Sex Ratio of a Northeastern Coyote, *Canis latrans*, Population Using Non-invasive Molecular Techniques

Chanda Essona Bennett

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Abstract

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Large carnivores (order Carnivora) play an important role in the ecosystem and their presence or absence can affect the abundance, distribution, and behavior of prey species and conspecifics. As such, carnivore conservation and management hinges on fully understanding carnivore ecology and potential community impacts. Studying adult sex ratio, as well as other demographic parameters, is key to interpreting and predicting carnivore population dynamics and future viability. Traditional research methods, however, have a variety of hanidcaps that can hinder efficient analysis of large carnivore studies (i.e. ethical concerns, low sample sizes, learned trap avoidance problems, and gender biases). To avoid the limitations of traditional research methods, I evaluated and developed a protocol that would non-invasively identify male and female coyotes using feces in an attempt to address population-level questions regarding coyote population dynamics in lower New York State. Two methods were tested for sex identification efficacy: ZFX/ZFY gene amplification and mitochondiral+SRY gene amplification. While the former is the ideal method of choice, the latter was most successful with coyote tissue and fecal samples. All tissue and 80% of fecal DNA extracts amplified under the double-primer sexing regime. Of the amplifiable fecal samples, 71% were identified as female and 29% as male. These preliminary results suggest that the coyote populations in lower New York may significantly be in favor of females. If future investigations of

this study confirm the preliminary female-biased sex ratio, coyote populations in lower New York could still be in its early stage of establishment despite the 20-years since first colonization. Future phases of this project will incorporate a variety of sampling and molecular technique alterations or additions to further address the growth potential of newly establishing Northeastern coyote populations in a non-invasive manner.

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Introduction

Like much of conservation and wildlife management, carnivore research has long been shaped by aesthetic, ecological, economic and political considerations. As a charismatic megafauna, carnivores in general elicit great sympathy, support, and interest from the public sector. Large mammalian carnivores (order Carnivora), in particular, have been idolized as symbols of strength, courage, and intelligence in both literary and artistic forms. Different aspects of carnivore behavior and form have been incorporated into many cultures as supplements to human expressions and spirituality (i.e. Rastafarian ideology, Hindu religion, Native American and African spirituality; Kellert et. al. 1996, Weber and Rabinowitz 1996). In turn, conservation directed toward carnivores, particularly the larger species, often attracts considerable funding toward research and management. Such attention tends to foster an umbrella-effect that results in protecting habitat and sympatric species that would not garner support on their own (Noss et. al. 1996).

Yet, despite this added attention and adoration, many carnivore populations have experienced drastic declines in number due to a variety of anthropogenic pressures such as habitat deforestation and degradation, overharvesting, and persecution. A majority of the large carnivore species, such as the tiger (*Panthera tigris*) and the African wild dog (*Lycaon pictus*), have been listed as endangered worldwide. In North America alone, several populations of large carnivores were extirpated from a substantial portion of their original ranges by the 1920's, including the grey wolf (*Canis lupus*), mountain lion (*Felis*

concolor) and grizzly bear (*Ursus arctos*) (Ginsberg and Macdonald 1990, Berger 1999, Murphy et. al. 1999).

Although a few carnivore populations are growing and recolonizing historical ranges (wolf: *Canis lupus; Mladenoff et. al. 1995*), or growing and establishing novel ranges (coyote: *Canis latrans*; Parker 1995, Gittleman and Gompper 2001), the future viability of many large carnivores (e.g. the African wild dog: *Lycaon pictus*, Creel and Creel 1996; and the Ethiopian wolf: *Canis simensis,* Fanshawe et. al. 1991), is of great concern. Because of such a threat, there is a continuing need to investigate and understand carnivore ecology and behavior particularly with growing conservation interest in reintroduction regimes and biodiversity restoration. In this study, I evaluated and developed techniques that would enable me to study the growth potential of a newly colonizing Northeastern coyote population in New York State using a non-invasive molecular analysis tool. The remainder of this document further describes the importance of carnivore research, the study methods implemented, the results and the concluding implications.

Carnivores in the ecosystem

The success of conservation action plans often relies on people's attitudes, perception and fears of carnivores in nature. An important challenge to carnivore conservation is mediating the human-carnivore conflict that arises when such animals are perceived as threats to people, livestock, and game. Fear for personal and domestic animal (i.e. pets) safety plays a major role in shaping the public view of predators and their potential

conservation (Kellert 1985); many are adamant against actions to restore carnivores in the ecosystem or to preserve the remaining populations. Such fear, and even panic, will escalate as large carnivores begin to inhabit regions with high human density. Similarly, predation by large carnivores on livestock can pose substantial monetary losses to a farmer in any given year. According to Knowlton et. al. (1999), coyote depredation on livestock can be responsible for as much as 30% of losses. In contrast, in areas implementing depredation control regimes losses tend to range between 1 and 6%. In addition, hunters and carnivores compete for many of the same prey species such as moose, caribou, deer and turkey. The combination of depredation on livestock and game species elicited great animosity towards the predator and facilitated the development of State and Federal predator control programs that decimated many carnivore populations (Knowlton et. al. 1999).

Not only are carnivores in direct competition with humans for prey and space, but they are also at risk of overharvesting for trade, meat, medicine, and trophies. According to the online retailer, www.furs.com, average prices for new designer fur coats from carnivores such as the raccoon, coyote, and lynx, range from $2000-$7000 each. Long dusters made from fisher pelt can be sold for as much as $25,000. On the other hand, some fur products are desired for traditional purposes. For example, in China, newlyweds desire red panda fur hats for good luck (Wei et. al. 1999). Recreational hunting is also a big pastime where permit-bearing trophy hunters take animals mainly for ornamental purposes (Lewis et. al. 1997). From a homeopathic point of view, bile from the gallbladders of bears and bones from tigers are considered valuable

commodities to potentially treat human ailments (Weber and Rabinowitz 1996). Yet while current predator control regimes and overharvesting do contribute to the carnivore problem at hand, ultimately the greatest threat to the future viability of carnivores may be habitat loss.

Due to their large spatial requirement, many carnivore populations suffer from range restrictions as a result of fragmented and degraded habitats, which is of concern given their important function in nature. Ecologically, large carnivores can greatly affect the organization of biotic communities. As predators, they impose a top-down pressure often limiting the abundance and distribution of prey species of reptiles, birds, mammals and insects (Franzmann & Schwartz 1986; Gasaway, et. al. 1992; Wright et. al. 1994; Terborgh 1987; Terborgh et. al. 1999, Post et. al. 1999, Hayes and Harestad 2000). Where predation has been relaxed or carnivores locally extirpated, a trophic cascade can occur, potentially freeing mesocarnivores and herbivores from natural predation thereby indirectly impacting the biomass of vegetation (Estes and Duggin 1995). In some cases, the presence or absence of such predation pressure can influence the behavior and/or demographics of the prey species (van Schaik and van Noordwijk 1985, Hunter & Skinner 1998; Berger 1999, Berger et. al. 2001). Recolonizing or expanding populations of carnivores, such as those of wolves and coyotes, will also have an impact on community structure, particularly for those prey species unfamiliar with native but novel predators (Berger et. al. 2001, Gittleman and Gompper 2001).

Given the many factors that threaten carnivore existence, their important roles in the ecosystem, and their function as flagship species, carnivore conservation and research is of the utmost priority. In essence, the effective management of carnivores hinges on fully understanding their biology, ecological influence and population dynamics. Focusing on demographic parameters, such as population size, age structure, and adult sex ratio, is key to addressing the potential growth of carnivore populations. For example, the adult sex ratio directly contributes to the population's birth rate. By monitoring the temporal fluctuations in the proportion of males to females, we can gain a better understanding of how populations will respond to short-term changes in their environment.

Research opportunities

A host of traditional research methods have been used for carnivore research including hands-off observation studies, radio-tracking, trapping, harvest information, and tissue sampling. These techniques provided the means by which to address sex ratio and other demographic issues, as well as systematic and behavioral questions concerning carnivore ecology. However, traditional research methods can be difficult to implement when trying to focus on large carnivores. Populations of large carnivores are typically characterized by low population density and large home ranges, with individuals that are often elusive and cryptic. Such trends can obstruct the efficient analysis of populational patterns. Mills (1996) argues that modern equipment and novel strategies can circumvent difficulties in traditional carnivore research, although he also acknowledges the pitfalls associated with trying to capture and census large carnivores. Trapping, for example, may be based towards those individuals more susceptible to capture. Similarly, most

studies measuring sex ratio have depended on information from harvested carnivores, which can potentially be biased towards a single sex (Parker 1995). Other traditional approaches, such as ground transect methods for censusing, can be time consuming, labor intensive, and expensive (Mills 1996). Research on free-ranging jaguars (*Panthera onca*) and red pandas (*Ailurus fulgens*) are cases in point. Past studies have often resulted in very small samples sizes despite extensive trapping efforts (Rabinowitz and Nottingham 1986, Crawshaw and Quigley 1991, Wei et. al. 1999).

Advances in molecular genetics, such as the polymerase chain reaction (PCR), have opened the door for the use of non-invasive, less stressful techniques in studying carnivores. Contemporary molecular genetics methods using fecal samples is an alternative research methodology and can potentially address similar questions of relatedness, abundance, population size, diet and distribution, as did traditional techniques (Lehman et. al. 1992, Morin et. al. 1992, Tinkel et. al. 1995, Taberlet et. al. 1996, Kohn et. al. 1999, Ernest et. al. 2000, Mowat and Stobeck 2000). Depending on the species, feces are often abundant and easy to acquire and store. For example, even though jaguars are difficult to capture and census, a large number of scat samples can be collected for analysis in a relatively short period of time (Rabinowitz and Nottingham 1986, Taberlet. al. 1997). A small amount of excrement from the host can contain sloughed intestinal cells from which DNA can be extracted, though often in quantities too low for analysis (Albaugh et. al. 1992; Constable et. al. 1995). The advantage of the PCR process is that it can produce millions of copies of a portion of DNA. Because this technique amplifies DNA whether in small or large quantities, it is even useful when the

original sample contains degraded or contaminated DNA, as is often the case with scat. DNA from the extraction and PCR procedure can be manipulated and analyzed in ways similar to how one would use extracts obtained from blood or tissue medium.

Despite the advantages of fecal DNA analysis, there are challenges associated with the technique that warrant further consideration. Extracted fecal DNA often harbors inhibitory substances that can hinder the amplification PCR process. On the other hand, even successfully amplified products could still yield low amounts of DNA, which may impede effective analysis. In addition, potential contaminates from ingested materials could provide false positive results during analysis. Although overcoming these constraints can be a long and arduous task, recent studies have successfully extracted and amplified DNA from degraded fecal samples from various mammalian species including seal (Reed et. al. 1997), mountain lion (Ernest et. al. 2000) and bear (Mowat & Strobeck 2000) for species, sex and individual identification analysis. However, few studies have attempted to explore whether non-invasive techniques can also be used to monitor fluctuations in sex ratios of a population (Kohn et. al. 1999).

Many aspects of the behavior and ecology of the coyote makes it a particularly good and interesting model to work with when considering issues related to carnivore ecology. For example, coyotes are viewed as a direct competitor with man over livestock and game and can have an impact on sympatric carnivores and prey species. Monitoring coyote populations can potentially be done by through fecal analysis in order to explore the adult sex ratio dynamics of large carnivores.

Overview of coyote ecology

Prior to European settlement, the coyote was restricted to the grassy plains of the mid-west. By the 1800's, however, rapid land conversions facilitated the continental radiation of the coyote north and eastward resulting in its novel colonization of northern North America. Coyotes have been established in upper New York State since the 1940's but it wasn't until the 1980's were coyotes present in the lower Hudson area (including densely populated suburban counties of Westechester, Putnam, and Rockland; Parker 1995; Gittleman and Gompper 2001; Fener 2001, J. Brady, pers. comm). Currently, coyotes are found in relatively high densities throughout much of their historic and colonizing range.

As generalist predators, the coyote diet can consist of a variety of species of birds, reptiles, and mammals (Parker 1995) in New York this new predator could greatly impact the abundance and distribution of local conspecifics—such as the red fox—and prey populations—such as the snowshoe hare and white-tailed deer (Moore and Miller 1986, Crooks and Soulé 1999). Therefore, it is important that we understand the dynamics and growth potential of this newly establishing carnivore. Investigating the coyote adult sex ratio is key to reaching this goal.

While demographic studies of coyote populations in their historic range suggest that coyotes exhibit an overall balanced adult sex ratio (1:1) (Parker 1995, Kohn et. al. 1999), studies in the Northeast suggest that observed male and female biases can vary depending on exploitation intensity (i.e. predation control efforts), food availability (i.e. fluctuations

in prey abundance), habitat type, and mobility, and hence dispersal, of a particular sex (Parker 1995). What remains most intriguing is the potential for coyote populations to exhibit different sex ratios depending on establishment stage. Some evidence from traditional capture and censusing studies support the theory that deviations from a 50:50 in favor of females may be typical of newly colonizing populations (Parker 1995). This bias has been attributed to a combination of non-mutually exclusive factors: an increased reproduction rate, at birth female bias, and/or greater dispersal ability of females. On the other hand, Moore and Millar (1984) found that sex ratios in colonizing coyote populations of eastern New Brunswick and Nova Scotia tended to favor males perhaps due to the ability for males to disperse into vacant territories. Lastly, there remains the potential for shifts in the adult coyote sex ratio as colonies become established over time, although this has not been extensively investigated (Parker 1995). The lower Hudson coyote population represents an exemplary site to investigate adult sex ratios for it is home to a relatively new coyote population.

The first step in assessing the sex ratio of the lower Hudson coyote population requires identifying the sex of individual animals. Unfortunately, coyote research faces many of the same difficulties associated with large-scale traditional studies of carnivores that can hinder observational and census efforts: i.e. ethical issues surrounding leg hold traps, learned avoidance of traps, harvesting biases by hunters and trappers. As suggested by Parker (1995), past traditional attempts at identifying coyote adult sex ratio could have experienced sampling biases that skewed the observation, i.e. more males taking advantage of bait (i.e. carcasses) than females. Since non-invasive techniques have been

implemented and successful in the past for other coyote populations (Kohn et. al. 1999), molecular analysis of coyote fecal samples offers a promising alternative to study the coyote in the lower Hudson region.

My goal was to evaluate and develop an efficient sexing protocol that would effectively identify male and female coyotes using fecal samples in a non-invasive study and to use this information to address population-level questions about coyotes inhabiting the lower New York area. For mammals, sex identification methods have generally focused on two types of conserved gene sequences: 1) the zinc finger protein gene homologous to both the X- and Y-chromosomes (ZFX/ZFY) which requires restriction enzyme digestion for diagnostic sexing; & 2) the sex determining region Y gene (SRY). Adult sex ratio of the lower Hudson coyote population can potentially be assessed from this study's sex identification protocol.

Methods

Study Site

Fecal samples were collected at Black Rock Forest, a 1500 ha preserve in the Hudson Highlands, New York, on the west side of the Hudson River (fig. 1). The forest is managed by the Black Rock Forest Consortium, which is comprised of 22 private and public educational and research institutions, including Columbia University and the American Museum of Natural History. While recently acquired by the Black Rock Forest Preserve, it has remained relatively unmanipulated since the turn of the 20th

large forested areas to the highly populated New York City suburbs. The preserve's landscape itself is continuous with land from the West Point military base and that of

Figure 1. Map of New York State indicating location of Black Rock Forest area.

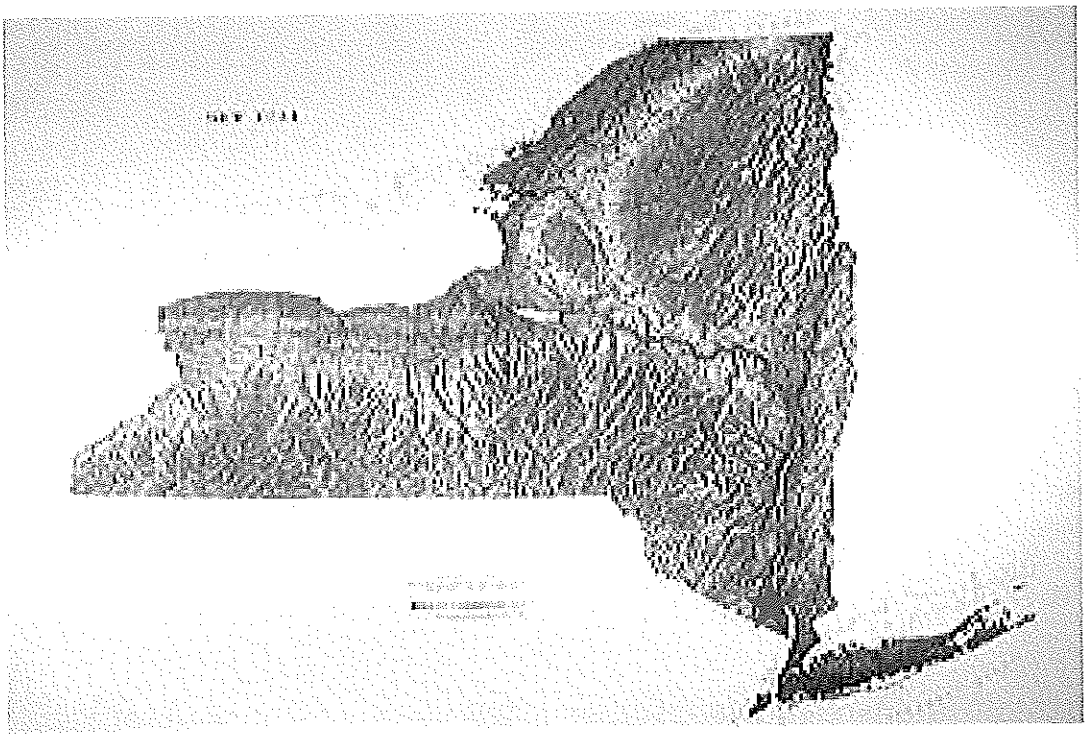
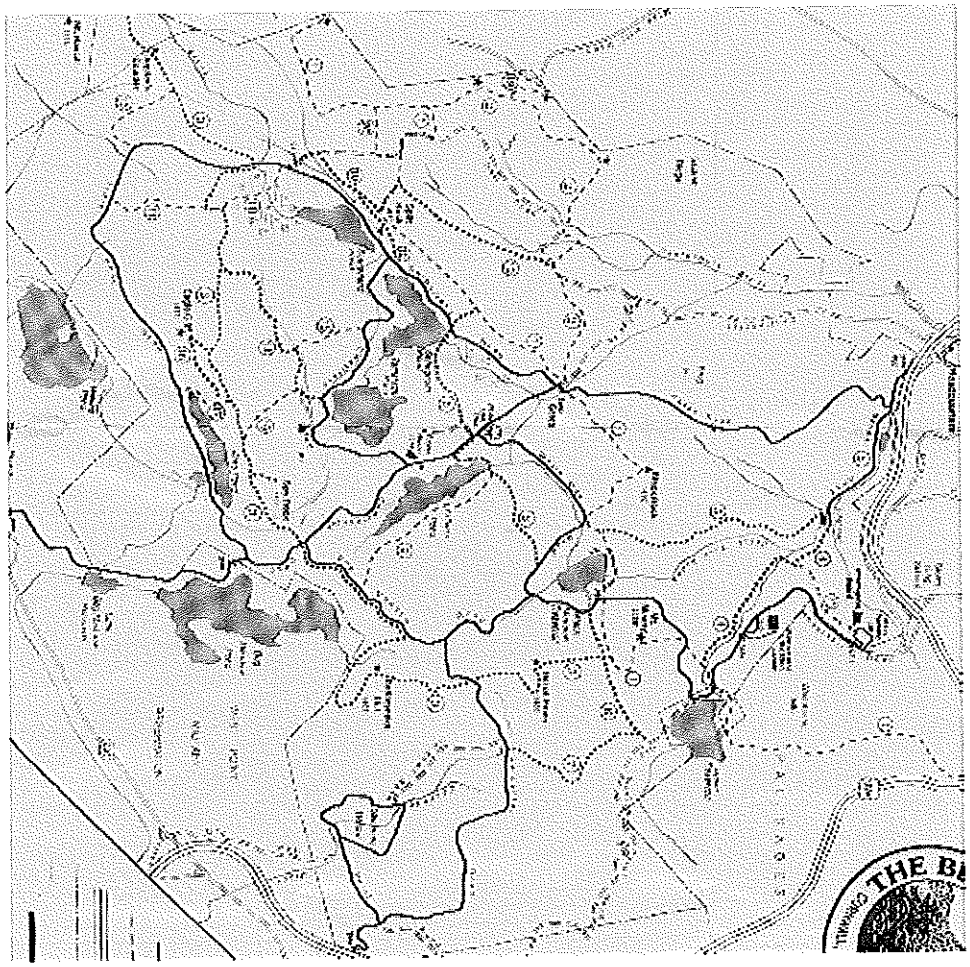


Figure 2. Hiking trails located in Black Rock Forest.



Harriman and Palisades State Parks. Black Rock Forest is home to a diverse biological community, numerous ponds and wetlands and is open to the public and their domestic pets for limited recreational activities. Coyotes were first observed in the forest in the late 1980's (John Brady, pers. comm.) and have remained a stable presence since then. While no feral dogs have been observed in the forest since coyote colonization, other carnivores do inhabit Black Rock Forest including red and gray foxes, raccoons, skunks, weasels, fisher, and feral cat. Occasional transients include bobcat, bear, and mink. though in low numbers.

Fecal Collection and Sampling

The hiking trails in Black Rock Forest are used by coyotes to traverse multiple habitats throughout the preserve (fig. 2). Since trails are heavily used and cover the majority of the preserve, they were selected as transects for fecal sample collections. Coyote scat were readily available on these transects. Unlike scent-marking by urination, defecation rates are not subject to differing defecation rates due to social status, gender, or season (Gese and Ruff 1997). However, females may be under-represented if they infrequently use transects during the collection period, particularly if pups are present. Fecal samples were chosen based on overall dog-like appearance—typically slender and 10.16cm in length containing hair or bone (fig. 3). Non-coyote fecal samples may potentially be collected if they conform to the stereotypical appearance. However, fecal mimics should be found in fewer quantities as certain transects surveyed will be in habitats less likely used by conspecifics. The most probable sampling biases would result not in the

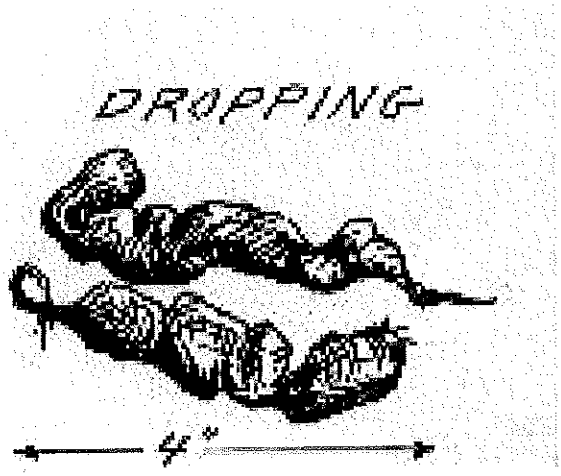
inclusion of scats from non-coyotes but in the exclusion of atypically formed coyote fecal samples.

A total of 8 tissue and 26 fecal coyote samples were used in this study: Twenty-three of the fecal and 2 of the tissue samples were collected between January and December 2000 from Black Rock Forest. The other tissue samples were collected from coyotes from the Albany Pine Bush. Fecal and tissue samples were placed in a zip lock bag, labeled and preserved in a –20°C freezer. The remaining three fecal samples collected in August 1999, dried and stored small brown paper bags at room temperature. Frequent transect coverage insured the collection of fresh fecal samples. Tissue samples, tongue or heart, were obtained from hunted carcasses or road killed animals. These samples were of known sex: 5 males and 3 females. Sex identification of the fecal samples were unknown and therefore required additional analysis.

DNA extraction

DNA was extracted from fecal, tissue, and blood samples following methods from Fernando et. al. (2000). In brief, approximately 50mg of the outer layer of frozen feces (20mg from tissue and 5μL from blood) was added to 1mL of digestion buffer and 20μL

Figure 3. Illustration of stereotypical coyote fecal formation.



of 10mg/mL Proteinase K. After a brief vortex and an overnight incubation at 65°C, the sample was centrifuged at 13k for 2 minutes. After 400μL of the supernatant was removed into new microcentrifuges the extract was then purified with 800mL of 25:24:1 phenol/chloroform/isoamyl alcohol, incubated for 10-15 minutes and centrifuged again. The remainder of the extraction protocol followed a modified version of the Qiagen DNAeasy kit (Qiagen, Chatham, NY) using the buffers provided. The aqueous supernatant was extracted once more and added to 1mL of the Buffer AL solution found in the kit. Approximately 700μL of the solution was filtered through the Qiagen spin columns twice. The extract was then washed twice with Buffer AW1 and AW2, respectively, and eluted with 100mL of warm Buffer AE. Lastly, the DNA elution was spun dry and resuspended with 40μL of warm Buffer AE.

PCR amplification

DNA was amplified from both tissue and fecal samples using 3μL of the extract in a 25μL reaction volume containing 3.0mM $MgCl_2$, 67.6mM Tris, 16.6mM $(NH_4)_2SO_4$, 0.4mM of each dNTP, 1.6mg mL^{-1} of bovine serum albumin, 0.6μM SRY f, 0.6μM SRY R, 0.4μM 12s F, 0.4μM 12s R, and 1 unit of TAQ. Amplification was done in the Perkin Elmer 9600 thermocycler as follows: hot start at 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 1 minute, and a final extension at 72°C for 2 minutes. All PCR reactions included a negative DNA control and two positive controls: a tissue and fecal sample of known origin. Fragments were separated following electrophoresis from an ethidium bromide 3% polyacrylamide gel in 1x TBE buffer and visualized under ultraviolet light.

Sex identification

The sex identification of coyote extracts was carried out using two different methods.

ZFX/ZFY assay: The first assay tested the use and efficiency of primers that would simultaneously amplify a canid zinc finger protein gene found on both the X- and the Y-chromosomes designed by García-Muro et. al. (1997): ZFX/Y P1-5ez and ZFX/Y P2-3ez (table 1).

The product is expected to be 902 base pairs in length. In addition, new ZFX/Y primers were designed from canid ZFX & ZFY sequences obtained from Genbank (*Canis familiaris* ZFX partial gene: AB027764; *C. familiaris* ZFY partial gene: AF027766) to target a smaller base pair fragment since DNA extracts were obtained from degraded fecal samples and size of amplifiable fragment remained questionable: ZFX/Y cbF and ZFX/Y cbR (table 1).

The product was expected to be ~110 base pairs. ZFX/Y fragments from both primer pairs were then subject to digestion by the Hae III or Taq 1 restriction enzymes (New England Labs) following packaging instructions and as suggested by García-Muro et. al. (1997).

SRY assay: In the second assay, the sex-determining region (SRY), which codes for the testis-determining factor, was targeted because of its specific link to the mammalian Y-

chromosome. SRY nuclear primers developed by Oliver et. al. (1999) was modified for use in this study to produce a 107 base pair fragment. Since the SRY will only amplify in the presence of the male Y-chromosome, a single-banded produce would represent males and females would not produce a band. However, the absence of a product in the reaction is not confirmation of a female since it could also result from a reaction malfunction (i.e. absence of DNA or extract inhibitors). To control for this, the 12s region of the mitochondrial DNA was also targeted during the SRY reaction. This acts as a safeguard against false female identification as was successfully done using hairs from brown bears (Taberlet et. al. 1993). This step is especially advantageous since thousands of copies of mtDNA are present in every diploid cell (as opposed to two copies of nuclear DNA) and mtDNA is easier to recover from degraded samples. Every reaction is expected to produce a mtDNA product with males producing an extra SRY product. A 181 base pair segment of the 12s gene was targeted using modified universal mammalian primers already developed (R. DeSalle, unpublished): 12s609mam, 12sanc790, SRYf, and SRYr.

Table 1. Primers tested on coyote tissue and fecal DNA extracts for amplification.

Primer	Sequence	DNA fragment	Tested on:	Reference
ZFX/ ZFY (X- & Y-linked Zinc Finger Protein)	*5ez:* 5' ATAATCACATGGAGAGCCACAAGCT 3' *3ez:* 5' GCACTTCTTTGGTATCTGAGAAAGT 3'	~ 900 base pairs	Dogs, Humans, Mice, Goats Whales, Lizards	García-Muro et. al. 1997
ZFX/ZFY homemade	*CB f:* 5' TGGCGGTCCACA 3' *CB r:* 5' TAGAAGAGTCTG 3'	160 base pairs	Coyote	Genbank aligned sequences AF027765 & Af027766
SRY (Y-linked Sex determining region)	*F:* 5' CTCGCGATCAAAGG 3' *R:* 5' TTCGGCTTCTGTAA 3'	107 base pairs	Coyote	Oliver et. al. 1999
12S (mitochondrial DNA)	*609MAM:* 5' GGAGCCTGTTCTRTAATCGAYAAACCC 3' *ANC790:* 5' GGTTAGAAAATGTAGCCCATT 3'	~190 base pairs	Various mammals	DeSalle Lab Primer

Results

Dried vs. Frozen scat

The extraction protocol successfully recovered DNA from all three dried and most of the frozen (21 of 26) fecal samples analyzed in this study. Despite this accomplishment, frozen samples generated better amplifiable products than the dried samples; products from frozen extracts consistently provided stronger stained bands indicating a greater quantity of the DNA fragment.

Sex identification

ZFX/ZFY assay

Amplification of the ZFX/ZFY locus on the sex chromosomes using primers from the literature (García-Muro et. al. 1997) successfully produced a 900 base pairs product only from tissue and blood samples (fig. 4a). Targeted fragments were not produced from any of the included fecal samples. The two designed primers, ZFX/Y cbF and ZFX/Y cbR, worked on all sample types and produced bands approximately 110 base pairs long (fig. 4b). Despite the amplification success, the Hae III restriction enzyme failed to digest the products and thus failed to make the distinction between males (cutting to produce a double-banded product) and females (leaving a single-band product).

SRY assay

The combination mtDNA and SRY sexing method was successful for both tissue and fecal derived DNA extracts (fig. 5a and b). All tissue of the DNA extracts, which were obtained from individuals of known sex, successfully produced the appropriate 12s

Figure 4: Zinc-finger protein products from tissue (T) and fecal (F) DNA extracts: a. ZFX/ZFY amplification products prior to restriction digest using primers developed by García-Muro et. al. (1997). Lanes L= Ladder, Tm 1-3 = products from known males, Tf = product from known female. F 1-3= products from fecal samples of unknown sex; b. ZFX/ZFY amplification after restriction digest using designed primers. Digestion did not occur with Hae III giving diagnostic double bands for males. Lanes L= Ladder, Tm1= product from known male, Tf= product from known female, F 1-2= products from fecal samples of unknown sex.

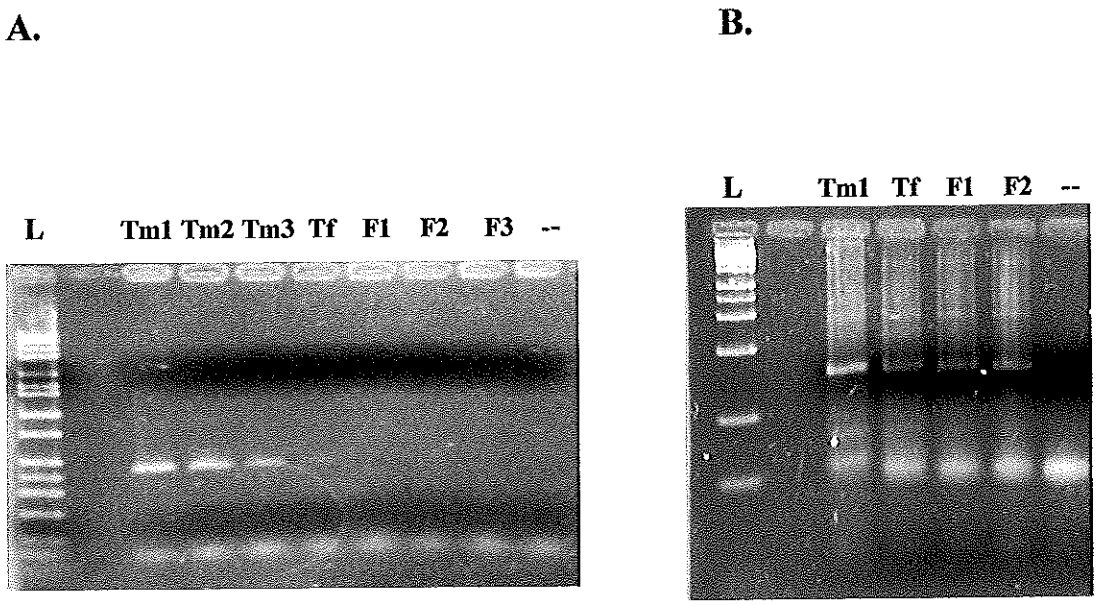
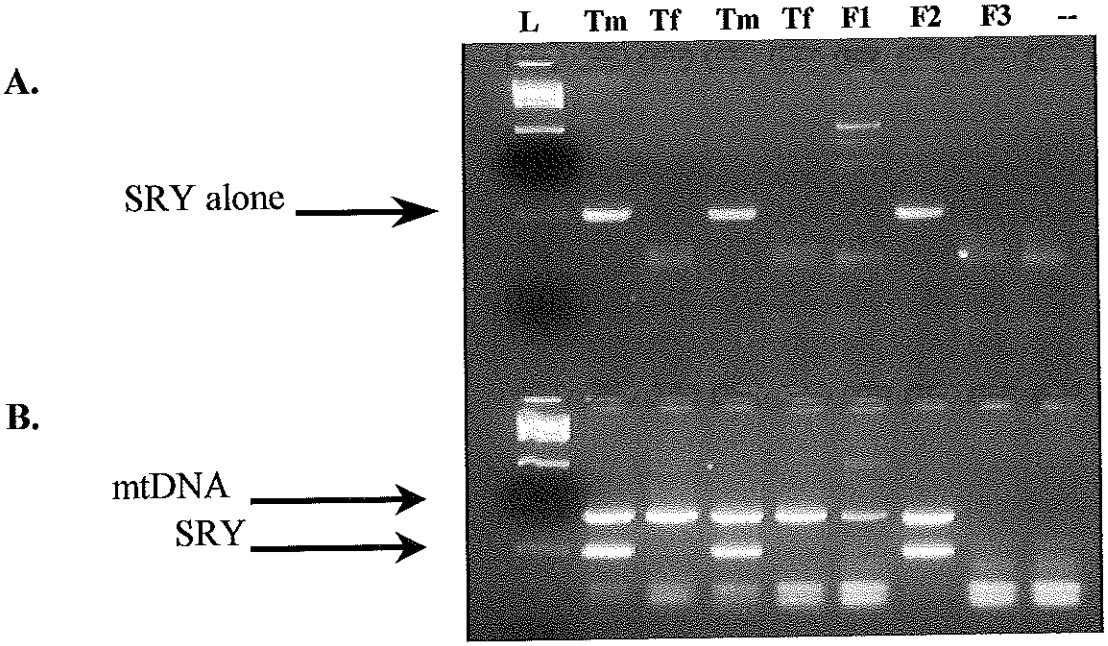


Figure 5: Gender identification of coyote tissue (T) and fecal (F) samples by amplification of the 12S and SRY genes: a. SRY amplification alone. Lanes L= ladder, Tm= products confirmed from 2 known males, Tf= products confirmed from 2 known females, F 1-3= products from fecal samples of unknown sex but identified as 2 females and 1 male; b. 12S and SRY amplification. Lanes L= ladder, Tm= products confirmed from 2 known males, Tf= products confirmed from 2 known females, F 1-3= products from fecal samples of unknown sex but identified as 1 male, 1 female, and 1 undetermined.



mtDNA and/or SRY products depending on the sex. Females were characterized by the presence of only one PCR product (181 base pair 12s fragment) while the males showed a diagnostic two-banded product (a 181 base pair 12s and a 100 base pair SRY fragment). Of the 26 fecal samples collected for analysis, 80% were successfully sexed using the double-primer protocol (table 2). While most of the samples were sexed on the first attempt, 50% of them required either 2 additional extractions from the original fecal sample or reamplifications from the extracts in order to yield a trustworthy product. Five fecal DNA samples remained of unknown sex despite multiple extraction and reamplification endeavors. These 5 samples were excluded from the study for one of two reasons: 1) absence of both mtDNA and SRY products, or 2) barely visible bands for credible assessment (fig.4b: F3).

Population-level analysis

As was expected, the sexing method produced 5 male and 3 female samples from the tissue. Of the amplifiable fecal samples, 71% were identified as female and 29% as male (table 2). The females were characterized by only one PCR product (181bp 12s DNA fragment) while the males showed a diagnostic two-banded product (181bp 12s DNA and 100bp SRY fragments).

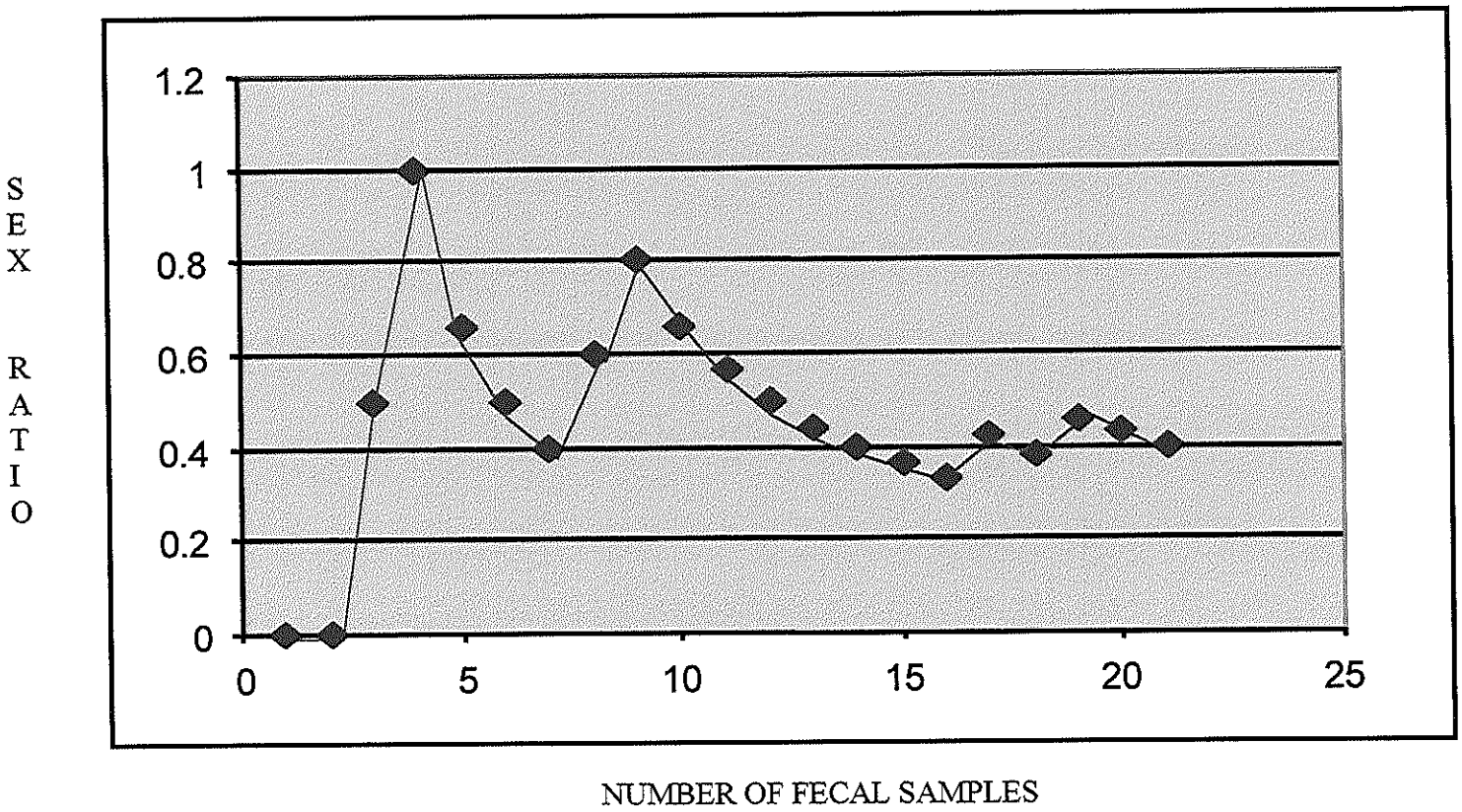
If each of the 26 fecal sample analyzed in this study originated from a different individual, the preliminary results from this study indicate that the Black Rock Forest population is heavily female-biased, with a sex ratio of about 3:1. As more fecal samples

are added to the study, the sex ratio should potentially hone in on the true value, as reflected in fig. 6.

Table 2: 12s mtDNA and SRY amplification results from 34 tissue and fecal DNA extracts.

	No. of Samples	Males	Females	Unknowns
Tissue	8	5	3	0
Feces	26	6	15	5
Total	34	11	16	5

Figure 6: January –December 2000 preliminary adult sex ratio (male:female) estimation from Black Rock Forest coyote population with increasing fecal sample size.



Discussion

A multitude of sexing options are available for tissue, blood, or fecal analysis for a wide range of mammalian species (Richard et. al. 1994, Kohn et. al. 1995, García-Muro et. al. 1997, Reed et. al. 1997, Kohn et. al. 1999). Targeting the zinc-finger protein gene homologous to both the X- and Y-chromosomes is the ideal approach for accurate sexing; the process incorporates a natural internal positive control mechanism that targets only the chromosomes for diagnostically identifying samples originating from males and females. Although the ZFX/ZFY genes have been amplified from blood (García-Muro et. al. 1997) and fecal samples (Wasser et. al. 1997), present application of this technique was not successful with the samples analyzed in this study. With further optimization, the designed ZFX/ZFY assay could prove to be an effective tool for identifying sex from fecal samples depending on the presence or absence of a particular digested product.

In the interim, the 12s mtDNA and SRY primer combination was the most reliable sexing tool for samples in this study. A similar double-primer protocol was used on fecal extracts from seals (Reed et. al. 1997) and fecal and hair extracts from bears (Kohn et. al. 1995) but has not, that I am aware of, been used with canid samples. The presence of the mtDNA fragment in every reaction was a successful internal positive control for the diagnostic identification of female samples in the absence of an SRY fragment. Its success is due, in part, to the small fragments targeted by both primer pairs. Since fecal DNA is typically heavily degraded, targeting shorter genomic fragments during the PCR process increases the probability of procuring amplified product.

Molecular scatology remains a generally applicable and inexpensive tool for the noninvasive study of carnivores in the wild. Fecal analysis is of great value since scat is readily accessible and contains intestinal cells (a reliable source of DNA) sloughed off during defecation. Of the available extraction techniques that use Chelex resin (Ernest et. al. 2000), magnetic beads (Flagstad et. al. 1999), or commercial kits (Wasser et. al. 1997, Kohn et. al. 1999), the most suitable protocol for the tissue and fecal (both dried and frozen) samples in this study was the phenol:chloroform and Qiagen kit combination as performed by Fernando et. al. (2000). Time spent optimizing the extraction protocol minimized the intensity of procedural complications such as contamination and inhibition. Recovering DNA from fecal samples may not always be successful on the first attempt; especially if epithelial cells are patchily distributed on scat or storage protocol affect cell longevity. As suggested by Kohn et. al. (1995), multiple extracts from different portions of the same sample was necessary in order to increase the possibility of obtaining fecal DNA for analysis. Those fecal samples that could not yield DNA may have lacked intestinal cells due to environmental degradation or were heavily contaminated with PCR inhibitors not removed during the extraction procedure. If samples still failed after multiple extractions or amplifying trials, they were discarded from the analysis.

Population-level analysis

Sex ratios of coyote populations have been shown to take on one of three general characteristics: 1) a stable population with a sex ratio of 1:1 (Parker 1995); 2) a female-biased population in areas where predator control regimes exist (females may compensate for increased mortality by skewing at-birth sex ratio towards females; Parker 1995); and

3) a male-biased sex ratio in colonizing populations (Moore and Millar 1984). Male-biased ratios have been attributed to the greater propensity for males to disperse into open habitats although sampling biases (i.e. males more susceptible to trapping than females) may skew observational results (Moore and Millar 1984, Parker 1995). While sex ratios can fluctuate depending on certain parameters such as prey abundance or dispersal ability, Parker (1995) suggests that there may be a pattern associated with progressive changes in coyote adult sex ratio as establishing population age. For example, if coyote population growth was subject to density dependent limitations, changes in the proportion of males to females as young populations age may serve to regulate population growth (although other issues such as food and space availability may factor into population maintenance).

If Parker's (1995) hypothesis holds, a scenario might be that a founding population would favor males due to their greater propensity to disperse. Females within this new colony would produce female-biased litters which in turn causes a short-term shift in the sex ratio to favor females. The sex ratio would level out as the population aged and birth ratio returned to equilibrium. This might explain the overall temporal adult sex ratio shift from a male-biased to female-biased sex ratio before reaching a 50:50 male:female ratio as observed in Nova Scotia between the 1970's and the 1990's (Parker 1995). If future investigations of this study confirms the preliminary female-biased sex ratio, the coyote population in Black Rock Forest, and hence that of the lower Hudson, would still be in an early stage of establishment (despite its 20 year presence) and could take another 30 years (according to Nova Scotia patterns) before the population reaches a 1:1 adult sex ratio.

Estimating the adult sex ratio using the results from this study's sex identification protocol can be fruitful as long as certain assumptions are met: 1) defecations rates do not differ between sexes or social status; 2) collected scat originated from *Canis latrans*; 3) the sample size is large enough to represent Black Rock Forest; 4) the sex ratio during the period of scat collection accurately reflects the true sex ratio; 5) each fecal sample represents an individual coyote or all individuals are equally sampled; and 6) the resulting sex ratio of the Black Rock Forest coyote population accurately represents that of the entire lower Hudson population.

The results of this pilot study should be reviewed with caution, as many of the assumptions noted above require further investigation. In future phases of this study more samples should be incorporated into the analysis; as the sample size increases, variance between samples minimizes and focuses in on the observed sex ratio (fig. 6). In addition, molecular sequencing of fecal extracts should be integrated into the project to address three main logistical and sampling concerns: 1) distinguishing coyote samples from prey and conspecifics (although this is unlikely a serious concern but needs verification nonetheless; Kohn et. al. 1999), 2) avoiding sub-sampling of individuals by identifying different genotypes through microsatellite analysis (Kohn et. al. 1999), and 3) identifying transient from resident population members. The latter concern can be addressed by collecting fecal samples during the late winter season, January-March, at which time transients are potentially absent from Black Rock Forest and pups are not

years (according to Nova Scotia patterns) before the population reaches a 1:1 adult sex ratio.

Ultimately, adult sex ratio can be assessed using the results from this study's sex identification protocol as long as certain assumptions are met: 1) defecations rates do not differ between sexes or social status; 2) collected scat originated from *Canis latrans*; 3) the sample size is large enough to represent Black Rock Forest; 4) the sex ratio during the period of scat collection accurately reflects the true sex ratio; 5) each fecal sample represents an individual coyote or all individuals are equally sampled; and 6) the resulting sex ratio of the Black Rock Forest coyote population accurately represents that of the entire lower Hudson population.

The results of this pilot study should be reviewed with caution, as many of the assumptions noted above require further investigation. In future phases of this study more samples should be incorporated into the analysis; as the sample size increases, variance between samples minimizes and focuses in on the observed sex ratio (fig. 6). In addition, molecular sequencing of fecal extracts should be integrated into the project to address three main logistical and sampling concerns: 1) distinguishing coyote samples from prey and conspecifics (although this is unlikely a serious concern but needs verification nonetheless; Kohn et. al. 1999), 2) avoiding sub-sampling of individuals by identifying different genotypes through microsatellite analysis (Kohn et. al. 1999), and 3) identifying transient from resident population members. The latter concern can be addressed by collecting fecal samples during the late winter season, January-March, at

which time transients are potentially absent from Black Rock Forest and pups are not being sampled. As such, fecal samples in this study, with the exclusion of 3 that were obtained in August, were collected during the late winter (January-March 2000) season.

Lastly, a New York State multi-population comparison of coyote populations of differing establishing ages will be incorporated into future research and could shed light on the dynamics of sex ratio change following colonization as mentioned by Parker (1995). Since coyotes moved from northern New York southward to the lower Hudson, populations from the north, central and south may exhibit differing demographic patterns, as a function of their colonization age and growth rate. Once this is done, one could ask: 1) where does the Black Rock Forest coyote population fit in the colonizing sex ratio scheme, and 2) if all three New York State coyote population zones (north, central, and south) followed the same sex ratio fluctuation pattern, how rapidly do sex ratios change assuming that there are differences)? Additional coyote fecal and tissue samples have been obtained from the Albany Pine Bush, colonized in the 1950's and 1960's (Sezeringhaus 1974, Fener 2001), and the Adirondacks, colonized in the 1930's and 1940's (Sezeringhaus 1974, Fener 2001), and await analysis.

The double-primer sex identification protocol described in this study has diverse applications for studying coyote population biology. In particular, the growth potential of coyote populations in the lower Hudson might be assessed using fecal molecular analysis techniques. With further temporal and spatial considerations, non-invasive methods can be used to reveal demographic patterns of newly establishing carnivore populations. As

such, wildlife conservationists and managers alike should give serious consideration to the advantages of indirect molecular techniques for use independently of or in concert with future traditional research projects.

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