



# Factors influencing red wolf–coyote hybridization in eastern North Carolina, USA



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## ABSTRACT

Understanding the mechanisms that govern interspecific hybridization is vital to mitigating its impacts on endangered species. Research suggests that behavioral mechanisms such as mate choice and social disruption can regulate the rate at which hybridizing species interbreed. We investigated hybridization events between endangered red wolves (*Canis rufus*) and coyotes (*Canis latrans*) in eastern North Carolina to evaluate potential factors that may promote hybridization between these species. Specifically, we examined spatial location, breeding experience, breeder origin (captive vs wild), breeder ancestry (pure vs hybrid), and past history of the animal. There were over four times (126 vs 30) as many red wolf litters as hybrid litters over a 13 year time period. Over half of the hybridization events followed the disruption of a stable breeding pair of red wolves due to mortality of one or both breeders. Of these 69% were due to anthropogenic causes, primarily gunshot mortality prior to the red wolf breeding season. Both male and female red wolves interbred with coyotes, although a majority (90%) of the events we observed involved females. Wolves that produced hybrid litters tended to be young, first-time breeders with slightly higher levels of coyote ancestry. Only 16% of the hybrid litters were produced in the inner core of the red wolf recovery area. Our results suggest that disruption of stable breeding pairs of red wolves facilitates hybridization, jeopardizing future recovery of the red wolf. They also indicate the importance of behavioral forces, especially social stability, in regulating hybridization.

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## 1. Introduction

Human activity has the potential to disrupt dynamics between hybridizing species, which can cause hybridization and introgression to emerge as conservation threats (Rhymer and Simberloff, 1996; Allendorf et al., 2001). As with any conservation problem, developing solutions requires recognizing the mechanisms that influence the process. For hybridization, that requires understanding the mechanisms that cause previously reproductively isolated species to interbreed. Species introductions (Rhymer and Simberloff, 1996; Stigall, 2010), habitat destruction and ecological homogenization (Seehausen et al., 2008; Crispo et al., 2011), and the spread of domesticated species (Likre et al., 2010; Champagnon et al., 2012) have been implicated as processes that may facilitate these shifts.

Still, these forces primarily influence whether hybridizing species come into contact, not necessarily whether individuals will interbreed. There is increasing recognition that behavioral

processes such as mate choice (Pfennig, 2007; Reyer, 2008; Svedin et al., 2008; Gilman and Behm, 2011; Robbins et al., 2014), interspecific competition (Wolf et al., 2001; Krosby and Rohwer, 2010; Sacks et al., 2011), and Allee effects (Lode et al., 2005) can influence the rate of hybridization. The potential for behavioral forces to moderate hybridization and introgression may be a critical factor that would influence conservation schemes.

Understanding the mechanisms that govern interspecific mating is vital to recovery of species threatened by hybridization, especially the critically endangered red wolf (*Canis rufus*) in eastern North Carolina. Historically red wolves were distributed across eastern North America, but overharvest, habitat destruction, and hybridization with coyotes (*Canis latrans*) led to extinction in the wild by 1980 (Paradiso and Nowak, 1972; Nowak, 2002; USFWS, 1990). Beginning in 1987, captive red wolves were reintroduced into eastern North Carolina and today a population of about 80–100 individuals occupies the 600,000 hectare Albemarle Peninsula (Phillips and Parker, 1988; Phillips et al., 2003; Bartel and Rabon, 2013).

At the same time, coyotes expanded their range into North Carolina (Hill et al., 1987) and in 1993 the first hybridization event between a reintroduced red wolf and a coyote was detected (Phillips et al., 2003). A subsequent population viability analysis

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suggested that hybridization was the greatest threat to red wolf recovery (Kelly et al., 1999). This led to the development of an aggressive adaptive management plan by the US Fish and Wildlife Service (USFWS) and the Red Wolf Recovery Implementation Team (RWRIT, Stoskopf et al., 2005) to limit hybridization and introgression. The genetic composition of the population is managed by an active monitoring program combined with genetic testing to remove hybrid individuals from the landscape (Stoskopf et al., 2005; USFWS, 2007; Bartel and Rabon, 2013).

Such aggressive practices have been implemented based on the hypothesis that a small red wolf population would be genetically swamped by coyotes without human intervention (Kelly et al., 1999). This is predicated on the assumption that when sympatric, red wolves and coyotes will breed indiscriminately. However, this assumption has not been tested empirically. Fredrickson and Hedrick (2006) modeled red wolf viability and found that positive assortative mating and aggressive interactions between the species were the most important factors in maintaining population viability. They developed hypothetical values for those parameters because empirical estimates did not exist. USFWS field biologists have observed red wolves displacing and occasionally killing coyotes and hybrids (USFWS, 2007). Otherwise, there is little understanding of how mate choice and social structure influence interactions between these species. Given the importance of social dynamics on the ecology of mammalian carnivores, there is potential for behavioral processes such as mate choice, social structure, and competition to limit hybridization (Rutledge et al., 2010; Sacks et al., 2011; Shurtliff, 2011). Conversely, disrupting these social systems may in turn influence reproductive patterns (Brainerd et al., 2008; Borg et al., in press) and hybridization rates (Rutledge et al., 2010).

We examined breeding records and individual histories of red wolves involved in hybridization events from 2001 to 2013 to elucidate factors that facilitate interbreeding between these species. Specifically, we asked the following questions: (1) Do age, prior breeding experience, and origin of the wolf influence the likelihood of hybridization, (2) are individuals with mixed red wolf/coyote ancestry more likely to hybridize, (3) are hybridization events evenly distributed across the recovery area, and (4) are hybrid litters produced under particular scenarios or breeder histories? If breeding between these species is indiscriminate, we would expect young dispersing red wolves to be the most likely individuals to encounter and breed with a coyote. Breeding opportunities within wolf packs are often restricted to a dominant breeding pair, which forces individuals in search of mates to disperse outside the pack (Mech and Boitani, 2003; Sparkman et al., 2012). In this system dispersing would increase the likelihood of an individual encountering a coyote considering that wolf packs are known to exclude coyotes within their range (USFWS, 2007). This is similar to observations of eastern wolf (*Canis lycaon*) packs in southern Canada (Benson and Patterson, 2013). We predicted that hybridization would increase from east to west, since the western portion of the study area has the fewest wolves, least stringent management, and closest proximity to the mainland coyote population. Also, we hypothesized that individuals with mixed red wolf/coyote ancestry would be involved in more hybridization events. By examining the characteristics and history of red wolves responsible for hybridization events with coyotes, we can better understand the mechanisms that govern hybridization, aiding recovery of this species.

## 2. Materials and methods

### 2.1. Genetic monitoring

Every spring USFWS biologists track female red wolves to locate active dens that contain pups. Blood samples are collected from

pups and genetic testing is conducted to assess their ancestry and place them within the red wolf pedigree (Adams, 2006; Bohling et al., 2013). Since the implementation of the adaptive management plan in 2000 monitoring of red wolf dens and genetic testing of captured canids, including pups, became standard, which improved detectability of hybridization events (Stoskopf et al., 2005; USFWS, 2007). As a result, we only considered hybridization events that have occurred since 2000 for this study. USFWS biologists possessed permits for capturing and handling red wolves that have been jointly issued by the USFWS, Association of Zoos and Aquariums Reintroduction Scientific Advisory Group, and IUCN Species Survival Commission Reintroduction Specialist Group.

Parentage for red wolf and hybrid litters was determined following the methods of Adams (2006) and Bohling et al. (2013). To summarize, each pup was genotyped at 17 microsatellite loci and assigned to red wolf parents using genetic and field data, allowing for  $\leq 1$  mismatch for a parent pair. Based on this pedigree we were able to estimate an individual's ancestry by averaging the amount of red wolf ancestry possessed by the parents as traced through the pedigree. In the case of hybrid litters, typically only the red wolf parent was identified, although in several situations the non-red wolf parent was later captured and determined to be a parent using genetic analysis. Several hybrid litters were detected when hybrid offspring were captured as juveniles and later assigned to a red wolf parent. Three hybrid litters fit this scenario; thus, the exact size of those litters could not be determined.

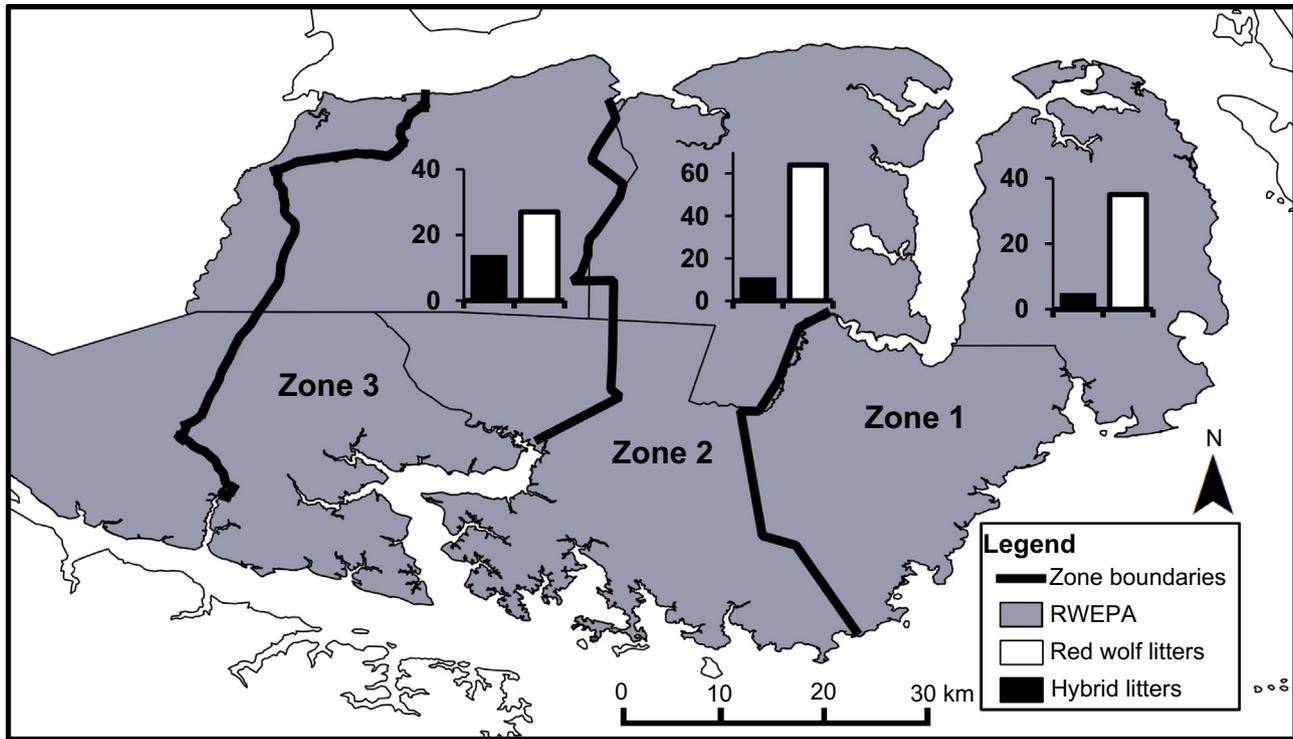
### 2.2. Location

The adaptive management plan divided the peninsula into three zones with different management goals (Stoskopf et al., 2005) (Fig. 1). Zone 1, the easternmost portion of the peninsula, serves as the core red wolf population and coyotes and hybrids captured in this area are euthanized. In Zone 2, directly west of Zone 1, hybrid individuals are euthanized but coyotes are sterilized under the hypothesis that sterile individuals would serve as territorial placeholders that discourage undetected coyotes from dispersing into the peninsula (Bartel and Rabon, 2013). Zone 3 is the furthest west section and falls at the junction of the peninsula and the mainland. Management practices in Zone 3 vary, but many sections of this area are managed similarly to Zone 2. This entire region has been designated as the Red Wolf Experimental Population Area (RWEPA).

We classified each hybrid and red wolf litter to a Zone based upon where it was detected (Zone 1, 2, or 3) and used a  $\chi^2$ -squared test of independence to evaluate the distribution of each type of litter across all three zones. As noted, some hybrids were discovered as adults. In these situations, once the red wolf parent was identified via genetic testing we assigned the location of these litters according to the home range of that red wolf during the prior breeding season.

### 2.3. Breeder experience

To examine the impact of breeder experience on hybridization we compared both the age and prior breeding experience of red wolves that produced hybrid and red wolf litters. For breeding experience we classified each litter according to whether it was produced by a first-time breeder or an experienced breeder. This was only performed for females since the sample size of male red wolves was low (see Section 3.1). We defined first-time breeder as any individual producing its first known litter of pups, regardless of whether it was a hybrid or red wolf litter. An individual was considered an experienced breeder once it had produced a second litter. We compared the proportion of total red wolf and hybrid litters that were born to experienced breeders using a  $\chi^2$



**Fig. 1.** Map of the Red Wolf Experimental Population Area (RWEPA) and the spatial distribution of hybrid and red wolf litters from 2001 to 2013. The thick black lines indicate the boundaries of the management zones 1, 2, and 3. The number of hybrid and red wolf litters per zone indicated by the graphs within each zone boundary. White columns represent red wolf litters, black hybrid litters.

test of independence. The effect of breeder age was measured by comparing the overall age of individuals responsible for hybridization events to the age of individuals producing red wolf litters. For three females the year of birth was unknown (Table 1).

#### 2.4. Breeder origin

Over the past two decades the USFWS has released captive individuals and individuals raised in island-propagation programs to augment the wolf population. The island-propagation program consists of releasing captive red wolves to isolated, unoccupied barrier islands in the southeastern US so they can acclimate to the wild (Phillips et al., 2003). We hypothesized that the unfamiliarity of captive and island-raised individuals with the RWEPA would inhibit their ability to locate red wolf mates, increasing the likelihood that they would encounter and breed with a coyote. Using the pedigree we were able to determine the origin of each red wolf (wild, captive, and island-born) and whether it produced any litters. We also noted wolves produced through cross-fostering, which involves integrating recently born captive pups into wild litters to provide an input of genetic diversity (Bartel and Rabon, 2013).

#### 2.5. Breeder ancestry

The hybridization event that occurred in 1993 between a female red wolf and male coyote resulted in introgression of coyote genetic material into the red wolf population. Two F1 hybrid males from this event bred with female red wolves and their offspring backcrossed with the red wolf population. The USFWS and RWRIT decided that any individual possessing >87.5% red wolf ancestry based on pedigree assignment would remain in the population (Stoskopf et al., 2005). A large segment of population now consists of these backcrossed individuals that have a slight amount of

known coyote ancestry (87.5–98% red wolf) (Adams, 2006; Bohling et al., 2013). Our goal was to determine whether these admixed individuals had a higher propensity to hybridize than ‘pure’ wolves. We calculated the average ancestry of individuals involved in hybridization events using the pedigree and compared that to the ancestry of individuals producing red wolf litters using a two-tailed *t*-test. We applied an arcsin transformation to ancestry values prior to analysis.

Even though red wolves and coyotes hybridize, there are noticeable differences in morphology, behavior, and ecology between the species (Phillips and Henry, 1992; Nowak, 2002; Phillips et al., 2003; Hinton and Chamberlain, 2014). Hybrids are intermediate between the two species; thus, we predicted that a red wolf would be more likely to select a hybrid as a mate than a coyote. However, since aggressive management practices remove hybrids from the landscape, we hypothesized that the production of hybrid litters would be primarily driven by interbreeding with coyotes. Since the non-red wolf parent was identified for several hybrid litters, we evaluated the ancestry of these animals using two methods to determine if they were coyotes or individuals of mixed ancestry. First, ancestry of non-red wolf individuals was assessed using the Bayesian clustering program STRUCTURE 2.3 (Pritchard et al., 2000; Falush et al., 2003). We used four known genetic groups as training sets: coyotes ( $N=94$ ) from North Carolina, Virginia, and Texas; gray wolves ( $N=38$ ) (*Canis lupus*) from Idaho and Alaska; domestic dogs ( $N=28$ ); and founders of the captive red wolf population ( $N=17$ ). Our previous work (Bohling and Waits, 2011; Bohling et al., 2013) showed that these four groups form distinct clusters when applied to STRUCTURE in a free-clustering framework. In the STRUCTURE analysis, individuals from our four known genetic groups were assigned a POPFLAG designation of 1 and the ‘Use Population Information’ option was used as the ancestry model with the default parameter settings and the correlated allele frequency model. Unknown individuals were given the

**Table 1**  
Characteristics of the individual wolves involved in hybridization events.

Year	Wolf <sup>a</sup>	Sex <sup>b</sup>	% Red wolf ancestry <sup>c</sup>	Origin <sup>d</sup>	Year born	First breeding event	Litter size	Zone <sup>h</sup>	Coyote ID <sup>i</sup>	History <sup>j</sup>
2001	11100	F	100	Wild <sup>e</sup>	NA <sup>f</sup>	Yes	6	2		Wolf dispersed
2001	11055	F	75	Wild	NA	No	3	3	30145	No previous history <sup>k</sup>
2001	10884	F	100	Wild	1995	Yes	9	3		No previous history
2001	11049	F	87.5	Wild	1999	Yes	6	3	20271	No previous history
2002	10947	M	75	Wild	NA	No	5	1	30214	Mistaken identity <sup>l</sup>
2002	11168	M	87.5	Wild	NA	Yes	NA <sup>g</sup>	1	30218	Breeder killed-poison
2002	11231	F	87.5	Wild	NA	No	NA	3	30272	Breeder killed-gunshot
2002	11030	F	100	Wild	1999	Yes	6	3	30205	Mistaken identity
2003	11030	F	100	Wild	1999	Yes	5	3	30205	Mistaken identity
2005	11037	F	87.5	Wild	1999	No	9	1		Breeder killed-gunshot
2005	11049	F	87.5	Wild	1999	No	6	3	20290	Breeder killed-gunshot
2006	11132	F	87.5	Wild	2001	No	9	2	20377	Breeder killed-natural
2006	11163	F	93.75	Wild	2001	Yes	6	2	20375	Breeder killed-trap injury
2006	11049	F	87.5	Wild	1999	No	1	3		Breeder killed-gunshot
2006	11248	F	100	Wild	2003	Yes	5	3		Breeder killed-gunshot
2006	11148	F	87.5	Wild	2001	Yes	6	3		Wolf displaced
2007	11323	F	96.9	Wild	2004	Yes	2	3		Coyote displaced
2008	11541	F	100	Wild	2006	Yes	4	1		Breeder killed-mange
2008	11517	F	100	Wild	2006	Yes	7	2		Breeder killed-gunshot
2008	11301	M	93.75	Wild	2004	No	NA	3	20493	Coyote displaced
2009	11440	F	93.75	Wild	2005	Yes	6	2		No previous history
2009	11429	F	96.9	Wild	2005	Yes	6	3		Breeder killed-gunshot
2010	11298	F	87.5	Wild	2003	No	8	2		Breeder killed-intraspecific mortality
2011	11779	F	90	Wild	2009	Yes	5	2		Previous breeder disappeared
2011	11630	F	96.9	Wild	2007	No	6	2		No previous history
2011	11725	F	100	Island	2007	Yes	5	2		No previous history
2012	11725	F	100	Island	2007	No	4	2		No previous history
2013	11837	F	100	Wild	2010	Yes	4	2		Breeder killed-gunshot
2013	11693	F	96.9	Wild	2008	No	6	3		Previous breeder disappeared
2013	11819	F	93.8	Wild	2010	Yes	4	1		Breeder killed-gunshot

<sup>a</sup> Identification number of the individual wolf involved in the hybridization event.

<sup>b</sup> F – female; M – male.

<sup>c</sup> Based on the red wolf pedigree.

<sup>d</sup> Birth location of individual.

<sup>e</sup> Wild-born individual from the reintroduced population in North Carolina.

<sup>f</sup> For these individuals the age of birth could not be determined.

<sup>g</sup> These litters were detected after USFWS biologists captured the hybrids as juveniles and genetic testing assigned parents.

<sup>h</sup> Refers to the management zone that the litter was discovered.

<sup>i</sup> The individual that bred with the red wolf was unable to be determined for all litters.

<sup>j</sup> History of the location and/or red wolf prior to the production of the hybrid litters.

<sup>k</sup> No significant history was recorded for these individuals.

<sup>l</sup> The individual the red wolf had paired with had been erroneously identified as a red wolf.

POPFLAG = 0 designation, which means their genotypes were not used to estimate allele frequencies. The number of populations was set to four with a burn-in period of 100 000 reps followed by 1 000 000 MCMC repetitions.

For each genetic cluster STRUCTURE produces a probability value that an individual's genotype originated from that group. We considered an individual 'admixed' using two different criteria following Bohling and Waits (2011). If an individual had a probability of assignment  $\geq 0.1$  for two or more species, we considered that evidence of admixture under our 'Relaxed' criterion. STRUCTURE estimates credibility intervals around each probability value. If the 90% credibility intervals for the values of two or more genetic groups did not overlap zero, we considered this strong evidence of admixture under our 'Conservative' criterion. Our reasoning was to provide two perspectives for interpreting individual ancestry (Bohling and Waits, 2011; Latch et al., 2011). A concern with comparing these results with the red wolf pedigree is that the pedigree is based on theoretical expectations of inheritance while the STRUCTURE results are based on model-estimates using genetic data. To examine if there was any bias in using ancestry estimates based on the pedigree, we compared ancestry estimates produced by both methods for all red wolves involved in hybrid and wolf reproductive events using a paired *t*-test.

Miller et al. (2003) developed a maximum-likelihood assignment test specifically for this red wolf–coyote system. This Canid

Assignment Test (CAT) uses genotypes of founders of the captive red wolf population and coyotes from Texas, Virginia, and North Carolina to simulate genotypes of red wolves, coyotes, F1 and F2 hybrids, and F1  $\times$  backcrosses with each parental species. Maximum-likelihood tests are then used to estimate the probability of an individual's genotype originating from those six classes. This test is used by the USFWS in conjunction with the pedigree assignment in management decisions and we included those results for another perspective on ancestry.

## 2.6. Breeder history

We assessed breeder history for each red wolf involved in known hybridization events. The information we collected focused on qualitative descriptions of specific events experienced by the wolves and/or the pack they were associated with. These events were documented by USFWS biologists as they monitored individual wolves over the course of the main period of dispersal and breeding (October–March). Examples of significant events included but were not limited to dispersal, displacement, formation of a new pack, disruption of breeding pairs, and mortality of an associated breeder. In the case of mortalities, a necropsy was conducted to determine whether the cause of mortality was natural or anthropogenic.

### 3. Results

#### 3.1. Location

From 2001 to 2013, 30 hybrid litters and 126 red wolf litters were documented by USFWS biologists and confirmed via genetic testing (Fig. 2). The mean number of hybrid litters per year was 2.2 ( $\pm 1.4\sigma$ ) whereas the mean number of red wolf litters was 9.7 ( $\pm 1.7\sigma$ ). The average number of pups were higher in hybrid litters (5.5) than red wolf litters (4.2) ( $p = 0.001$ ). In a majority of the cases (27 of 30) a female was confirmed as the red wolf parent of a hybrid litter. A total of 23 female red wolves were involved in hybridization events; most only produced one hybrid litter. Two females were responsible for two hybrid litters each and another was responsible for three. The three male red wolves that produced hybrid litters were responsible for one each.

A majority of the hybrid litters were found in Zones 3 ( $n = 14$ ) and 2 ( $n = 11$ ), with the lowest number in Zone 1 ( $n = 5$ ) (Fig. 1). Conversely, red wolf litters were more evenly distributed (Zone 1 = 35, Zone 2 = 64, Zone 3 = 27). The distribution of red wolf and hybrid litters across the zones was significantly different ( $\chi^2 = 8.05$ ,  $p = 0.02$ ). All red wolves that produced hybrid litters were originally born in the wild except for one female wolf that was born in the island propagation program. Most of the females responsible for red wolf litters were also wild-born ( $n = 43$ ), but four cross-fostered and three island-born females also produced litters. Among males that produced red wolf litters, 47 were wild-born, six island-born, one captive-born, and one cross-fostered.

#### 3.2. Breeder experience

Since a majority of the hybrid litters were produced by females, we only examined breeding age and experience for female red wolves. Among hybrid litters, 16 of 27 involved a female breeding for the first time. This was a significantly higher ( $\chi^2 = 8.8$ ,  $p = 0.003$ ) than the proportion of red wolves litters that involved a first-time breeding female (35 out of 120). Across all litters, the average age of female red wolves responsible for hybrid litters (4.2 years  $\pm 1.6\sigma$ ) was less than the average age of those responsible for red wolf litters (5.2 years  $\pm 2\sigma$ ;  $p = 0.007$ ).

#### 3.3. Breeder ancestry

There was no significance difference between the values of ancestry produced by the pedigree and STRUCTURE for individual red wolves ( $p = 0.89$ ). Thus, we report results from the pedigree. Due to the low number of males involved in hybrid litters, we

restricted comparisons to female wolves to ensure adequate sample sizes. The average red wolf ancestry of females involved in hybrid events was 0.939 while those involved in red wolf litters had an average of 0.963. Following data transformation, these values were not significantly different ( $t = 2.02$ ,  $p = 0.128$ ). For males, the average amount of red wolf ancestry was 0.947 for those that produced red wolf litters and 0.854 for those producing hybrid litters, but we did not test statistical significance due to low sample size.

The suspected non-red wolf parent was identified by USFWS biologists using field observations for 16 hybrid litters. Two litters involved the same non-red wolf parent: a male that had been mistakenly identified as a red wolf based on morphological examination in the field. Genotypes were obtained for 10 of these non-red wolf parents. Based on the STRUCTURE results, only three individuals appeared to be pure coyotes using the 0.9  $q$ -value threshold (Fig. 3). Two additional individuals also appeared to be coyotes, for they had  $q$ -values of 0.81 and 0.77, respectively, for the coyote cluster and no other  $q$ -value  $> 0.1$ . For these five individuals the credibility interval surrounding the  $q$ -value for only the coyote cluster did not overlap zero. Four of these individuals we classified as coyotes by the CAT; one, 30272, was classified as a coyote  $\times$  F1 backcross (Table 2).

Two individuals had  $q$ -values indicative of F1 coyote–red wolf hybrids (Fig. 3). Using the red wolf pedigree, we were able to identify the red wolf and coyote parent for both of these individuals, confirming that they were F1 hybrids. Individual 30205 was the only individual for which the credibility intervals for the coyote and red wolf cluster did not overlap zero. Both individuals were classified as F1 hybrids by the CAT (Table 2). Two other individuals appeared to be F1  $\times$  red wolf backcrosses (75%), which were confirmed using the pedigree, and one (30145) produced credibility intervals for two clusters that did not overlap zero. The CAT also classified these individuals as F1  $\times$  red wolf backcrosses. One individual appeared to be a F1 coyote–dog hybrid  $\times$  coyote backcross ( $\sim 75\%$  coyote,  $\sim 25\%$  dog) using the ‘Relaxed’ criterion. To the CAT, which does not incorporate dog ancestry, this individual was a coyote.

#### 3.4. Breeder history

Seven of the 30 hybrid litters had no notable previous history associated with the red wolf responsible for the litter (Table 1). Only two instances resulting in hybrid litters involved red wolves that were considered ‘dispersers’. One was a female red wolf that had been displaced by a neighboring female wolf and settled with a non-red wolf mate. The other was a female that exhibited wide movement patterns during the breeding season and never paired

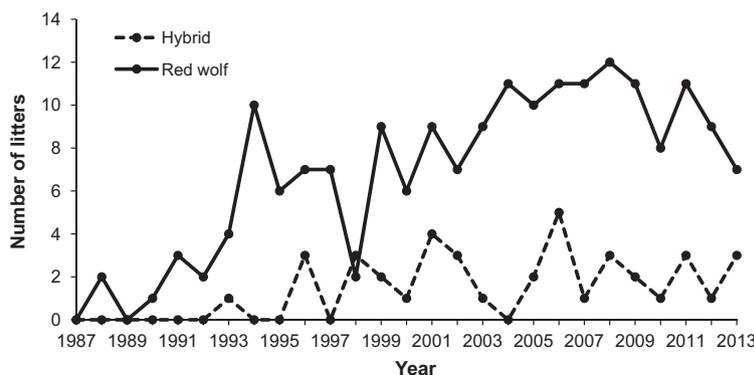
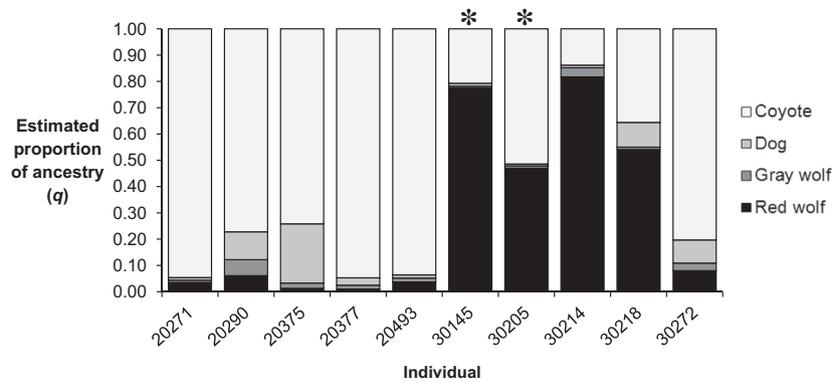


Fig. 2. Number of red wolf and hybrid litters produced each year since the reintroduction of red wolves into North Carolina. Beginning in 2000 the FWS implemented an adaptive management program to genetically monitor the population and reduce hybridization. As a result, for this study we focused solely on litters produced after 2001.



**Fig. 3.** STRUCTURE ancestry coefficients for the non-red wolf individuals responsible for hybridization events. Each bar represents an individual ancestry composition and each color reflects the ancestry value assigned to each species. This analysis was conducted with a dataset containing 17 polymorphic microsatellite loci. For individuals with a \* above their bar plot, the STRUCTURE 90% credibility intervals did not overlap zero for two genetic groups. Individuals 30205 and 30218 were determined to be F1 coyote–red wolf hybrid using the pedigree. Individuals 30145 and 30214 were determined to be F1 × red wolf backcrosses using the pedigree.

**Table 2**

Results from the Canid Assignment Test (CAT) for non-red wolf individuals responsible for hybridization events. This test uses maximum-likelihood statistics to evaluate the probability of an individual belonging to six separate ancestry classes. The six classes are the two parental species (red wolf, coyote), F1 and F2 hybrid, and F1 backcrosses with each parental species. Values indicated the probability (*p*) that an individual’s genotype can be excluded from that ancestry class. The most likely class is indicated by the term ‘LMI’. Coyotes Specific Alleles (CSA) refers to number of alleles possessed by an individual that are found only in coyotes and not the founders of the red wolf population.

Individual	Most likely classification	Maximum-likelihood probability of assignment						CSA
		Red wolf	Coyote	F1	RWBC <sup>a</sup>	CoyBC <sup>b</sup>	F2	
20271	Coyote	0.004	LMI	0.004	0.004	0.072	0.004	11
20290	Coyote	0.004	LMI	0.004	0.004	0.0112	0.008	10
20375	Coyote	0.004	LMI	0.004	0.004	0.028	0.004	16
20377	Coyote	0.004	LMI	0.004	0.004	0.012	0.004	13
20493	Coyote	0.004	LMI	0.004	0.004	0.124	0.004	10
30145	RWBC	0.004	0.004	0.004	LMI	0.004	0.052	3
30205	F1	0.004	0.004	LMI	0.004	0.024	0.04	5
30214	RWBC	0.004	0.004	0.004	LMI	0.004	0.02	2
30218	F1	0.004	0.004	LMI	0.004	0.036	0.048	5
30272	CoyBC	0.004	0.044	0.004	0.004	LMI	0.036	10

<sup>a</sup> Red wolf × F1 hybrid backcross.

<sup>b</sup> Coyote × F1 hybrid backcross.

with a male red wolf. Three hybridization events were the result of a red wolf pairing with an individual that was previously believed to be a red wolf until genetic testing revealed otherwise. The previous history of these females was unknown. All three of these cases occurred in 2002 and 2003, soon after the implementation of the new management plan and strict genetic testing. Two of these cases involved the same female red wolf and male non-red wolf.

Half of the hybrid litters (16 of 30) followed the disruption of a stable breeding pair of red wolves prior to or during the breeding season. Most of these litters (*n* = 9) followed the death of one or both members of a breeding pair of red wolves by gunshot. Another two followed the death of a red wolf breeder by either poison or a trap injury. Two hybrid litters followed the “disappearance” of a breeder, meaning the FWS has lost radio contact with a breeding member of a red wolf pack. Five hybrid litters followed the disruption of a breeding pair by natural causes. One involved a female wolf whose male partner died naturally after a foreign object punctured a lung, causing a systemic lung infection. Another occurred when a female wolf mated with a non-red wolf after both the breeders in her pack died of mange. The third case involved a female whose mate was killed by another wolf. In two other situations a female red wolf had been paired with a sterilized coyote that was subsequently displaced or killed by a red wolf from another pack; these female red wolves then mated with non-red wolves.

To summarize, excluding the seven cases with no known history, 78% (18/23) of the hybrid events followed disruption of social groups. Of these, 61% (11/18) followed anthropogenic mortality,

which could be an underestimate if the two individuals that “disappeared” had a similar fate. In situations of breeder disruption either the remaining breeder (*n* = 7) or a previously unmated member of the pack (*n* = 9) interbred with a non-red wolf.

#### 4. Discussion

Exploring individual records of hybridization events in this red wolf–coyote system provides insights into mechanisms that may regulate interactions between these two species. There are several observations we can draw from the wolves involved in these events. First, a large number (90%) of hybridization events involved female red wolves. Based on genetic data, researchers have hypothesized that introgression between wolves and coyotes predominantly occurred when male wolves bred with female coyotes (Lehman et al., 1991; Mozón et al., 2014); our findings refute that hypothesis and support the genetic results of Hailer and Leonard (2008). It also matches the predictions of Wirtz (1999) that hybridization is driven by females from the rarer species selecting heterospecific mates from the more common species.

Ultimately, it appears that hybridization events tend to follow the disruption of stable breeding pairs of wolves, frequently due to anthropogenic actions such as gunshot mortality. In this system canids begin establishing pair bonds during a period that corresponds with the onset of hunting seasons for large mammals. The elimination of red wolf breeders during the breeding season forces reproductively active red wolves to quickly locate another

mate. A higher percentage of hybrid than red wolf litters were produced by first-time female breeders, which is likely due to the low natural turnover in red wolf breeders from year to year (Sparkman et al., 2011). We hypothesize that as stable red wolf pairs dissolve a social 'release' occurs for young wolves providing them with the opportunity to breed, which echoes findings from other wolf populations (Brainerd et al., 2008). The inexperience of these animals coupled with the timing of pair dissolution during the breeding season may facilitate selection of a heterospecific mate.

Such findings correspond with those from other mammalian carnivore social systems that have demonstrated sensitivity to disruption due to human activity (Tuytens and Macdonald, 2000; Loveridge et al., 2007; Packer et al., 2009; Wallach et al., 2009; Rutledge et al., 2010; Davidson et al., 2011; Borg et al., in press). By pooling data from multiple wolf populations across Europe and North America, Brainerd et al. (2008) found breeder loss facilitated pack dissolution and abandonment of territory. Disrupting stable social systems in canids has cascading effects on intraspecific and, as shown by this red wolf system and wolves in southeastern Canada (Rutledge et al., 2010, 2011), interspecific interactions. Management of wolves must take into account the consequences of breeding pair dissolution for these events can facilitate genetic introgression into small populations.

The disparity between the number of red wolf and hybrid litters suggests that red wolf pairings are more common than those between red wolves and coyotes, which is encouraging considering coyotes outnumber wolves in portions of the RWEPA (USFWS, 2007; Bohling, 2011; Bartel and Rabon, 2013). Such patterns are heavily influenced by human management, which impacts our interpretation of these results. Regardless, the low number of hybridization events that could be attributed to naturally dispersing individuals suggests that even when sympatric these species do not breed randomly and red wolves seek conspecific mates. This is despite the fact that the red wolf population is increasingly inbred (Brzeski et al., 2014) and genetic diversity has declined over time (Bohling et al., 2013). Inbreeding avoidance can facilitate selection of a heterospecific mate (Palmer and Edmunds, 2000; Gee, 2003). In pack-forming canids inbreeding avoidance is linked with dispersal from natal packs (Sillero-Zubiri et al., 1996; vonHoldt et al., 2008; Sparkman et al., 2012), which could facilitate hybridization if dispersing increases the probability of interacting with heterospecifics. Despite the abundance of coyotes and limited gene pool, hybridization does not appear to be fueled by dispersing red wolves, which indicates some form of assortative mating may be operating in this system.

Many of the non-red wolf parents involved in these hybridization events had mixed ancestry. With the removal of hybrids from the landscape, we expected most hybridization events to involve coyotes; these results, however, suggest that red wolves may be selecting for admixed mates over pure coyotes if available. Both traditional trapping (USFWS, 2007; Bartel and Rabon, 2013) and non-invasive genetic sampling (Adams et al., 2007; Bohling, 2011; Bohling and Waits, 2011) reveal that coyotes vastly outnumber hybrids in this landscape, yet admixed individuals were responsible for 40% of the hybridization events when the non-wolf parent was identified. In hybrid systems in which there is a morphological, behavioral, ecological, or size disparity between the parental groups suggest, hybrids can serve as a bridge between the two parental groups and facilitate introgression (Dowling and Secor, 1997; Goodman et al., 1999; Seehausen et al., 2008; Duvernell and Schaefer, 2014). If red wolves are preferentially selecting admixed mates in this landscape, then efforts to remove hybrids must remain a management priority.

Characterizing the wolves involved in hybridization events is difficult due to the circumstances that surround these events. Any observed patterns could simply be a result of a random

artificial selection due to the stochastic nature of anthropogenic mortality. For example, wolves involved in hybridization events tended to have slightly higher levels of coyote ancestry. However, this was not a strong pattern and could be a random artifact. Low sample size of hybridization events relative to red wolf litters, although good for red wolf conservation, limits our ability to draw inferences. Ascertainment bias may also influence the results since the breeding habits of red wolves that are not monitored are difficult to determine. This may explain the overwhelming bias toward female red wolves producing hybrid litters: USFWS biologists monitor female wolves as they begin to localize around a den site so they can locate the pups but do not do the same for males. Questions regarding the impact of genetic ancestry, breeding experience, and sex on hybridization could be best answered with mate choice experiments.

The realization that hybridization tends to follow the disruption of stable breeding pairs is important for management of the red wolf population. There has been a dramatic increase in the amount of gunshot and overall human-caused mortality over the past decade (Bartel and Rabon, 2013). For the adaptive management plan to be successful, more effort must be placed in reducing the number of red wolves killed by gunshot to facilitate efforts to restrict hybridization. A recent agreement between the North Carolina Wildlife Resources Commission and several environmental groups to limit coyote hunting in the RWEPA is a positive step toward achieving this goal (SEL, 2014).

Interpreting the spatial distribution of hybridization events and their significance for red wolf conservation is more complex. Trapping surveys indicate that wolves and coyotes are not evenly distributed across the RWEPA (USFWS, 2007; Bartel and Rabon, 2013). Non-invasive genetic surveys suggest that over 80% of the canids in Zone 1 are red wolves, whereas coyotes composed 70% of the population in Zone 3 (Bohling, 2011). Zone 2 is close to a 50–50 split between the two species. Thus, the abundance of hybridization events in Zone 3 could be simply a result of an abundance of coyotes and the inability of wolves to locate conspecifics, which matched our initial expectations and the classic 'desperation hypothesis' (Hubbs, 1955).

The distribution of hybrid events may also be linked to human social factors and land use practices. Zone 1 is predominantly composed of protected areas under public ownership and the first reintroductions occurred in this area, meaning the USFWS has longstanding relationships with many of the landowners. Expansion of the red wolf population westward has mainly occurred on private lands. Many of these landowners did not anticipate wolf colonization of their property, which has created friction between local communities and the USFWS as was highlighted in an independent review of the red wolf program (WMI, 2014). This conflict, combined with lack of protected refuges for wolves, lack of awareness among the hunting community, and proximity to the mainland coyote population, likely facilitates breeding pair disruption and the spatial pattern of hybridization events.

The management approach taken by the USFWS places value on protecting the genetic 'purity' of the population by limiting hybridization. Such efforts hinge on the commitment to protect a species that today is populated by descendants from just a few founders. The taxonomy and ancestry of these founders has been the source of controversy and some genetic studies suggest the red wolf is a relatively recent product of hybridization between coyotes and gray wolves (Lehman et al., 1991; Roy et al., 1996; vonHoldt et al., 2011). An alternative perspective is that red wolves align closely with eastern wolves in southeastern Canada, which represent a North American-derived wolf sharing a close evolutionary relationship with coyotes (Wilson et al., 2000; Hedrick et al., 2002; Kyle et al., 2006; Rutledge et al., 2012). The debate concerning these alternative hypotheses is ongoing (vonHoldt et al., 2011;

Rutledge et al., 2012). This uncertainty has fueled debate regarding the efficacy of investing resources in the protection of a population potentially derived from hybridization (Gittleman and Pimm, 1991; O'Brien and Mayr, 1991; Jenks and Wayne, 1992; vonHoldt et al., 2011). Further complicating matters is the perception that red wolves have a high propensity to hybridize, leading some to suggest management should be lessened allowing the population to assimilate with the surrounding coyote population (Kyle et al., 2006).

Our results provide a new perspective in this debate by revealing the extent to which human factors, both positive and negative, govern hybridization dynamics in this system. The opposing forces of management designed to maintain the red wolf gene pool and human disruption eroding reproductive boundaries impact our perception of potential red wolf recovery. The current introduced population has remained morphologically (Hinton and Chamberlain, 2014) and genetically (Adams et al., 2007; Bohling et al., 2013) distinct from the resident coyote population. We must recognize that human management as a principle factor in maintaining this pattern. At this point, though, management may operate as a zero-sum game, with facilitation of red wolf reproduction counteracting the impacts of pack disruption. In this case, the infrequency and stochasticity of hybridization events and low number of hybrids across this landscape (Adams et al., 2007; Bohling, 2011; Bohling and Waits, 2011) suggest behavioral processes play a role in maintaining the distinctiveness of the red wolf population.

Research has demonstrated the importance of behavioral forces in regulating interspecific hybridization and species boundaries (Wolf et al., 2001; Pfennig, 2007; Reyer, 2008; Svedin et al., 2008; Krosby and Rohwer, 2010; Sacks et al., 2011; Robbins et al., 2014). Even in the face of introgression, boundaries between parental groups can be maintained through behavioral mechanisms. Our findings mirror those of eastern wolves around Algonquin Park in which hunting disrupted pack dynamics and facilitated coyote introgression (Rutledge et al., 2010, 2011). Following restrictions on hunting in areas surrounding the park, the social dynamics of the population were restored and introgression became less frequent. Reducing manipulation, especially pack disruption, would reveal whether red wolves are capable of maintaining their genetic profile as eastern wolves have. Decisions regarding the future of red wolf management must account for the fact that human activity can facilitate hybridization and in turn impacts our interpretation of the relationship of this species to other canids.

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