estimated nDNA pairwise F_{ST} among *Canis* species and populations grouped by U.S.A. State calculated using package hierfstat in R.

17 Loci	Mexican wolf	Gray Wolf	Domestic dog	Red wolf	Reference coyote	TX Mainland	Galveston Island
Mexican wolf	--						
Gray wolf	0.3169	--					
Domestic dog	0.3437	0.1062	--				
Red wolf	0.3634	0.1678	0.1791	--			
Reference coyote	0.2670	0.0879	0.1215	0.1397	--		
TX Mainland	0.3067	0.1475	0.1721	0.1500	0.0878	--	
Galveston Island	0.3031	0.1361	0.1684	0.1472	0.0700	0.0973	--

Table 1.1.3. Samples with private alleles (NPA) and the percentage of red wolf ancestry estimated using STRUCTURE.

Galveston Island	Red wolf ancestry (%)	NPA	Mainland Texas	Red wolf ancestry (%)	NPA
023	14.7	1	556	33.1	1
289	9.5	1	932	5.7	2
316	24.5	1			
500	8.7	1			
672	11.5	1			
759	1.1	1			

1.8 Literature Cited

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2 The natural history, ecology, and conservation of *Canis* in the southeastern United States: a systematic review

2.1 Abstract

Two recent studies rediscovered red wolf alleles that have persisted in coyote populations decades after they were extirpated. This prompted us to do a systematic review of red wolf natural history, ecology, life history traits, and their complicated relationship with coyotes. Here we talk about the similarities and differences between these closely related species, and outline areas where information is lacking in the primary literature for directions of future research.

2.2 Introduction

The red wolf is protected under the United States Endangered Species Act 1973 (ESA) and is arguably the most imperiled wolf species in the world. In the 1970s, the last known red wolves were believed to have resided in the agricultural-marsh complexes of southeastern Texas and southwestern Louisiana, USA. The population was purposely removed from the wild by 1980 by the United States Fish and Wildlife Service (USFWS) over concerns of extinction from disease and hybridization with expanding coyote populations. These efforts led to a robust red wolf captive breeding program and eventual reintroductions into coastal North Carolina. Although declared extinct from the wild, there continued to be reports of red wolves in rural regions of coastal Texas and Louisiana (Heppenheimer et al. 2018a) as coyotes expanded their populations into the red wolf's former range. Although previous efforts to detect surviving red wolves were unsuccessful, two recent independent studies discovered substantial amounts of red wolf

ancestry in coyote populations occurring along the coastal areas of Texas and Louisiana (Murphy et al. 2018; Heppenheimer et al. 2018a). Both studies discovered red wolf alleles that could represent a reservoir of previously lost red wolf ancestry. The existence of red wolf “ghost” alleles suggests that substantial red wolf ancestry has naturally persisted in some isolated areas for over 40 years through means not yet well understood. The ESA lays out a clear path to mitigate key threats to the red wolf, but it lacks explicit policy providing protection for admixed individuals (e.g., red wolf-coyote hybrids) that serve as a reservoir for extinct genetic variation (Haig & Allendorf 2006; Placyk et al. 2012; Fitzpatrick et al. 2015). These recent discoveries along the American Gulf Coast bolster the need to adapt and implement a hybrid policy under the ESA to prevent further reductions of the remnant wild red wolf gene pool (Haig & Allendorf 2006; vonHoldt et al. 2018).

Additionally, there are considerable gaps in our knowledge about red wolf natural history that makes understanding the wolf’s historic role in the southeastern United States (hereafter southeast) difficult (Hinton et al. 2013). This is complicated by hybridization with coyotes and the contemporary complete range overlap of southeast coyotes and red wolves. Nearly all research (e.g., ecological, genetic, management) is limited to small remnant and declining populations that were replaced by coyotes along the Gulf Coast of Texas and Louisiana (Nowak 1967; Riley & McBride 1971; Paradiso & Nowak 1972), the captive breeding program (Kalinowski et al. 1999; Rabon & Waddell 2010; USFWS 2013; Rabon 2014), and the reintroduced North Carolina population (Sparkman et al. 2011a, 2011b, 2012; Hinton & Chamberlain 2014; Bohling & Waits 2015; Brzeski et al. 2015; Hinton et al. 2015, 2016, 2017; Sparkman et al. 2017). Recently, the USFWS has

been tasked with the duty to update their red wolf recovery plan. Therefore, a comprehensive review of red wolf natural history, ecology, life history traits, and its relationship with coyotes will help our ability to differentiate between the species and their respective niches in the southeast to enact efficient management and conservation strategies for the endangered red wolf.

Our objectives are to review and synthesize the primary literature of red wolves and their relationship with coyotes in the southeast, to provide a succinct record of similarities, and more importantly differences in historic ranges, morphology, food habits, reproduction, and competition between the two species. We used this information to highlight gaps in our current knowledge about *Canis* in the southeast for future studies and to make inferences about the southeast based on closely related taxa in other parts of North America. We do not review red wolf taxonomy, as that has been done (Hinton et al. 2013; Waples et al. 2018; NAS 2019) and the red wolf is listed as a legally distinct species, but instead focus on historical accounts, geographic range, and phenotypic variation when discussing the taxonomy of *Canis* in the southeast.

2.3 Natural History of *Canis* in the southeastern United States

Historical and fossil records

The earliest recognized *Canis* fossils are from western North America dated to the late Hemphillian era (6 Ma) (Nowak 1979; Tedford et al. 2009). Members of the Genus *Canis* spread across North America and eventually migrated into South America after the opening of the Panamanian Isthmus (3 Ma) and into Eurasia across the Bering land bridge (2 Ma) (Nowak 2002; Tedford et al. 2009; Chambers et al. 2012). In North

America, *Canis* followed an evolutionary progression of increasing size from a small fox sized species *Canis ferox* to the largest known species *Canis dirus*. The small coyote-sized animal, *Canis lepophagus* is believed to be the common ancestor (2-4 Ma) of *Canis latrans* and of a small wolf-like animal, *Canis edwardii* (synonymous with *Canis priscolotrans*) (Nowak 1979; Nowak 2002; Tedford et al. 2009). The first wolf-sized canid *Canis armbrusteri* appeared in the late Irvingtonian Era (0.5 Ma) thought to have evolved from *Canis edwardii* or an ancestor and ranged across North America including the southeast. Later during the late Rancholabrean era (0.011 Ma) the large wolf *Canis dirus* replaced *Canis armbrusteri* and had a distribution throughout North and South America (Nowak 1979; Nowak 2002; Tedford et al. 2009). The enormous size of *C. dirus* opened ecological space for a smaller canid to occupy during the late Pleistocene, and *C. latrans* is recorded in the fossil record in eastern North America, where evidence suggests they were larger and more robust than they are today (Meachen & Samuels 2012; Meachen et al. 2014; Tomiya & Meachen 2018). At the end of the Pleistocene (0.010 Ma), both *Canis dirus* and *Canis latrans* disappeared from the east but a small wolf persisted (Nowak 2002).

The fossil record is still unclear where the red wolf of eastern North America originated, but multiple hypotheses have been proposed: 1) they migrated from Eurasia from an ancient ancestor of *Canis lupus*, possibly *Canis mosbachensis*, became isolated by glaciation and speciated (Nowak 2002), 2) they are the product of recent hybridization between the Eurasian *C. lupus* and North American *C. latrans*, or 3) they evolved during the Late Pleistocene from a population of *C. latrans* or a close ancestor in the eastern United States (Nowak 1979; Nowak 2002; Chambers et al. 2012).

After *C. dirus* and *C. latrans* disappeared from eastern North America, the modern coyote ranged throughout western North America (Bekoff 1977), evolving differential characteristics such as reduced body size and teeth structure where they were sympatric with *C. lupus*, reflecting their separate ecological niches (Nowak 1979). During the Pleistocene, *C. lupus* was mainly a Holarctic species (except for a far-removed lineage in the southwest, *Canis lupus baileyi*). However, during the last Ice Age, glaciers forced their populations south into central and eastern North America, where fossils of *Canis lupus* can be found alongside *C. dirus* (Nowak 1979; Graham & Lundelius 1984; Nowak 2002). At this time *C. lupus* populations would have also met with *C. rufus* populations. These two species, although distinct, are close both in size and morphology, thus making it possible they mated during the late Pleistocene / early Holocene (Chambers et al. 2012). However, as glaciers receded, *C. lupus* followed their larger prey (e.g., moose, caribou) north to areas such as the Great Lakes Region or eastern Canada leaving *C. rufus* in the southeast, but possibly bringing with them *C. rufus* traits from adaptive introgression (Nowak 2002; Hedrick 2013).

C. rufus and *C. latrans* remained allopatric for most of their history, however, where their ranges met in the central United States, limited hybridization may have continued to occur leading to incomplete lineage sorting, as *Canis rufus* was smallest on the western edge of their range (Goldman 1937, 1944). *C. latrans* returned to eastern North America in the 19th and early 20th centuries as *C. rufus* populations were extirpated and forest cover was converted into agriculture (Ramankutty & Foley 1999; Nowak 2002). Since the 21st century, *C. rufus* is only present on the Albemarle Peninsula of

North Carolina, while *C. latrans* are ubiquitous across most of North and Central America.

Historical range of the red wolf

The Pre-Columbian (10,000 Bp – 1600) red wolf inhabited the southeastern United States from the Atlantic coast, west to central Texas, north to the Ohio River Valley, northern Pennsylvania, and southern New York, and south to the Gulf of Mexico and southern Florida (Nowak 1979, 2002, 2003). The earliest known scientific descriptions of the red wolf occurred during the late-18th and early-19th centuries when geographic variation among the wolves of North America was first being examined (Say 1823). The two earliest forms to be described were the eastern form, *Canis niger*, in the Florida region (Bartman 1791) and a Texas form now referred to as *Canis rufus rufus* (Say 1823). During the mid-20th century, both red wolf forms were combined into a single species, *C. niger*, and for the first time, a third form was described as the Mississippi Valley wolf (*Canis rufus gregoryi*) (Goldman 1937, 1944).

The red wolf was under threat almost immediately after North America was settled by Europeans as they were most likely the first North American wolf species encountered. Their extirpation from the northern portion of their historic range is not well documented. For instance, there are no historic names applicable to wolves east of the Mississippi River and north of Georgia, as none were published (Rhoads 1903; Nowak 1967, 1972). From the few available collections of State and County records, it appears the red wolf perished from the most northern regions of its range by the 20th century. In New Jersey, there are no records of wolves after 1800, although they survived on the

fringes of Pennsylvania until 1890 (Rhoads 1903), and the forests of Illinois and Indiana served as the last wolf stronghold in the northern range into the 20th century. In the south, red wolves were extirpated from South Carolina by 1860 and retreated to the mountains of Virginia, North Carolina, and Georgia before perishing by the early 1900s (Kellogg 1937; Nowak 1972). By 1920, red wolves were extirpated from the south Atlantic States as well as most other parts of their range (Nowak 1972; USFWS 1990). In the late 1930s, only two populations existed, one in the Ozark and Ouachita Mountains of Arkansas, eastern Oklahoma, and southern Missouri, and the other in the extensive river bottom forests and coastal regions of southern Louisiana and southeast Texas (Nowak 1972; USFWS 1990).

The last remaining red wolves were forced into marginal habitat along the Mississippi River Basin down to the Gulf Coast region of Texas and Louisiana where the greatest number of wolves occupied the coastal prairies and inland forests of the Gulf Coast states (Nowak 1972). Here in the western part of their range, red wolves survived into the mid-20th century (Riley & McBride 1972), but local extirpation promoted isolated hybridization with expanding coyote populations, especially in the states of Texas (Paradiso & Nowak 1971; Riley & McBride 1972), Louisiana (Nowak 1967; Goertz et al. 1975), Arkansas (Gipson et al. 1974), Missouri (Elder & Hayden 1977), and Oklahoma (Freeman & Shaw 1979). By the 1970s, red wolves were extirpated throughout most of their range and approximately only 100 individuals were believed to be left and the species nomenclature was updated to *Canis rufus*. (McCarley 1959; McCarley 1962; Pimlott 1965).

Recognizing that the red wolf was at risk of extinction, the USFWS included red wolves in the first cohort of species listed as endangered under the ESA (Carley 1975; McCarley et al. 1979; USFWS 1990; Hinton et al. 2013). Concerned the last known red wolf populations would become genetically swamped due to red wolf hybridization with coyotes, the USFWS established the Red Wolf Recovery Program to capture the last remaining red wolves in Texas and Louisiana to preserve the species via a captive breeding program (USFWS 1989; Phillips et al. 2003; Hinton et al. 2013). They captured over 240 canids from 1973-1980 of which 43 met the morphological standards to be considered red wolves (Table 1); of which only 14 became the founders of all contemporary red wolves (USFWS 1990). After 1980, the red wolf was declared extinct in the wild, becoming the first species to be purposely extirpated in the wild to save it from extinction (Phillips et al. 2003; Hinton et al. 2013). Red wolves were reintroduced as a nonessential experimental population to Alligator National Wildlife Refuge on Albemarle Peninsula, North Carolina in 1988, becoming the only place where a wild population exists today (Hinton et al. 2013). The population grew to 140 in the early 2000s but due in part to anthropogenic mortality events (Bohling et al. 2015; Hinton et al. 2015), the population has declined to as few as 9 wild wolves in July 2020 (USFWS 2021).

2.4 *Canis* ecology in the southeastern United States

Morphology

The use of morphometrics is a valuable tool in exploring morphological differences among closely related taxa and can provide insights into a species' ecology. For example, skull morphology and dentition are strongly associated with feeding

adaptations. The Biological Survey, a large mid-20th century survey focused on canids in North America, reported three distinct members of *Canis*: the coyote, the gray wolf, and the red wolf, consistent with prior taxonomic findings (Goldman 1937, 1944; Jackson 1951). These initial efforts led to further research focused on developing diagnostic characteristics using cranial measurements and multivariate statistics (Lawrence & Bossert 1967; Atkins & Dillion 1971; Paradiso & Nowak 1971; Gipson et al. 1974; Elder & Hayden 1977; Nowak 1979, 2002). All these studies successfully separated early red wolf populations from the other species of North America *Canis*, although with conflicting conclusions. Lawrence and Bossert (1967) concluded that red wolves were distinct morphologically and more similar to *C. lupus* than to *C. latrans*, a conclusion that contrasted with Goldman's (1937, 1944) conclusion that red wolves more closely approached *C. latrans* in morphology than *C. lupus*. Similarly, Paradiso & Nowak (1971) reported that the red wolf approaches the gray wolf in size but is more like the coyote in tooth structure. Using multivariate analyses evaluating interspecific and intraspecific variation in skull and dental characters, Nowak (1979, 2002) compared modern *Canis* to fossil specimens to conduct the most comprehensive taxonomic analysis on North American *Canis* and concluded that the consistent separation and lack of overlap in space among the skulls of red wolves, gray wolves, and coyotes showed that the red wolf was a morphologically unique lineage dating back to the early Pleistocene. Despite the differing conclusions, all studies agreed the red wolf had morphological measurements between those of gray wolves and coyotes.

Additional morphological characteristics of the red wolf are largely described based on the founders of the captive breeding program, reports mostly from Texas and

Louisiana, and of historic or fossil museum specimens (Riley & McBride 1972; Nowak 1979, 2002; Chambers et al. 2012; Hinton & Chamberlain 2014). Given most contemporary measurements are solely based on individuals from the western edge of the red wolf's historic range, the reported morphological variation likely does not represent the complete historic variation of the species.

Unlike the red wolf, coyotes show great variation in size and morphology across their range. In general, western coyote populations are the smallest, followed by southeastern and northeastern populations, respectively (Hinton et al. 2019). This variation in body size could be the result of different selective pressures as well as hybridization with other *Canis* species (Hinton et al. 2018; Heppenheimer et al. 2018b). For example, northeastern coyote phenotypes are influenced by hybridization with Eastern wolf populations in Ontario (Hinton et al. 2018). However, the genetic structure of coyote populations indicates northeastern coyotes only occur north of the Virginia/North Carolina border (Bozarth et al. 2011; Heppenheimer et al. 2018a, b; Hody & Kays 2018), suggesting changes in phenotypes of southeastern coyote populations occur due to factors not influenced by northeastern coyotes.

Red wolves and coyotes are both sexually dimorphic, with males larger in size and weight than females. The red wolf can range from 16 to 39 kg (kilograms) with average weights from 20 to 28 kg (Table 2) (Riley & McBride 1972; Paradiso & Nowak 1972; Davies 1974; Hinton & Chamberlain 2014). The coyote rarely exceeds 18 kilograms, but maximum body mass can range between 20.2-25.9 kg. Total lengths of red wolves from the Gulf Coast are between 120-165 cm (Paradiso & Nowak 1972; Riley &

McBride 1972; Davis 1974). In North Carolina, the average body lengths of red wolves were 106 (75.0-125.0 cm), and tail length 36.4 (25.8-48.0 cm) for an average body length of 142.8 cm (Hinton & Chamberlain 2014). Body size in North Carolina may be impacted by inbreeding depression (Brzeski et al. 2014) or founder effects associated with the captive breeding population bottleneck. Throughout their range coyotes are about 100-150 cm in total length (Bekoff & Gese 2003; Trani & Chapman 2007). Red wolf ear length for both males and females reported by Riley and McBride (1972) was between 11.4-13.9 cm and reported by Hinton and Chamberlain (2014) 11.0 cm (9.0-12.9 cm). Compared to coyotes 8.6-12.2 cm and 9.9 (8.0-12.8) reported respectively. Red wolf hindfoot length 20.3-24.9 cm (Riley & McBride 1972) and 22.3 cm (17.0-27.0 cm) (Hinton & Chamberlain 2014). Coyote hindfoot length 17.7-21.6 cm (Riley & McBride 1972) and 18.7 cm (16.4-22.5 cm) (Hinton & Chamberlain 2014).

Characterizing detailed morphological differences between red wolves and coyotes is important when examining potential hybrids and their ecological role now on the landscape in the absence of red wolves. In other hybrid systems, intermediate sized hybrid offspring were ecologically different from their parents (Skovrind et al. 2019). Red wolf-coyote hybrids represent an intermediate size canid on the landscape (Riley & McBride 1972; Hinton & Chamberlain 2014), which may behave different and impact their ecological communities differently than coyotes, thus helping us understand the historic role of the red wolf in the southeast.

Melanism

Historically (1630-1880) melanism, the darkening of the body caused by excessive production of melanin, used to be a diagnostic feature in distinguishing red wolves from coyotes (Nowak 1967; Paradiso & Nowak 1972; Gipson 1976; Trani & Chapman 2007). The dominant variant which causes melanism in *Canis* is the K^B allele (CBD103) and is believed to originate in domestic dogs (Ollivier et al. 2013). The average age of the K^B allele is estimated to be 47,000 years ago (13,000-121,000), (Hedrick 2013; Ollivier et al. 2013) which overlaps with estimated dog domestication 15,000-40,000 bp (Vila et al. 1997; Savolainen et al. 2002). In wild *Canis*, melanism has been linked with habitat type (Jolicoeur 1959; Gipson et al. 2002; Anderson et al. 2009), likely for concealment, and in gray wolves has been associated with a decrease in aggressive behavior (vonHoldt et al. 2020). The trait associated with melanism in gray wolves has been traced to the K^B mutation in the melanocortin pathway that appeared 12,000-120,000 years ago (Anderson et al. 2009). Schweizer et al. (2018) linked this mutation to introgression with early Native American dogs in the Northwestern Territories or possibly Yukon, Canada. This is consistent with the distribution of melanistic wolves in western North America reported by Anderson et al. (2009). The same 3 base pair deletion at the K^B site has been found in other *Canis* populations including the Golden Jackal (*Canis aureus*) (Ambarli & Bilgin 2013; Galov et al. 2015) and European wolf populations (Caniglia et al. 2013; Randi et al. 2014), all consistent with introgression with domestic dogs (Ollivier et al. 2013).

Melanism in coyotes is not as straightforward. Melanism caused by the K^B mutation is virtually nonexistent in western coyotes where gray wolves, dogs, and coyotes overlap, but present in eastern populations where eastern wolves and coyotes co-occur (Rutledge et al. 2009). One hypothesis is the K^B allele is found in coyotes in the northeast because it is a natural hybrid zone between gray wolves, eastern wolves, and coyotes. If true, it explains why melanism is almost nonexistent in western coyote populations but does not explain why melanism is common in southeastern coyote populations.

Coat coloration can vary widely in red wolves which have been described as gray, black, red, and yellow (Riley & McBride 1972), but they are usually a mixture of cinnamon-buff, cinnamon, tawny, or reddish-buff interspersed with gray and black (Howell 1921; Riley & McBride 1972; Trani & Chapman 2007). Today, there are no cases of melanism in the extant red wolf population, which is a direct result of a population bottleneck where this adaption was lost (Hinton et al. 2015). In coyotes, the banded nature of their hair shows great variation in color, ranging from almost pure gray to rufous, but coyotes are typically a blend of gray mixed with a reddish tint (Bekoff & Gese 2003; Schmultz et al. 2007). Melanism is a relatively new phenomenon in coyotes, and it appears more common in southeastern populations than elsewhere in their range (Gipson 1976; Mowry & Edge 2014; Caudill 2015). The frequency of melanism in southeastern coyote populations is unknown, but multiple studies have reported it occurs in approximately 10% of the population (Gipson 1976; Mowry & Edge 2014; Caudill 2015). Early reports of melanistic coyotes started in the late 1800s, in the states of Alabama (Howell 1921), Ohio (Preble 1942), Arkansas (Black 1936; Dellinger & Black

1940; Gipson 1976), and Tennessee (Rhoads 1896; Kellogg 1939). These early reports were from areas known to have been inhabited by melanistic red wolves (Gipson 1975).

These results suggest melanism in southeastern coyotes could be the result of hybridization with red wolves rather than domestic dogs. In other mammals, mutation at different regions of the genome has resulted in similar color variation. For example, melanism in rock pocket mice (*Chaetodipus intermedius*) has been shown to evolve in two different populations independently from mutations at different genes (Nachman et al. 2003). Further research is necessary to understand the genes related to melanism in southeastern coyote populations and its link to red wolf hybridization.

Food habits

The red wolf and coyote are both generalist opportunistic predators (McVey et al. 2013), and therefore their diets overlap (McVey et al. 2013). Diets of carnivores are important to understand as predator food habits can have compounding impacts on prey populations (Hairston et al. 1960; Paine 1969; Mills et al. 1993; Schmitz et al. 2000). Diets of generalist carnivores are highly dependent on spatial and temporal differences (McVey et al. 2013). Therefore, sympatric diet studies reduce spatial and temporal variability and provide individual data on differences between species. The data on the diet of the red wolf is limited due to the limited range in which they exist (McVey et al. 2013). Conversely, coyote diets have been extensively studied throughout their western range (Murie 1945; Bekoff 1977; Andelt et al. 1987) as well as in the southeast (Gipson 1974; Michaelson 1975; Hall 1979; Smith & Kennedy 1983; Wooding 1984; Lee 1986;

Blanton & Hill 1989; Hoerath 1990; Wagner 1993; Wilson 1993; Thornton et al. 2004; Schrecengost et al. 2008; Watts & Frick-Ruppert 2018).

Although gray wolves are known to consume large ungulate prey, many of which are of European origin (Young 1944; Chambers et al. 2012), red wolves are small to medium mammal specialists. In Texas, they were reported to feed on nutria (*Myocastor coypus*), swamp rabbit (*Sylvilagus aquaticus*), cottontail rabbit (*Sylvilagus floridanus*), rice rat (*Oryzomys palustris*), cotton rat (*Sigmodon hispidus*), muskrat (*Ondatra zibethicus*), and raccoon (*Procyon lotor*) (USFWS 1982), they also killed hogs (*Sus scrofa*) and white-tailed deer (*Odocoileus virginianus*). Some of these prey items seemed less important in North Carolina, where Phillips et al. (2003) found white-tailed deer, raccoons, and marsh rabbits (*Sylvilagus palustris*) constituted 86% of their diet. Additionally, scats from red wolves that were translocated to Cape Romain National Wildlife Refuge, South Carolina contained fox squirrels (*Sciurus niger*), American coot (*Fulica americana*), as well as other birds and small mammals (USFWS 1982). In North Carolina, red wolf packs consumed mostly mammalian prey. Dellinger et al. (2011a) found white-tailed deer accounted for 37-66% of pack diet depending on the metric of diet composition. The difference in diet between the states is likely attributed to prey availability in the habitat they occupy. North Carolina is the only space where red wolves and coyotes coexist and there have been multiple diet studies on these species (Phillips et al. 2003; Dellinger et al. 2011a; McVey et al. 2013; Hinton et al. 2017), and in general red wolves are less omnivorous than coyotes (USFWS 1982; Andelt & Knowlton 1987; Chamberlain & Leopold 1999; Schecengost et al. 2008; McVey et al. 2013; Hinton et al. 2017; Watts & Frick-Ruppert 2018).

Across their range, coyotes exhibit frequency-dependent switching strategies (Patterson et al. 1998; Bartel & Knowlton 2004) because their moderate body size permits broad use of prey (Gese et al. 1996a; O'Donoghue et al. 1998a; Lingle 2000; Cherry et al. 2016b; Benson et al. 2017). When prey is abundant, they switch their diet based on optimal foraging models (MacCracken & Hansen 1987) but in unpredictable environments, they feed opportunistically (Boutin & Cluff 1987). There is considerable overlap in the types of food items coyotes consume throughout the southeast (Schencengost et al. 2008). For instance, southeast coyotes feed on small and large mammals, fruit and other plant material, arthropods, a variety of birds, and many other organisms (Chamberlain et al. 1999; Thornton et al. 2004; Schrecengost et al. 2008; Kilgo et al. 2012; McVey et al. 2013; Swingen et al. 2015; Watts & Frick-Ruppert 2018). Coyotes can kill adult white-tailed deer (Gompper 2002; Kays et al. 2010), but they prey proportionally more on neonates (Huebschman et al. 1997; Patterson & Messier 2000; Smith et al. 2003; Berger et al. 2008; Kilgo et al. 2010; Kilgo et al. 2012; Hinton et al. 2017). In urban habitats, they are adept at exploiting human-mediated resources and will readily consume garbage, dog food, carcasses, and other human-related items (Bekoff & Gese 2003; Cherry et al. 2016). Coyotes will overwhelmingly forage on fruits and arthropods in the spring and summer months, where Schecengost et al. (2008) found coyotes diets in South Carolina were dominated by plant matter from May to November 2005 and June through July 2006. In North Carolina, McVey et al. (2013) reported fruit and arthropods seemed relatively unimportant, however, they noted they collected scats that consisted entirely of orthopterans or persimmon and blackberry seeds but couldn't include in their analysis because the lack of fecal matter prevented usable DNA for

species identification, suggesting they underestimated arthropods and vegetation in coyote diets.

Red wolf and coyote diets overlap to a degree that suggests the coyote may have partially filled the niche historically occupied by red wolves across the southeast (Hill et al. 1987; Thurber & Peterson 1991; Gompper 2002; McVey et al. 2013; Cherry et al. 2016). However, replacing the ecological conditions that red wolves provided is not as easy as replacing one canid with another. It is important to consider the differences in diet and habits of prey and prey populations when assessing niche realizations. Part of the coyotes' considerable success in human-dominated landscapes compared to other canids can be attributed to its flexible mesopredator feeding strategy. This feeding strategy, although successful, does not resemble an apex predator of top-down feeding strategies attributed to larger canids and apex predators. Although coyotes do limit some mesopredators such as red foxes (Sargeant et al. 1987; Harrison & Sherburne 1989; Gese et al. 1996; Crooks & Soule 1999; Gehrt & Clark 2003), evidence suggest that they do not limit others such as raccoons (Gehrt & Clark 2003) and in fact, their mesopredator nature may have compounding effects on species such as ground-nesting birds (Rogers & Caro 1998). Evidence in North Carolina shows red wolves do prey upon mesopredators such as raccoons (Phillips et al. 2003; McVey et al. 2013; Hinton et al. 2017). Additional studies need to focus on the indirect effects red wolves have on mesopredators through avoidance strategies and how this may benefit other species (mussels, crabs, birds), thus more clearly elucidating niche partitioning between red wolves and coyotes.

2.5 Life history traits of *Canis* in the southeast

Life history traits are an adaptive suite of demographic variables associated with age-specific survivorship, fecundity, and generation times as fundamental determinants of population performance (Pianka 1970; Roff 1992; Stears 1992). In large carnivores, mating and social structure have a significant influence on population dynamics such as habitat selection, reproduction, and behavior. Canids have multiple phases of social organizations throughout their life histories, classified as transients, solitary residents, resident mated pairs, and packs (Bekoff & Wells 1986). There are subtle but important life history differences between red wolves and coyotes including home range size, competition, reproduction, and behavior.

Space use

Red wolf space use was not extensively studied before their extirpation from the southeast. Therefore, understanding how the reintroduced population distributes itself as it expands is crucial to understanding how a natural population would distribute itself on the landscape (Hinton et al. 2016). However, since European settlement, the eastern United States has been transformed from vast open forests and savannahs vegetated with southern oaks, pine, and hickories (Delcourt et al. 1993; Carroll et al. 2002), to a mixture of agriculture, *Pinus* plantations, and urban development (Burkett et al. 2000; Conner & Hartsell 2002; Sohl & Sayler 2008). Additionally, the reintroduced population is genetically isolated from a small coastal area that included Liberty, Chambers, Jefferson, Brazoria, Galveston, and Harris counties in Texas, and Cameron and Calcasieu Parishes in southwestern Louisiana (Riley & McBride 1972). The habitat in these counties is not

represented of the entire species historic range. When red wolves were monitored by Russell and Shaw (1972), the highest densities were found along marsh edges and in the prairie and rice fields, few were found in the woodlands. Investigating these factors is important to understand if red wolf space use in North Carolina is an innate response to marsh and prairie habitats or a product of local adaptation from where the last red wolves were trapped.

Before reintroduction, a strong requirement by the USFWS was “a large area of habitat of at least 170,000 acres in size, preferably dedicated units of National Wildlife Refuge lands and National Park Service properties.” This requirement limited the amount of available space for the species in the southeast. However, based on all available space use studies from North Carolina, red wolves have a strong selection for agriculture lands over large contiguous forested habitats available on USFWS National Wildlife Refuges (Hinton & Chamberlain 2010; Chadwick et al. 2010; Dellinger et al. 2013; Karlin et al. 2016; Hinton et al. 2016). If agricultural land was indeed preferred this would expand available red wolf habitat as much of the land use in the southeast is used for agriculture (Ramankutty & Foley 1999; Sohl & Sayler 2008) and suggest the factors limiting red wolves may be more socioeconomic than ecological.

Coyotes are typically associated with open grasslands and native prairie ecosystems that make up their historic range in western North America (Nowak 1978; Voight & Berg 1987; Gompper 2002; Kamler et al. 2005). The conversion of eastern forests to agriculture created suitable habitat for coyotes to expand their populations east (Gipson 1974; Gompper 2002; Jantz 2011). This created interest in their space use,

habitat selection, and impacts on their new ecosystem. Coyote space use varies across their range (Bekoff 1982), but in the southeast they generally select for more open habitats because these likely facilitate their cursorial hunting strategy (Kleiman & Eisenberg 1973; Kaufman & Fleharty 1974; Kamler & Gipson 2000; Schrecengost et al. 2009; Jantz 2011; Hinton et al. 2015; Cherry et al. 2017).

In North Carolina, both red wolves and coyotes select similar habitat, most notably in or on the edge of agriculture (Hinton et al. 2015, 2016). Even though they select for similar habitat, red wolf average home range size (64.8 km^2) is significantly larger than the coyotes (27.2 km^2) (Hinton et al. 2015, 2016). Because of this, some authors have suggested that coyotes can occupy marginal habitat in habitats that the larger red wolf cannot (Gese et al. 2015; Murray et al. 2015). These studies did not consider the benefits transient individuals have on maintaining population dynamics. Transients of both species are excluded from resident's territories and use similar edge habitats and road networks to bide in marginal habitats adjacent to residents, waiting for territory and mating opportunities. When human-caused mortality was low (1990-2004) resident red wolves accounted for approximately 30% of the population (Hinton 2014; Hinton et al. 2016), and red wolves typically took over vacant areas following resident breeder deaths (Hinton et al. 2015; Gese & Terletzky 2015; Hinton et al. 2017). However, increased anthropogenic mortality from accidental take and purposeful poaching have decreased red wolf populations allowing for coyote encroachment. Today, coyotes have saturated the red wolf recovery area and red wolf populations are likely too low to recover lost territory based on current management strategies.

COMPETITION: Territorial Defense

Information on competitive interactions and territory defense is often difficult to observe (Gese 2001), but despite this challenge, territorial behaviors have been well documented for gray wolves (Peters & Mech 1975; Rothman & Mech 1979; Harrington & Mech 1978a, 1978b, 1979, 1983; Harrington 1987), and coyotes (Camenzind 1978; Barrette & Messier 1980; Bowen & Cowan 1980; Gese & Ruff 1997, 1998). Information on red wolf competitive interactions is limited, but here we attempt to summarize the available information and make inferences based on populations of closely related taxa.

In Yellowstone National Park, gray wolves exhibit density-dependent regulated survival through intraspecific competition between packs (Benson & Patterson 2013; vonHoldt et al. 2020; Schell 2020), and adult survival is greatly influenced by intraspecific aggression independent of prey availability (Cubaynes et al. 2014). In North Carolina, Phillips et al. (2003) reported red wolf intraspecific aggression in five instances that resulted in the death of a newly reintroduced wolf and a single case of a wild-born female who was killed from competition with her own pack. Even near peak red wolf recovery (2005) intraspecific aggression only accounted for 5% of mortality (USFWS 2007).

Likewise, coyotes actively defend territory through scent marking and howling, but the killing of conspecifics is rare (Phillips & Henry 1972; Fedriani et al. 2000; Nelson et al. 2010). Gese (2001) reported most instances of territorial defense by coyotes ended

at home range boundaries and rarely ended in mortality (but see Okoniewski 1982; Patterson & Messier 2001). Camenzind (1978) recorded two instances of coyotes killing pups of residents but reported intraspecific competition does not appear to be a main source of mortality. Studies documented that the dominant male is usually the aggressor towards an intruder, but numerical advantages played a significant role in an intraspecific encounter (Gese 2001). In western North America, gray wolves and coyotes do not exhibit spatial segregation as their home ranges often overlap (Paquet 1991; Thurber et al. 1992; Arjo & Pletscher 2000; Berger & Gese 2007; Merkle et al. 2009; Benson & Patterson 2013). This is likely because of resource partitioning (Atwood & Gese 2008, 2010) and reproductive isolation between gray wolves and coyotes (Garcia-Moreno et al. 1996; Pilgrim et al. 1998; Kyle et al. 2006; Benson & Patterson 2013). Gray wolves will kill coyotes where their ranges overlap (Arjo & Pletscher 2000; Merkle et al. 2009), but this aggression is usually over limited food resources such as carcasses and not over territory or mates (Paquet 1991; Berger & Gese 2007; Atwood & Gese 2010). Prior to European settlement in eastern North America, eastern and red wolf ranges had little overlap with coyotes (Nowak 2002; Murray & Waits 2007). Today in Ontario, eastern wolves, coyotes, and their hybrids exhibit high degrees of spatial segregation where their ranges meet regardless of genotype (Benson & Patterson 2013). This is likely due to a lack of reproductive isolation and shared resource use (Benson & Patterson 2013; Gese & Terletzky 2015), where each canid is a direct competitor. Benson and Patterson (2013) documented rare cases of eastern wolves killing coyotes or hybrids near pack boundaries, suggesting aggression was over territory and not limited resources like carcasses. Aggressive interactions have been observed between red wolves and coyotes (Henry

1995, 1998). In fourteen years of red wolf recovery, Gese & Terletzky (2015) documented red wolves displacing sterile coyote placeholders 37% of the time but didn't report any interspecific mortalities.

Roth et al. (2008) modeled coyote impact on red wolf recovery and found that coyotes have a substantial impact on red wolf numbers through competition including consumption of limited food resources, occupying limited space, and thwarting the establishment of territories. These results suggest that red wolves and coyotes are direct competitors and home ranges have limited overlap. Species with larger body size are generally dominate over smaller species, although a numerical advantage in the smaller species can override benefits of larger body size (Carbyn 1982; Dekker 1983, 1989; Harrison et al. 1989; Arjo & Pletscher 1999; Gese 2001; Roth et al. 2008). Merkle et al. (2009) reported 18 instances (9%) in which coyotes prevailed over gray wolves at carcasses in Yellowstone National Park, and at Northern Madison Study Area of Montana where wolf packs are generally smaller in size, 47% of agonistic interactions at carcass sites resulted in coyote dominance (Merkle et al. 2009).

The competitive relationship between red wolves and coyotes is complex and delicate because interspecific introgression over time could lead to the genomic extinction of red wolves in North Carolina. Recovery of the red wolf population would be difficult as coyotes have since saturated the red wolf recovery area and available habitat is becoming increasingly fragmented (Dellinger et al. 2013; Murray et al. 2015; Gese et al. 2015). However, new adaptive management strategies could assist the dwindling population. A coyote hunting ban in the red wolf recovery area offered some relief for the

endangered species because red wolves were being confused for coyotes leading to increased shooting deaths and pack disbandment (Murray et al. 2015). However, under unexploited conditions, coyote group size is known to increase, and a transient red wolf is unlikely to successfully displace a pack of resident coyotes or hybrids (Benson & Patterson 2013). So, additional management including the use of sterile placeholders or coyote removal may also be necessary for successful red wolf recovery.

COMPETITION: Interspecific interactions: Trophic cascades

In multi-predator ecosystems, intraguild competition can be a driving force in community structure (Schoener 1983; Merkle et al. 2009), where the removal of top predators can induce trophic cascades by affecting the behavior and abundance of smaller predators (Oksanen et al. 1981; Estes et al. 2011; Flagel et al. 2017) and lead to ecological shifts and ecosystem instability (Newsome & Ripple 2015). For example, without the threat of predation, ungulate herbivores can increase in numbers and decrease the biomass of easily browsed taxa (Martinez del Rio et al. 2011). Top-down effects, where top predators limit the populations of smaller predators, have recently been under investigation as top predators, like wolves, are recovering in parts of their former ranges. The removal of top predators has led to a phenomenon known as mesopredator release; increased populations of mid-sized predators (Crooks & Soule 1999). Since red wolves were extirpated, coyotes expanded their populations and became the dominant canid in the southeast (Gier 1975; Parker 1995; Hody & Kays 2018). Coyote populations are less affected by bottom-up effects than wolves because their feeding habits and moderate body size permits broad use of prey (Gese et al. 1996a; O'Donoghue et al. 1998a; Lingle

2000; Cherry et al. 2016b; Benson et al. 2017), allowing coyotes to persist at high densities and exert intense predation pressure on their main prey (Ripple et al. 2013). Here we assess the impact coyotes have on trophic cascades after release from competition in other parts of North America and make inferences about how coyotes impact community organization in the southeast.

Prior to European settlement, coyotes were reportedly uncommon or absent in wolf dominated landscapes (Gier 1975; Schullery & Whittlesey 1992; Parker 1995; Hody & Kays 2018). However, after wolves were removed from much of North America, coyote populations erupted in abundance and expanded their range (Parker 1995; Hody & Kays 2018). Ritchie & Johnson (2009) found a change in top predator abundance can lead to a fourfold change in mesopredator abundance, which can influence landscape level shifts in community organization. In the West, increases in coyotes have at least partially resulted in decreases of their main prey, most notably rabbits and hares (Ripple et al. 2013) as well as decreases in red foxes throughout parts of North America from direct killing and avoidance behavior (Voigt & Earle 1983; Major & Sherburne 1987; Harrison et al. 1989; Fedriana et al. 2000). Ripple et al. (2013) identified 12 endangered species or species of concern that coyotes directly affect, two, the least tern (*Sterna antillarum*) and whooping crane (*Grus americana*) are native to the southeast (Drewien et al. 1985; Atwood & Masey 1988; Ripple et al. 2013).

Flagel et al. (2017) found that the return of wolves to the Great Lakes Region created refugia for smaller carnivores and snowshoe hares because wolves suppress coyote populations (Stenlund 1955; Pimlott & Joslin 1968; Berg & Chesness 1978; Fuller

& Keith 1981; Thurber et al. 1992; O'Donoghue et al. 1997; Ballard et al. 2001; Berger & Gese 2007; Levi & Wilmers 2012). Red foxes used high wolf use areas almost exclusively and reduced the abundances of deer mice in these areas (Flagel et al. 2017). This reduced granivory in high wolf use areas led to an increase in seedling recruitment (Chandler et al. 2020). Moreover, snowshoe hare populations were positively correlated with high wolf use areas which may positively affect the endangered Canadian lynx (*Lynx canadensis*) (Ripple et al. 2013; Flagel et al. 2017).

One main difference between red wolves and coyotes are their body sizes (Hinton & Chamberlain 2014). Carbone et al. (1999) determined that 21.5 kg was the mass threshold at which predators switch from feeding on small prey (less than half of predator mass) to large prey (near predator mass). On average red wolves are above this threshold (Riley & McBride 1972; Paradiso & Nowak 1972; Davies 1974; Hinton & Chamberlain 2014), which suggests they pursue large prey. To persist on large prey requires larger home ranges and territories (Carbone et al. 2005), and therefore leads to lower population densities, especially in species that defend territories (Ritchie & Johnson 2009). Coyotes tend to be below this threshold, have smaller home ranges, and pursue a broad variety of prey, suggesting coyotes can persist at higher densities than red wolves in the same ecosystems. Higher population densities of coyotes would lead to an increase in predation pressure and prevent populations of their main prey and competitors from recovering in numbers, leading to community reorganization. For example, removal of coyotes from western Texas led to a decrease in rodent species richness and diversity, but an increase in rodent density, biomass, jackrabbit density, and increase of a relative abundance of badgers, bobcats, and gray foxes (Henke 1992). Restoring an apex predator

can reinstate a landscape of fear indirectly impacting lower trophic levels. Based on these trends red wolf restoration may benefit southeastern communities by increasing biodiversity through suppressing coyote foraging behavior or directly excluding them. In the southeast, this may mean populations directly affected by coyotes may increase in abundance such as leporids and red foxes. Louis et al. (2020) found evidence that red foxes may be attracted to red wolf scat, suggesting red foxes seek refugia from coyotes in red wolf territory.

One recovery method for red wolf restoration may include looking for new reintroduction sites. Since coyotes occupy almost the entire historic range of the red wolf, coyote removal may be necessary before any reintroduction of wolves to prevent competition and introgression. We suggest additional research into how coyotes impact community structuring before and after removal as this may inform how the reestablishment of red wolves may benefit biodiversity. Additionally, new evidence suggests that red wolf-coyote hybrids persist in the southeast (Murphy et al. 2018; Heppenheimer et al. 2018a, 2020), and these hybrids may have larger body sizes than coyotes (Giordano & Pace 2000). Future research into the diet and home range sizes of these hybrid animals may help inform the historic influence red wolves had on southeastern ecosystems.

Reproduction

Canids are often social obligate monogamists (Kleiman 1967, 1977; Hennessy 2007), which is a unique reproduction strategy for mammal taxa. Red wolves and coyotes exhibit similar reproductive strategies. The reproduction statistics on red wolves is

limited to the captive breeding program and the reintroduced populations, and therefore, may not represent the historic variation of the species. In contrast, information on coyote reproduction is widespread but dependent on population, geographic location, exploitation, density, competition, and many other factors. Here we do our best to summarize the available information and make inferences between red wolves and coyotes. A few main differences in reproductive strategy we will focus on are 1) age of first reproduction, 2) age at dispersal, 3) helper behavior, and 4) assortative mating.

The breeding season for both red wolves and coyotes is December through March (Paradiso & Nowak 1972; Magee 2008). However, courtship behavior likely begins 2-3 months prior to successful copulation (Bekoff & Diamond 1976; Bekoff & Wells 1986). Both species have a gestation period of approximately 60 days (Paradiso & Nowak 1972; Bekoff & Wells 1986; Bekoff & Gese 2003), and pups are born starting in early April to early June (Paradiso & Nowak 1972). Based on available results, the average litter size for red wolves is just over four (Kalinowski et al. 1999; Rabon & Waddell 2010; USFWS 2013; Rabon 2014). In captivity, 688 pups were born to 157 litters through 1996, averaging just over 4 per litter (Kalinowski et al. 1999). Rabon & Waddell (2010) confirmed by reporting 792 offspring born to 192 litters from 1977-2006, averaging 4.15 pups per litter. In Texas, Paradiso & Nowak (1972) reported litter sizes between 2-10 with an average of 6.62 from 29 litters, however, most young died before six months old. Coyotes mean litter size is between 3 and 7 but is positively correlated with food availability and negatively correlated with population density (Knowlton 1972; Todd & Keith 1983; Andelt 1985; Knowlton & Gese 1995; Windberg 1995; Gese et al. 1996a, b; O'Donoghue et al. 1997).

Female red wolves were reported reproducing their first year in captivity, but males typically did not reproduce until their second year (Rabon 2014). In the wild, red wolves had a mean age of first reproduction at 3.51 years (Sparkman et al. 2012), only about 5% of wolves were reproductive their first year and about 50% were reproductive their second year (Sparkman et al. 2011b). In contrast, Sparkman et al. (2012) reported red wolf/coyote hybrids in North Carolina had a mean age of first reproduction at 2.43 years which is significantly earlier than red wolves. Both male and female coyotes can reproduce their first year (Gier 1968; Knowlton 1972; Rabon 2014). However, in Idaho no females reproduced their first year, but 80% reproduced their second year (Green et al. 2002). Green et al. (2002) determined that age of first reproduction was highly dependent on available resources and space. In exploited populations with low population densities first year coyotes may reproduce more often than in unexploited populations with high population densities. Because courtship and mate selection begin 2-3 months before successful copulation (Bekoff & Diamond 1976), individuals that reproduce their first year are likely replacing residents that died right before successful breeding took place. Reproduction in both species decreases after 8-9 years of age (Windberg 1995; Green et al. 2002; Sparkman et al. 2017).

Another difference between red wolves and coyotes is age at dispersal. Dispersal behavior may have a genetic component, and the variation in behavior may be due to genetic differences within and between the species (Sparkman et al. 2012). Wolves are social animals belonging to packs and complex social groups, compared to coyotes who are more asocial, existing in breeding pairs or small family groups (Phillips & Henry 1992). In North Carolina, Sparkman et al. (2011b) reported that 65% of red wolves

delayed dispersal for one or more years, 45% dispersed their second year, and 20% after that. Timing of dispersal for red wolves is somewhat affected by population density (Karlin & Chadwick 2012). As competition increased over limited resources red wolf pups dispersed later than adults (Karlin & Chadwick 2012). Comparatively, hybrids had a significantly earlier dispersal age than red wolves, where only 3% of hybrids dispersed at 2 years of age, and none later (Kanine & Mengak 2011; Sparkman et al. 2012). In Maine, Harrison (1992) reported 86% of coyotes dispersed their first year and 100% had departed prior to 1.5 years old. In Yellowstone National Park, delayed dispersal of coyotes was related to the presence of an abundant, clumped, defendable winter food resource (mainly elk carrion) (Bekoff & Wells 1982a, b). Additionally, Gese et al. (1996) reported dispersing coyotes were low-ranking pups or betas, with little access to ungulate carcasses, and those who stayed in the pack were high ranking individuals potentially with the objective of advancing to a breeding position. One factor affecting delayed dispersal and group size in coyotes is habitat saturation. Groups formed in areas of minimal exploitation and when available habitat was low, because the risk of injury from direct encounters with territorial residents was high (Andelt 1985).

What are the advantages and disadvantages of yearlings and pups delaying dispersal? Individuals that decide to delay dispersal and remain with breeding parents are often called helpers. Helping behavior between and within the species appears contingent on a variety of factors including but not limited to food availability, population density, and health or condition of helpers (Malcom & Marten 1982; Harrington et al. 1983; Clutton-Brock et al. 2001; Gusset & Macdonald 2009). Delayed dispersal for both red wolves and coyotes increased yearling survival, and in male red wolves increased the

probability of becoming reproductively successful (Sparkman et al. 2011b, 2017). In red wolves, the presence of helpers at low population densities was positively correlated with body mass and survival, but body mass was negatively affected at high population densities (Sparkman et al. 2011a), suggesting that helpers help to provision food for the pups but feed themselves first (Sparkman et al. 2011a). The presence of coyote helpers in Wyoming was not correlated with pup survival (Bekoff & Wells 1982). Helpers rarely fed pups (but see Ryden 1975) and their presence had no appreciable effect on juvenile weight (Bekoff & Wells 1982; Andelt 1985). However, the advantage to the helper was increased survival rate and increased likelihood of inheriting a breeding area with abundant food resources (Bekoff & Wells 1982), while the disadvantage was not being able to breed that year. Helping behavior is dependent on many factors, and the reliance on helpers likely differs between red wolves and coyotes. Research indicates coyotes delay dispersal less often than red wolves, do not help raise the pups, and do not reduce the amount of time parents spent at den sites (Bekoff & Wells 1982; Andelt 1985; Sparkman et al. 2012). Helping behavior and dispersal results in coyotes suggests packs are a secondary social class for the species and the main social class is mated pairs.

Today in North Carolina red wolves and coyotes are sympatric and occasionally hybridize. However, hybridization between the species is infrequent relative to the proportion of parental groups on the landscape (Bohling & Waits 2015; Bohling et al. 2016). Under natural conditions, red wolves sought conspecific mates resulting in little hybridization (Bohling & Waits 2015), even as the wolf population became increasingly inbred (Brzeski et al. 2014). When hybridization did occur, red wolves appeared to select hybrid individuals with mixed ancestry rather than pure coyotes (Bohling & Waits 2015),

indicating there is some form of assortative mating and selection against conspecifics (Bohling & Waits 2015). Consequently, this means when hybridization does occur, hybrids can act as a bridge between the two parental species possibly increasing hybridization (Dowling & Secor 1997; Goodman et al. 1999; Seehausen et al. 2008; Duvernell & Schaefer 2014; Bohling & Waits 2015).

Canids are obligate monogamists, and many female red wolves stay with the same mate until death (Kleiman 1967, 1977; Sparkman et al. 2017). Although we do not know the exact method of mate selection, female mate selection appears very important for reproductive success in these cooperative breeding species (Sparkman et al. 2011b, 2017). Hybridization in North Carolina is almost entirely one sided with female red wolves mating with male coyotes. Anthropogenic mortality during or immediately before the breeding season is the main source of pack disbandment, disruption of social structure, and leading cause of hybridization between red wolves and coyotes (Loveless et al. 2011; Hinton et al. 2015; Bohling & Waits 2015). However, assortative mating patterns provide evidence that red wolves can still be recovered across parts of its historic range even when saturated with coyotes. Once populations reach capacity, assortative mating will limit hybridization, and restoration will only require continued education and outreach.

2.6 Conclusion

The red wolf and coyote continue to hybridize in the wild, and evidence suggests there are populations of hybrids that remain in the southeast. This presents two different problems: 1) contemporary hybridization with wild red wolves is detrimental to the

genetic integrity of the species, but 2) historic hybridization could be important as hybrid individuals may be a reservoir of lost red wolf genetic material and therefore may warrant some protection. The ESA deals with hybrids that have ancestry from both a protected and unprotected species on a case-by-case basis (Haig & Allendorf 2006; Placyk et al. 2012; Fitzpatrick et al. 2015).

Currently, the extant red wolf population is not experiencing detrimental effects from inbreeding depression due to the founder effect or because there are no outbred individuals for comparison (Brzeski et al. 2014). However, given that inbreeding will likely increase (Brzeski et al. 2014) inbreeding depression continues to be a threat to red wolf viability. Genetic rescue through cross-fostering or genome editing tools could theoretically alleviate some of the concerns over inbreeding depression. But cross-fostering requires distantly related individuals and must be done in captivity which is difficult.

Here we summarized information about the endangered red wolf and its relationship with coyotes in the southeastern United States to continue the discussion about some hybrid populations in the southeast. As presented here there are still gaps in our knowledge about red wolf ecology prior to historic population declines, but hybrid populations represent a rare opportunity to learn about ecological impacts and natural history of a species thought to be regionally extinct. Additional information needs to be collected to better understand the extent of red wolf ancestry present in the southeast. Then the USFWS and stakeholders can make decisions regarding protections if applicable based on the best available science.

2.7 Figures



Figure 2.1. Range contraction of the red wolf, based off state and county records.

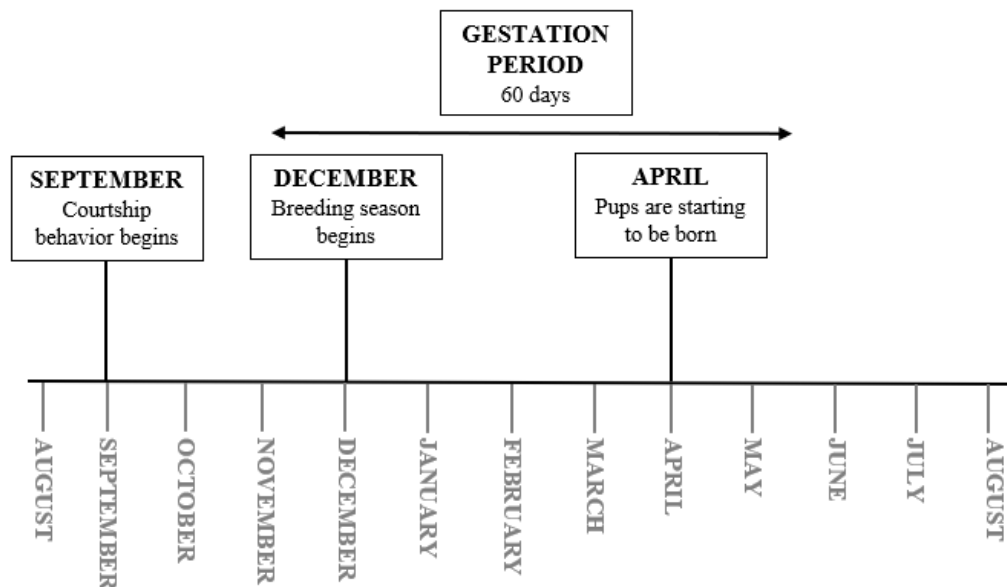


Figure 2.2. Timeline of the breeding cycle for wild canids.

2.8 Tables

Table 2.1. Minimal morphological standards to be considered a true red wolf by the USFWS in Texas and Louisiana.

lengths (mm)	Male	Female
Skull Length	215	210
Zygomatic breadth	110	110
Weight (kg)	22.5	19
Total length	1346	1295
Hind foot length	229	222
Ear length	120.6	114.3

Shoulder height	685.8	673.1
Brain/skull ratio	23	23.5

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A Appendix A

DNA from scat can be affected by climatic conditions and time since defecation (Ando et al. 2020). Therefore, prior to our sampling, we performed a pilot study to test the efficacy of different scat collection methods. Scats were collected in three different ways: swabbing with a cotton swab and place in Longmire buffer, wiping the exterior surface with toothpicks and dried with silica, and collecting whole scats place on ice or dried. All methods provide approximately the same quality sequences. However, swabbing with a cotton swab was the most efficient and cost-effective approach. We also collected whole scats for use in additional future analyses.

We attempted to determine time since defecation form the scat appearance (Ando et al. 2020). Wet scat was the freshest, dry scat but brown or black was next freshest, dry scat but white was old, scat that was mostly prey items without much fecal material was the oldest. We attempted to extract DNA from all levels of degradation. We had moderate to high success with the two freshest classes and low to very low success with the later classes. Therefore, during DNA extraction the freshest scats were extracted first followed by the older scats. However, for most sampling periods we performed a DNA extraction on all scats collected.