Using Molecular Tools to Examine Predator-prey Relationships in Newfoundland

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Authorization to Submit Dissertation

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Abstract

Predation is a central process in many ecological communities, but the relationships between predator and prey populations are highly variable as a result of the inherent complexity of predator-prey systems. In recent years, the use of molecular methods in ecology has rapidly increased, but the application of molecular tools in the study of predation remains underutilized. We were interested in using molecular tools to illuminate the interactions between two predators and a common prey species, caribou, in Newfoundland. We compared morphological and molecular methods of food habit analyses and used noninvasive sampling to estimate black bear and coyote abundances. We also utilized molecular tools and a statistical model to decrease the subjectivity associated with predator species identification at kill sites. Our research suggested that molecular methods detect prey species in a significantly higher percentage of predator scats. Although caribou were frequently detected in black bear and coyote scats, we determined that moose were the most common prey species for both predators. We also demonstrated that black bears and coyotes were the primary predators of caribou calves, and that predation by Canada lynx, red fox, and bald eagle was limited. In addition, our approach elucidated predator-specific kill site observations that should improve the accuracy of future predator species identifications at calf kills. We also found that the most cost-effective and efficient methods of sampling predator populations for the purpose of abundance estimation may vary between species and across study sites. Our study demonstrated that molecular tools can be expanded to illuminate complex predator-prey processes and inform conservation and management decisions.

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Dedication

I would like to thank my wife for her enduring love and support during the completion of this project and throughout our marriage. She inspired me to dream bigger and then provided the emotional support that allowed me to fulfill those dreams. She has continually put her own professional and personal interests aside, so that I could pursue my professional goals. During the last five years, she has sacrificed numerous potential weekend trips so that I could focus my attention on studying, reading, and writing. She is my best friend and everything I have accomplished is a direct result of her role in my life. My next challenge is to provide her with the same level of emotional and financial support necessary for her to achieve her goals and aspirations, which I pledge to do with all my being.

I would also like to thank my parents for the loving, Christian home they provided. The core of who I am and what I believe stems from this upbringing, which instilled a sense of responsibility and a kindness to others that has allowed me to succeed both professionally and personally.

Finally, I would like to thank the rest of my family and my wife's family, along with all our friends both near and far for all their support during the trials and tribulations of life.

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Chapter I – A Comparison of Morphological and Molecular Food Habit Analyses of Predator Scats

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Abstract

An understanding of species' food habits is required to make sound conservation and management decisions. Traditionally, morphological analyses of undigested hard parts from food items remaining in scats have been used to assess diets. More recently molecular analyses of scats have been used to identify plant and prey species DNA, but no studies have compared morphological and molecular analyses of food habits for large, terrestrial carnivores. We used molecular tools to determine the percentage of black bear and coyote scats that contained three common prey species (caribou, moose, and snowshoe hares) in Newfoundland and compared the results to a traditional morphological analysis. We found that a ranking of the relative prey frequencies was consistent between the two methods, but molecular methods tended to detect prey species in a greater percentage of scats for all prey species. However, there were individual scats in which a prey species was detected by morphological methods only, and we provide evidence that molecular methods could result in false negatives if prey DNA is not uniformly distributed throughout a scat or as a result of PCR inconsistency. We also determined that molecular methods could be implemented for less cost than morphological methods when a large number of samples are processed or if the cost of developing a molecular prey species identification test is excluded. We recommend that controlled feeding studies be performed to validate molecular methods and investigate the utility of molecular methods to estimate the proportions for all food items consumed.

Introduction

Knowledge of a species' resource requirements is particularly important for understanding habitat needs (Litvaitis 2000). In particular, the food habits of a species can provide information regarding how it consumes and/or competes with other members of its community (Klare *et al*. 2011). Examination of stomach contents and scats are the most common methods used to evaluate the food habits of terrestrial animals (Litvaitis 2000). Scat analysis is particularly attractive because of the ease of collection and implementation, and its non-destructive and noninvasive nature, particularly when studying rare or elusive species (Valentini *et al*. 2009) or species of conservation concern (Mills 1996).

Traditionally, hard part or morphological scat analysis entails the identification of undigested animal or plant matter (Casper *et al*. 2007a), such as bones, teeth, hair, feathers, scales, exoskeletons (Litvaitis 2000), otoliths (Casper *et al*. 2007b), and macro- or microscopic plant material (Valentini *et al*. 2009). Determining the proportion of scats containing a food item or the proportion of hard parts from a specific food item are the most common methods, but identifying the volume or mass of food parts from a specific food item in proportion to all food items, in conjunction with species-specific correction factors, provides a more accurate estimate of the contribution of food items to a species' diet (Klare *et al*. 2011). However, the accuracy of morphological methods are often limited by a lack of identifiable hard parts (Casper *et al*. 2007a), variable delays in the excretion of hard parts between food items (Casper *et al*. 2007a), and difficulty distinguishing between hard parts of closely related species (Spaulding 2000; Zeale *et al*. 2011). Recently employed molecular methods have the potential to limit or eliminate some of these challenges by permitting the

objective identification of food items from both soft and hard matter present in scats (Tollit *et al*. 2009).

Researchers have used molecular methods to study the food habits across a range of species. The scats of pinnipeds (Deagle *et al*. 2005; Casper *et al*. 2007a; Casper *et al*. 2007b; Matejusová *et al*. 2008; Tollit *et al*. 2009; Bowles *et al*. 2011), penguins (Deagle *et al*. 2007; Deagle *et al*. 2010), bottlenose dolphins (*Tursiops truncatus*) (Dunshea 2009), chamois (*Rupicapra rupicapra*) (Rayé *et al*. 2011), domestic sheep (Pegard *et al*. 2009), snow leopards (*Panthera uncia*) (Shehzad *et al*. 2012a), leopard cats (*Prionailurus bengalensis*) (Shehzad *et al*. 2012b), and bats (Zeale *et al*. 2011) have been analyzed with molecular methods. Valentini *et al*. (2009) demonstrated the utility of a DNA barcoding approach by identifying food habits of primarily herbivorous vertebrate and invertebrate species via a DNA fragment of the chloroplast genome (P6 loop of *trn*L (UAA) intron). Some researchers have followed a similar approach by utilizing restriction sites that are found across a broad range of species (Dunshea 2009; Pegard *et al*. 2009; Deagle *et al*. 2010; Rayé *et al*. 2011; Shehzad *et al*. 2012a; Shehzad *et al*. 2012b), but others have relied on species- or taxon-specific markers to identify known diet items (Matejusová *et al*. 2008; Bowles *et al*. 2011; Zeale *et al*. 2011).

To our knowledge, only three studies have compared the findings of morphological and molecular analyses. A feeding trial study of captive fur seals (*Arctocephalus* spp.) determined that molecular methods detected prey species sooner after feeding, more frequently, and over a more predictable time period in comparison to morphological methods (Casper *et al*. 2007a). However, in a study of wild Stellar sea lions (*Eumetopias jubatus*), molecular analyses failed to identify a prey species in 22% of scats, but were able to identify prey to species when morphological methods were limited to genus (Tollit *et al*. 2009). Tollit

et al. (2009) also reported significant differences in detection rates between the two methods for certain prey species. Molecular methods also provided greater species resolution in an insectivorous bat study and found several additional orders (Zeale *et al*. 2011). However, despite the importance of understanding the food habits of large, terrestrial carnivores (Mills *et al*. 1992), no studies have compared the results of morphological and molecular analyses in a terrestrial system containing a large, carnivorous species.

The island of Newfoundland, Canada, presents an excellent study system for comparing morphological and molecular diet analyses because of the relative simplicity of the predator-prey system. Black bears (*Ursus americanus*) depend heavily on vegetation, but also consume large amounts of meat, particularly in spring, when an abundance of winter killed moose (*Alces alces*) and caribou (*Rangifer tarandus*) and neonate calves are present. Coyotes (*Canis latrans*) only recently immigrated to Newfoundland in the 1980s, but have been confirmed as one of the major predators of caribou calves (Mahoney & Weir 2009; Lewis & Mahoney 2014; Mumma *et al*. 2014) and also consume moose and snowshoe hares (*Lepus americanus*). Given the recent decline (>66% since 1998) in Newfoundland's caribou population (Mahoney & Weir 2009), a better understanding of the food habits of the two major predators of caribou calves is warranted.

Our goals were to evaluate the feasibility of using molecular tools to detect several common prey items in scats of two large, terrestrial carnivores and determine how these results compare to morphological analyses. We hypothesized that molecular methods would identify a prey species in a greater percentage of scats, but that morphological methods would identify a prey species in some scats where molecular methods did not as a result of PCR failure or a lack of prey DNA uniformity throughout a scat. We evaluated both of these

possible explanations for molecular method failures and also compared costs between the two techniques.

Materials and Methods

Study Site

Newfoundland (111,390 km^2) is an island off Canada's eastern coast with a cool, maritime climate characterized by interspersed coniferous forest, windswept barrens, and peatland (McManus & Wood 1991). We selected three study sites (Fig. 1) inhabited by three of Newfoundland's caribou herds (La Poile, Northern Peninsula, and Middle Ridge), along with moose, snowshoe hares, and predatory black bears and coyotes (Rayl *et al*. 2014).

Scat Sampling

In 2009, black bear and coyote scats were collected along roads and in areas adjacent to roads in La Poile and the Northern Peninsula using a trained scat detection dog (MacKay *et al*. 2008). Scat sampling was spread across study areas to increase the number of habitat patches and individual predators sampled. Scat detection dog searches varied in distance (5-10 km) and search time (2-6 hr) depending on the number of scats found and the weather. Scats were placed in plastic bags by dog handlers wearing latex gloves and frozen at the end of each field day when possible. Prior to lab processing, scats were thawed and a total of ~0.5 ml of fecal material was removed from multiple locations on the outside of each scat (Stenglein *et al*. 2010) and placed in 2 ml collection tubes containing DETS buffer to prevent DNA degradation (Frantzen *et al*. 1998). Plastic bags and tubes were labeled with a sample number and date of collection, which linked samples to an electronic record of their GPS coordinates.

Predator Species Identification

Samples were extracted using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA) in a laboratory dedicated to low quantity and quality DNA samples, and extractions included a negative control. We used a mitochondrial DNA (mtDNA) control region fragment analysis test to identify each sample to species (Murphy *et al.* 2000; Dalen *et al.* 2004; Onorato *et al.* 2006; Mumma *et al.* 2014; De Barba *et al*. 2014a). Since the identification of each scat to species relies on sloughed epithelial cells from the GI tract of the predator, we assumed that prey DNA would exceed predator DNA; therefore, we considered a positive black bear or coyote result an indication of good sample quality and that prey DNA, if present, would have a high probability of amplification. We determined allele sizes using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and associated GeneMapper 3.7 software.

Morphological Prey Species Identification

All scats collected in 2009 from La Poile and the Northern Peninsula that tested positive for black bear or coyote were analysed using standard morphological analyses of undigested hard parts from food items remaining in scats (Reynolds & Aebischer 1991). Thawed scats were individually cleaned by washing in a 2.0-mm mesh sieve and then dried to a constant mass in a drying oven at 40–50°C for 24–48 hours. We placed the dried contents of a scat onto a 30 × 20 cm tray with a 3 x 6-cell grid at the bottom and evenly spread scat contents across the grid. We selected the food item part crossing or nearest to each grid cell intersection (n=10) to get a representative sample of scat content. We secured hair samples on a plastic cover slip between two glass slides clipped with four paper clamps. The hairs were placed within a conventional toaster oven at high power for 10 minutes to melt the cuticular

mosaic pattern onto the plastic cover slip and then removed from the plastic cover slip. Both the hair and cover slip were taped to a datasheet and examined under magnification. We examined the structures of the cuticle, medulla, and cross sections under a microscope and compared those to a reference collection of hairs representing various regions of the body from all local potential prey species. To aid in identification of other food items, we collected reference samples of ants, as well as potential vegetative food items (including leaves, berries, and seeds) at different phonological stages from our study sites. We also identified these and other remains (e.g., feathers) using relevant taxonomic keys and manuals. For comparisons between morphological and molecular techniques, we simply tallied whether or not a given prey item (caribou, moose, or snowshoe hare) occurred in a given scat.

Molecular Prey Species Identification

Three common prey species were selected for our molecular prey species identification test, caribou, moose, and snowshoe hare. Reference samples of each prey species were collected from Newfoundland (caribou = 4 hair, moose = 3 tissue, and snowshoe hare = 3 tissue). DNA was extracted from the hair samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) in a laboratory dedicated to low quality DNA samples. DNA was extracted from the tissue samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). Each extraction included a negative control to monitor for contamination of PCR reagents.

All sequences available for the target species at the cytochrome b region of mtDNA were downloaded from Genbank (www.ncbi.nlm.nih.gov/genbank/). These sequences, 8 caribou (accession numbers AY726672-8), 4 moose (accession numbers AY090099, AY245520, EF077657 and M98484), and 3 snowshoe hares (accession numbers AF010152,

AY292733 and LAU58932) were used to design primers that would target ~200 base pairs (bps) in caribou and moose and ~375 bps in snowshoe hare of the cytochrome b region. We then used these primers to generate sequence data from the reference prey individuals from our study area (Table 1). The PCR for sequencing caribou and moose contained 0.06 μM of primers NFCytb F and NFCytb R, 1.5 mM $MgCl_2$, 0.4 mM dNTPs, 1X Amplitaq gold PCR buffer, and 0.5 U of Amplitaq Gold DNA Polymerase (Applied Biosystems) in a final reaction volume of 10 μL. The thermal profile for this reaction was an initial denaturation step of 95 °C for 10 min, followed by 35 cycles of 95°C for 30s, 50°C for 30s and 72°C for 1 min. The PCR and thermal profile for sequencing snowshoe hare was the same as above except using primers LepusA F and LepusSeq R. Prior to sequencing, PCR product was cleaned using ExoSAP-IT (Affymetrix) according to the manufacturer's protocol. Sequencing was carried out in 10 μL reactions using BigDye Terminator v3.1 (Applied Biosystems). Sequencing products were cleaned using a BigDye XTerminator Purification Kit and then run on a 3130xl Genetic Analyzer (Applied Biosystems). We used the program Sequencher 4.7 (Genecodes Corporation) to edit and align the sequences with those from Genbank. The program MacClade 4.0 was used to determine the number of unique haplotypes (Maddison & Maddison 2003).

DNA isolated from scat samples can be degraded, which often hinders PCR amplification of longer DNA fragments (Kohn *et al*. 1995; Murphy *et al*. 2000). Thus, the goal was to design species-specific primers to amplify fragments ≤200 bps. The primer NFCytb F was designed to anneal to both caribou and moose DNA. A species-specific reverse primer was designed for each species from the aligned sequence data above to produce bands of different sizes when combined with the primer NFCytb F (Tarandus3 R and

Alces3 R, Table 1). The Tarandus3 R primer amplified a 105-base pair (bp) fragment while the Alces3 R primer amplified a 129-bp fragment (Table 1). A species-specific reverse primer was designed for snowshoe hare that produced a 179-bp fragment when combined with the primer LepusA R (Table 1).

All primers were multiplexed into a single PCR reaction to minimize the amount of time, and cost accrued when analysing samples. This PCR contained 0.57 μM of primer NFCytb F, 0.14 μM of primers Caribou3 R, Moose3 R, LepusA F and LepusA R, 0.5X Q solution and 1X Multiplex Mastermix (Qiagen Inc., Valencia, CA) in a final reaction volume of 7 μL. The thermal profile for this reaction was an initial denaturation step of 95°C for 10min, followed by 15 cycles of 94°C for 30s, touchdown starting at 65°C and decreasing by 0.7°C each cycle for 30s, 72°C for 1min, followed by 35 cycles of 94°C for 30s, 55°C for 30s and 72°C for 1min. The NFCytb F and LepusA F primers were labelled with 6-FAM dye. PCR products were run on a 3130xl Genetic Analyzer using Genescan 500 LIZ Size Standard (Applied Biosystems). The results were visualized using Genemapper 3.7 (Applied Biosystems).

Validation of Molecular Prey Species Identification Test

To validate the prey species identification test, samples of known species origin were collected for caribou (n=20 hair), moose (n=17 faecal pellets), and snowshoe hares (n=19 faecal pellets). The three tissue samples used for sequencing from moose and snowshoe hares were included in the validation. DNA was extracted from the hair samples using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA). DNA from the pellet samples was extracted using QIAamp Stool Kit (Qiagen Inc., Valencia, CA) with one protocol modification. For the moose, one pellet was washed with up to 3 mL of buffer ASL

depending upon the size and dryness of the sample. The goal was to completely submerge each pellet in buffer ASL in order to wash cells from the largest surface area possible. We removed 1.4 mL of buffer ASL from around the pellet and moved to the next step in the protocol. DNA extractions were performed in a laboratory dedicated to low quality DNA samples, and each extraction contained a negative control to test for reagent contamination. The DNA extracts were then tested using the molecular prey species identification test, and the results were cross-referenced with the known prey species of origin.

We used the molecular test to determine the presence of caribou, moose, and snowshoe hares in all scats that tested positive as black bear or coyote collected from La Poile and the Northern Peninsula study sites in 2009. All samples were tested in duplicate and samples with unclear results were retested up to 2 additional times to clarify the presence or absence of prey species. A positive prey species identification required a clear peak exceeding 200 fluorescent units, which resembled the shape of positive controls and distinguished itself from non-specific noise. We compared the results of the first two tests to determine the consistency of PCR results. Both molecular and morphological analyses were conducted blindly to the results of the other prey identification method to avoid the potential for bias.

Comparison of Morphological and Molecular Methods

We evaluated if a ranking of the relative prey frequencies was consistent between methods and used Z-tests with a Bonferroni correction for multiple tests in R (R Core Team, 2014) to evaluate if there were differences between morphological and molecular analyses of the percentage of scats containing each prey species (caribou, moose, or snowshoe hare). However, since the observations of our analyses were individual scats, we also conducted

McNemar's chi-squared tests with continuity corrections (Agresti 1990) in R (R Core Team, 2014) to identify if the paired morphological and molecular results differed between individual scats. To assess the potential for false positives and negatives, we tabulated the number of scats that agreed and disagreed between methods for each prey species.

We also compared the cost/sample associated with each method, which includes supply costs and technician costs assuming a pay rate of $15/hour. Since we relied on molecular predator species identification for both analyses, we present the molecular cost/sample with and without the cost of DNA extraction and predator species identification. Our estimates for the molecular analyses are based on processing samples in batches of ~20 during extraction and ~96 during PCR. We also provide the cost/sample with and without the cost of developing the molecular predator and prey species identification tests. Because development costs per sample decrease as the number of samples increases, we present the cost/sample for 100, 500, and 1000 samples.

Testing of Scat Uniformity

In 2012, scat detection dogs were transported via helicopter to remote locations within the Middle Ridge to locate black bear and coyote scats as part of a larger predator abundance survey. Five black bear and 5 coyote scats were selected to evaluate if prey DNA is spread uniformly throughout a scat. We sampled each scat from 2 exterior locations and 2 interior locations and then tested the four samples of each scat in duplicate. We evaluated the differences in prey species detection from the four samples of each individual scat.

Results

DNA Sequencing and validation of molecular prey species identification test

A 210-bp fragment of the cytochrome b region of mtDNA was successfully amplified and sequenced for the four caribou and three moose reference samples. We found a total of two unique haplotypes for both caribou and moose (see Data Accessibility). The 8 caribou sequences (accession numbers AY726672-8) and 4 moose sequences (accession numbers AY090099, AY245520, EF077657 and M98484) represented 4 distinct haplotypes for each species. Thus, we used a total of 6 haplotypes from each species to design the conserved forward primer and the species-specific reverse primers. We amplified and sequenced a 370-bp fragment of the cytochrome b region of mtDNA for the three snowshoe hare reference samples and found two unique haplotypes (see Data Accessibility). The three snowshoe hare sequences (accession numbers AF010152, AY292733 and LAU5893) represented two haplotypes one of which matched a haplotype from the reference samples. Therefore, three unique snowshoe hare haplotypes were used to design the species-specific primer pair.

All validation samples that produced PCR product (caribou n=20, moose n=16 and snowshoe hare n=20) showed bands at the correct species-specific size (Table 2). In addition, there was no cross-species amplification in the validation samples. All samples that failed to produce PCR product were fecal pellets, which is likely due to the DNA being degraded in these samples rather than a failure of the prey species identification test.

Comparison of Morphological and Molecular Results

We tested 140 black bear scats and 156 coyote scats for prey remains. All extraction and PCR controls were negative. Both methods identified moose as the most common prey item for black bears followed by caribou and snowshoe hares, although differences in the

percentages of scats containing moose and caribou were not significant within methods (Fig. 2). Moose was also the most common item for coyotes followed by snowshoe hares and caribou, but none of the percentages estimated via morphological methods were significantly different and moose and snowshoe hares were not significantly different for the molecular analysis (Fig. 3). However, we did find significant differences between morphological and molecular methods in the percentages of black bear scats containing caribou and moose (Fig. 2). For coyotes, molecular methods found a significantly higher percentage of scats that contained moose and snowshoe hares, but not caribou (Fig. 3). Overall, morphological analyses found at least one prey species in 34% of black bear scats and 78% of coyote scats, while molecular analyses detected at least one prey species in 70% and 90% of black bear and coyote scats, respectively.

Although the majority of scats agreed with regards to the presence of a specific prey species, there were a large number of discrepancies between methods. Morphological and molecular analyses were in agreement for 64%, 63%, and 92% of black bear scats regarding the presence of caribou, moose, and snowshoe hares, respectively (Table 3). Morphological and molecular analyses of coyote scats agreed for 68%, 62%, and 70% for caribou, moose, and snowshoe hares, respectively (Table 3). However, McNemar's chi-squared tests (Agresti 1990) indicated that the pairwise results of individual black bear scats were significantly (α=0.05) different for all 3 prey species and that the pairwise results of individual coyote scats were significantly different for moose and snowshoe hares. Positive molecular and negative morphological results were the more common disagreement (6%-31%) for scats from either predator, but there were also disagreements (<1%-13%) for negative molecular and positive morphological analyses (Table 3).

PCR Consistency and Scat Uniformity

We found that 16% (caribou), 11% (moose), and 3% (snowshoe hare) of duplicate PCR tests of black bear scats failed to produce the same prey species result. Duplicate PCR tests of coyote scats for caribou, moose, and snowshoe hare failed to produce the same result in 8%, 11%, and 6%, respectively. We also found that 0/5, 1/5, and 0/5 of black bear scats and 0/5, 1/5, and 1/5 of coyote scats failed to produce the same prey species result (caribou, moose, or snowshoe hare, respectively) across the 4 subsamples taken from different locations of the same scat.

Cost Comparison

We estimated the cost of morphological analysis at $23.60/sample (Table 4). The molecular analysis cost/sample, excluding method development, DNA extraction and predator species identification, was $2.48 (Table 4). When we include the molecular prey species development cost (~$2000), the per sample rate for analyzing 100, 500, and 1000 scat samples was $22.48, $6.48, and $4.48, respectively (Table 4). We also estimated the cumulative cost of DNA extraction, species identification, and prey species identification for a total of $19.25/sample (Table 4). If we add the development costs of the molecular predator and prey species identification tests, the total cost per sample rises to $59.25 for 100 samples, $27.25 for 500 samples, and $23.25 for 1000 samples (Table 4).

Discussion

Although morphological and molecular methods indicated identical trends in prey species rankings, there were a large number of discrepancies between the prey species detected for individual scats. Approximately one-third of comparisons differed between morphological and molecular results across all prey species (Table 3), and McNemar's chi-

squared tests (Agresti 1990) indicated that the pairwise scat results were different for all prey species except caribou in coyote scats.

The most common disagreement between methods were positive molecular and negative morphological results indicating that molecular methods had higher rates of detection; although, we also observed disagreements for negative molecular and positive morphological results (Table 3). Two out of three prey species were detected at significantly higher percentages for black bears and coyotes using molecular methods. Casper *et al*. (2007a) also found higher rates of prey detection with molecular methods, but another study reported lower rates of detection for molecular methods in comparison to morphological methods (Tollit *et al*. 2009). Tollit *et al*. (2009) attributed decreased detections to old scats with degraded DNA, which was likely less prevalent in our study since we only tested scats that were successfully identified as black bear or coyote via molecular analyses.

Several factors could contribute to false positives and negatives for both methods. Both false positives and negatives for morphological analysis of scats could be the result of assigning hard parts to the wrong species (Spaulding *et al*. 2000). This is especially problematic in systems with closely related species and often results in researchers grouping species by genus or higher taxonomic levels (Zabala *et al*. 2003; Tollit *et al*. 2009; Zeale *et al*. 2011). Alternatively, false negatives could result from the absence of hair, bone, or other hard parts in scats. This may be less likely to occur for small mammals where the entire carcass is consumed (Hewitt and Robbins 1996), but is more probable when portions of large mammals, such as the viscera, are consumed that include very little hair or bone. False positives could also occur for molecular analyses as a result of cross-contamination or via the occurrence of non-specific peaks on electropherograms. We limited the probability of false positives as a

result of cross-contamination by using negative controls during extractions and PCRs. We also were conservative in assigning prey species by retesting unclear samples and requiring strong (>200 florescent units) and clear peaks (only included peaks that had a similar shape exhibited by positive controls and distinguished themselves from non-specific noise). False negatives could be the result of prey DNA not being uniformly distributed throughout a scat or PCR inconsistency both of which we demonstrated by comparing duplicate results and testing different locations of black bear and coyote scats. However, we think that these sources of error were limited in our study since we sampled fecal material from multiple locations on a scat and tested samples in duplicate.

In our study, the per sample cost of morphological prey species identification exceeded that of molecular prey species identification if we assume that predator species identification via molecular methods was necessary for both food habit analyses (Table 4). However, morphological analyses were less expensive when processing <920 samples when DNA extraction, predator species identification, and development costs were included. We also acknowledge that the instrumentation necessary to perform molecular diet analyses is considerably more expensive than equipment needed for morphological analyses.

Although molecular methods were generally less expensive and had higher rates of detection, morphological analyses present several advantages. Most importantly, the ability to quantify the proportion of hard parts to specific prey species provides a means to more accurately assess the relative importance of different food items in a species' diet (Klare *et al*. 2011). In addition, the straightforward nature of hard part analysis makes it readily accessible to a wide range of researchers (Casper *et al*. 2007b), and morphological analyses are also capable of analyzing highly degraded scats that would be unlikely to have viable DNA (Tollit

et al. 2009). Another advantage of morphological analyses could be the ability to determine the age of individuals being consumed via differences in hard part size (Latham *et al*. 2013).

Despite certain advantages of morphological methods, we think our study demonstrates the utility of molecular methods to more consistently identify prey species and provides evidence that molecular methods have the potential to increase the accuracy and precision of food habit assessments. Next generation sequencing and DNA barcoding approaches have already shown great promise by permitting a single test for multiple prey and/or plant species that could be easily transferred across systems (Valentini *et al*. 2008, De Barba *et al*. 2014b). This approach would be particularly useful in systems where little is known about the food habits of a study organism and would likely increase the probability of detecting infrequent food items. Other molecular studies have shown great promise using real-time PCR to quantify the proportion of prey DNA in scats as an index of the proportion of prey in predator diets (Matejusova *et al*. 2008; Bowles *et al*. 2011), which requires extracting the entire scat or assuring that fecal material is homogenized prior to removing a subsample. Although morphological analyses have a similar approach by evaluating the proportion of prey items in scats, they can be compromised by differences in hard part digestibility, which creates the need for both predator- and prey-specific correction factors in order to estimate true proportions (Hewitt and Robbins 1996; Tollit *et al*. 1997). Further research is essential to validate molecular methods using controlled feeding trials, but ultimately molecular food habit analyses have the potential to provide increased insight into how species utilize and compete for food resources, which improves our ability answer ecological questions.

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Tables

Table 1 – DNA primers used during the development of the molecular prey identification test.

Primer Name	Dye	Primer Sequence 5’ to 3’	Frag Size (base pairs)
NFCytb F	6-FAM	ACCCACCCATTAATAAAAATTGT	
Tarandus3 R		GAATTAAGCAGATTCCTAG	105
Alces3 R		GAATAGTCCTGTAAGGATTTGTAAG	129
LepusA F	6-FAM	TTAACCACTCCCTAATTGAC	
LepusA R		TTACGTCTCGGCAGATATG	173
NFCytb2 R		TCGTCCTACATGTATAATA	210
LepusSeq R		TAGGGTTGTCCCAATGTAG	370

Table 2 – Known samples used during the development of the molecular prey identification test. Key – SS Hare = Snowshoe Hare

Species	Sample Type	# Samples	# Success
Caribou	plucked hair	20	20
Moose	tissue	3	3
	fecal pellet	17	13
SS hare	tissue	3	3
	fecal pellet	19	19

Table 3 – Percentage of agreement (+) and disagreement (-) between morphological and molecular analyses of black bear and coyote scats for caribou, moose, and snowshoe hare.

Predator	Analysis Method	---------->	Morphological	
Black Bear (n=140)	Molecular	Caribou	(+)	(-)
		(+)	(8%)	(28%)
		(-)	(8%)	(56%)
Black Bear (n=140)	Molecular	Moose	(+)	(-)
		(+)	(42%)	(31%)
		(-)	(6%)	(21%)
Black Bear (n=140)	Molecular	Snowshoe Hare	(+)	(-)
		(+)	(1%)	(6%)
		(-)	(>1%)	(91%)
Coyote (n=156)	Molecular	Caribou	(+)	(-)
		(+)	(22%)	(19%)
		(-)	(13%)	(46%)
Coyote (n=156)	Molecular	Moose	(+)	(-)
		(+)	(37%)	(32%)
		(-)	(6%)	(25%)
Coyote (n=156)	Molecular	Snowshoe Hare	(+)	(-)
		(+)	(35%)	(22%)
		(-)	(7%)	(35%)

Table 4 – The cost ($USD) of morphological food habit analysis and molecular food habit analysis of scats broken down by development cost, supply cost, technician (Tech) time, technician cost per sample, and cost/sample for 100, 500, and 1000 samples.

	Itemized Costs				Totals		
Analysis Method	Development Cost	Supply Cost	Tech Time (min)	Tech Cost ($15.00/hr)	Cost /Sample (100)	Cost /Sample (500)	Cost /Sample (1000)
Morphological Prey ID	NA	$1.10	90	$22.50	$23.60	$23.60	$23.60
Molecular Prey ID[1]	Not Included	$1.60	3.5	$0.88	$2.48	$2.48	$2.48
Molecular Prey ID[1]	$2,000[2]	$1.60	3.5	$0.88	$22.48	$6.48	$4.48
Molecular Predator and Prey ID[3]	Not Included	$8	45	$11.25	$19.25	$19.25	$19.25
Molecular Predator and Prey ID[3]	$4,000[4]	$8	45	$11.25	$59.25	$27.25	$23.25

[1]Does not include the cost of DNA extraction and molecular predator species identification
[2]Includes the cost of development for molecular prey species identification test
[3]Includes the cost of DNA extraction and molecular predator species identification
[4]Includes the cost of development for molecular predator and prey species identification tests

Figures

Fig. 1 – The location of our three study sites and associated calving grounds on the island of Newfoundland, Canada.

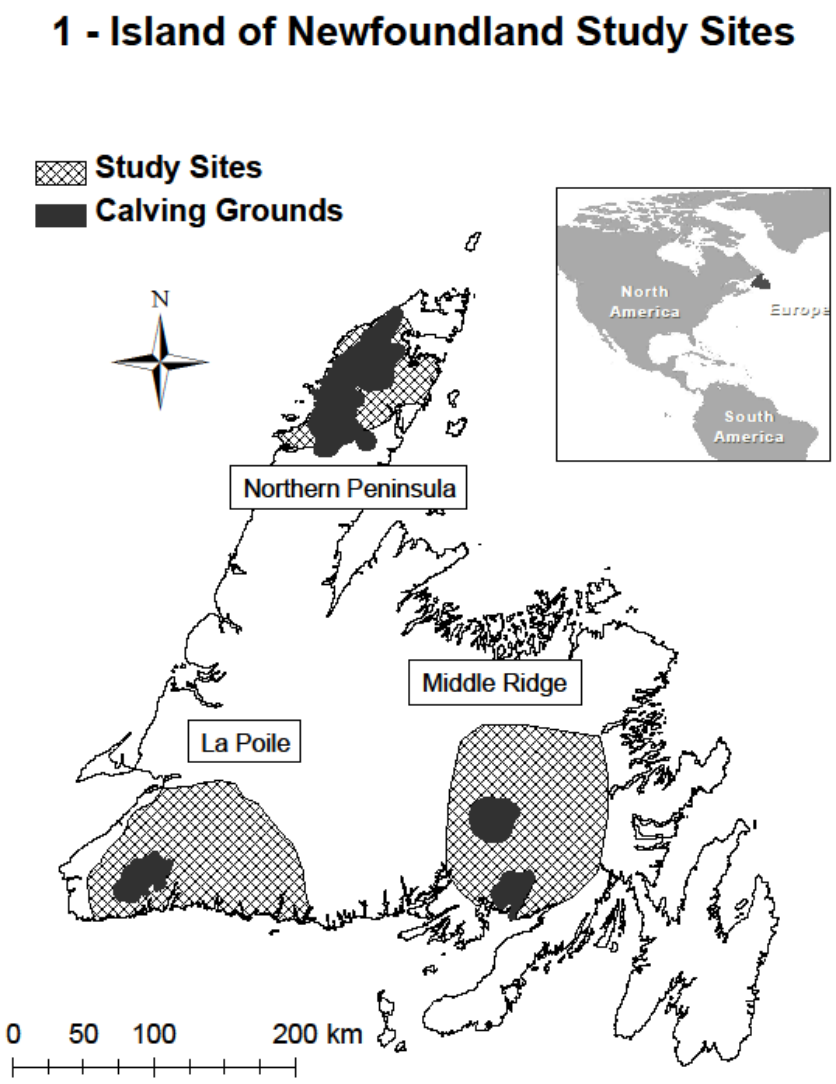


Fig. 2 – The percentage (and 95% CI) of black bear scats containing caribou, moose, and snowshoe hare as determined by morphological and molecular analyses.

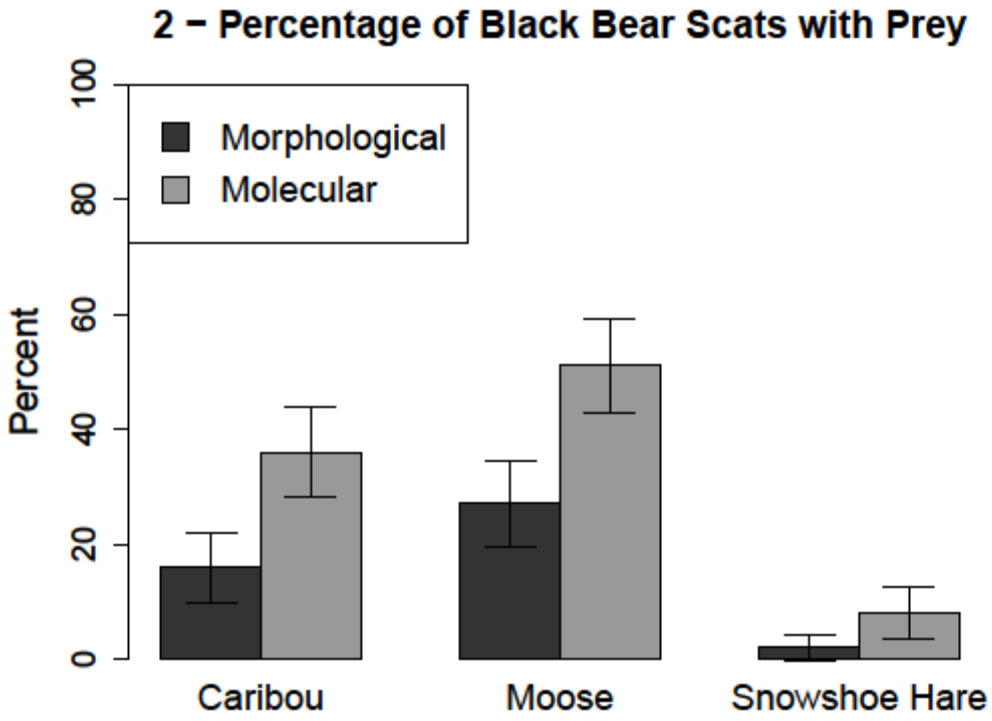
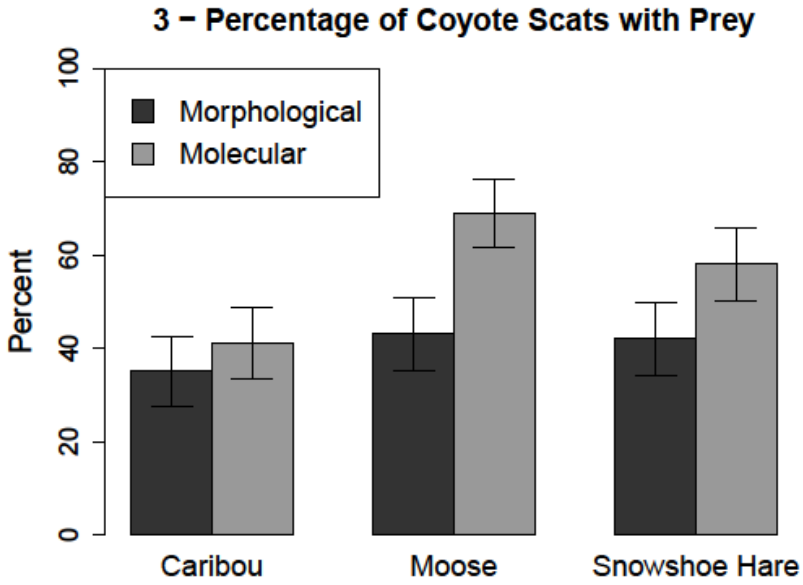


Fig. 3 – The percentage (and 95% CI) of coyote scats containing caribou, moose, and snowshoe hare as determined by morphological and molecular analyses.



Chapter II – Enhanced Understanding of Predator-prey Relationships Using Molecular Methods to Identify Predator Species, Individual, and Sex

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Abstract

Predator species identification is an important step in understanding predator-prey interactions, but predator identifications using kill site observations are often unreliable. We used molecular tools to analyze predator saliva, scat, and hair from caribou calf kills in Newfoundland, Canada to identify the predator species, individual and sex. We sampled DNA from 32 carcasses using cotton swabs to collect predator saliva. We used fragment length analysis and sequencing of mitochondrial DNA to distinguish between coyote, black bear, Canada lynx, and red fox and used nuclear DNA microsatellite analysis to identify individuals. We compared predator species detected using molecular tools to those assigned via field observations at each kill. We identified a predator species at 94% of carcasses using molecular methods, while observational methods assigned a predator species to 62.5% of kills. Molecular methods attributed 66.7% of kills to coyote and 33.3% to black bear, while observations assigned 40%, 45%, 10% and 5% to coyote, bear, lynx and fox, respectively. Individual identification was successful at 70% of kills where a predator species was identified. Only one individual was identified at each kill, but some individuals were found at multiple kills. Predator sex was predominantly male. We demonstrate the first large-scale evaluation of predator species, individual, and sex identification using molecular techniques

to extract DNA from swabs of wild prey carcasses. Our results indicate that kill site swabs (1) can be highly successful in identifying the predator species and individual responsible and (2) serve to inform and complement traditional methods.

Introduction

Predation is a central process in ecological communities, and the assemblages of predator and prey species can create an array of complex interactions (Prugh *et al.* 2005, Zager *et al.* 2006, Barnowe-Meyer *et al.* 2010). Accurately determining the predator species responsible for prey mortality is an important first step to understanding predator-specific roles in predator-prey systems. Directly observing predation is ideal, but such events are generally rare and only possible to observe for diurnal predators in open habitats (Blejwas *et al.* 2006). Alternatively, monitoring the survival of prey species or the predation habits of predator species via radio-collared individuals and performing site investigations can be an effective means of evaluating predator-prey interactions. The former requires identification of predator species from kill site observations using predator-specific kill site evidence, such as hair, scat, or species-specific killing or feeding characteristics (Onorato *et al.* 2006). However, there is often overlap between the killing and feeding characteristics between different predator species and variability in experience among field technicians making it difficult to ensure accurate and consistent predator species identification (Cozza *et al.* 1996).

Molecular methods present a promising alternative approach that could decrease the uncertainty of predator species identification. Molecular methods have been implemented extensively in wildlife research to answer questions regarding gene flow, social structure, hybridization, and population viability (DeYoung & Honeycutt 2005), but have only recently been used to identify predator species at kill sites. Predator scat and hair collected at elk (*Cervus elaphus*) kill sites were used to identify predator species (Onorato *et al.* 2006) and predator saliva collected from killing wounds on a threatened marsupial was used to identify feral cat predation (Glen *et al.* 2010). In addition, cotton swabs were used to sample predator

saliva from domestic sheep carcasses to differentiate between wild canid and feral dog predation (Williams *et al.* 2003, Sundquist *et al.* 2008, Caniglia *et al.* 2012).

Molecular predator species identification could also inform predator prey dynamics and management actions through the identification and sex of individual predators. For example, molecular methods were used to determine that two mountain lions (*Puma concolor*) preyed more frequently on an endangered bighorn sheep (*Ovis canadensis*) population and the authors suggested targeted removal of individual predators could decrease predation while not jeopardizing predator populations (Ernest *et al.* 2002). Targeted control efforts were also recommended by a study that used kill site swabs and telemetry data to identify specific male coyotes and breeding pairs as domestic sheep killers (Blejwas *et al.* 2006).

To further evaluate the potential of molecular methods for generating valuable data on predation at a large spatial scale, we applied these methods at caribou (*Rangifer tarandus*) calf kill sites in Newfoundland, Canada. The predator-prey system on the island of Newfoundland is an ideal model because of a changing multi-predator system, a dramatic increase in neonate predation, and a large proportion of unassigned kills (26%) using traditional field methods (Mahoney & Weir 2009). The Newfoundland caribou population has decreased by >66% since the late 1990s (Mahoney & Weir 2009) and an increase in calf predation, partially the result of a changing predator guild, contributed to the decline. In previous studies, the major predator of Newfoundland caribou calves was black bear (*Ursus armericanus*), but Canada lynx (*Lynx canadensis*) accounted for additional predation, and occasionally mortalities were attributed to red fox (*Vulpes vulpes*) and bald eagle (*Haliaeetus leucocephalus*) (Mahoney & Weir 2009). However, coyotes (*Canis latrans*) have become a significant predator of caribou

calves since their colonization of Newfoundland from mainland North America via sea ice in the 1980s (Trindade *et al.* 2011), and their impact on the caribou population may be underestimated given the large number of unassigned calf kills.

Our goal was to evaluate the power of molecular methods to study predation using the caribou predator-prey system in Newfoundland as our model. We were interested in the following research questions: 1) are molecular methods able to identify the predator species at more kill sites than field observation methods, 2) is there a difference in the proportion of predation attributed to each predator species between molecular and field observation methods, 3) are a majority of kills attributed to a small number of individual predators, and 4) do male predators prey on caribou calves more frequently than female predators? Based on the success of previous studies that used molecular methods to evaluate predator species at kill sites (Williams *et al.* 2003, Blejwas *et al.* 2006, Sundquist *et al.* 2008) and the large proportion of unassigned caribou calf kills in previous Newfoundland studies (Mahoney & Weir 2009), we predicted that molecular methods would identify the predator species at more kill sites than field observation methods and that the proportion of predation attributed to each predator species would differ between molecular and field observation methods. We also expected that male coyotes would be detected more frequently at kill sites than females based on studies of coyotes depredating domestic sheep (Blejwas *et al.* 2006).

Materials and Methods

Study Site

The island of Newfoundland (111,390 km^2) has a cool maritime climate and consists of coniferous forest interspersed by windswept barrens and peatland (McManus & Wood 1991). Caribou are the only native ungulate on Newfoundland and are widely distributed

across the island. The calving grounds of three caribou herds (La Poile, Middle Ridge, and Northern Peninsula) (Fig. 1) ranging from 500-1500 km^2 were selected for study.

Capture and Monitoring of Caribou Calves

From May 27th through June 1st, 2010, we hand-captured 92 one-to-three day old caribou calves across the three study sites (Fig. 1). Each calf was fitted with a 200g expandable, breakaway very high frequency (VHF) radio-collar containing a motion sensitive transmitter (Lotek Wireless Inc., New Market, ON, Canada; Telemetry Solutions, Concord, CA). Transmitter pulse rates increase for collars that are stationary for >4 hours signaling calf mortality or a slipped collar. Collar pulse rates were checked daily via fixed-wing and/or helicopter flights from the date of capture until June 11th and monitored every other day from June 12th until June 25th. From June 26th through July calves were checked weekly.

When a collar indicated calf mortality, we investigated the location for caribou calf remains and predator sign. For each calf mortality, trained field personnel evaluated kill site observations and assigned a black bear, coyote, Canada lynx, red fox, bald eagle, or unknown predator. Personnel experience varied between the nine biologists that evaluated kill sites from $\geq$30 years to 1 year, but all calf mortalities were assessed by multiple biologists both in the field and at a later date through the review of kill site images and site observations recorded using standard field protocols (Fig. S1 Supplementary Information). When present, predator scat and hair samples were collected, and carcass remains were sampled for predator saliva containing predator DNA using cotton swabs.

Predator Species Assignment Using Field Observations

In Newfoundland, caribou calf kills attributed to black bear typically consist of a skinned hide with a few bone fragments and chewed hoof tips. Distinguishing between

coyote and lynx kills is less clear. Throat trauma and a highly variable amount of calf remains are commonly seen for both predators, but calf remains at coyote kills are often pulled apart and spread over a larger area. The size and spacing of canine punctures provides another method of differentiating between the two predators. In addition, claw punctures on the dorsal surface of calf carcasses are suggested as evidence of a lynx kill, but the potential for talon punctures from bald eagles prevents the use of punctures alone as identifying characteristics. Characteristics of a fox kill are unclear and predator species assignments for kill sites consisting of buried front halves and decapitated heads remain uncertain.

Sample Collection for Molecular Analysis

Field personnel searched for predator scat and hair in the vicinity of the collar location out to roughly 25 m. Approximately 0.5 ml of total fecal material was collected from multiple locations on the lateral surface of each predator scat using small sticks collected from woody shrubs in the field (Stenglein *et al.* 2010) and placed in 2 ml collection tubes containing DETS buffer (Frantzen *et al.* 1998). Predator hair samples were placed in individual paper envelopes and stored collectively in sealed plastic bags containing silica desiccant (Roon *et al.* 2005). Field personnel wore sterile latex gloves for both scat and hair collection to prevent cross-contamination between kill sites and samples.

Sterile, cotton swabs were used to sample remains for residual predator DNA from saliva (Williams *et al.* 2003, Blejwas *et al.* 2006, Sundquist *et al.* 2008, Glen *et al.* 2010). We swabbed hemorrhaged and non-hemorrhaged wounds while wearing sterile latex gloves and avoided touching multiple wounds with the same gloves to prevent cross contamination between carcasses and wounds. We considered hemorrhaged wounds to be caused by the predator species, since hemorrhaging is an indication that the wound was inflicted while the

prey species was still alive. Non-hemorrhaged wounds were labeled as feeding wounds and recognized as potentially attributable to the predator or scavenger species. The collar, bones, hide, and other remaining tissues were also swabbed and labeled as feeding wounds when the majority of the carcass was consumed or when we found only the collar. Up to four different areas or tissues from each carcass were swabbed.

Swab Technique

We conducted a literature search and pilot study to determine the most effective swabbing and preservation method (Supplementary Information). We chose ethanol as our wetting agent to assist in lifting dried cells from tissues and to promote rapid drying for DNA preservation. We collected two ethanol-soaked swabs (A and B) from each area or tissue to provide a back-up sample in case of laboratory error. Immediately following collection, all swabs were placed in individual paper envelopes that were collectively stored in sealed plastic bags containing silica desiccant and stored at room temperature.

DNA Extraction and Species Identification

We extracted all samples in a laboratory dedicated to low quantity DNA samples and used the Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA) for scat samples and the Qiagen DNeasy tissue kit for hair and swab samples. When available, 10 follicles were used in each hair extraction and for all extraction batches a negative control was used to monitor for contamination. B swabs were only processed when we wanted to verify an A swab species identification or when all A swabs from a carcass failed to provide a species identification.

Species identification for all samples was conducted using a mitochondrial DNA (mtDNA) control region fragment analysis method. This test uses primers previously

reported for differentiating black bear, coyote, and two non-target species: brown bears (*Ursus arctos*) and wolves (*Canis lupis*) (Murphy *et al.* 2000, Onorato *et al.* 2006), plus an additional primer (H3R) designed to differentiate red fox (Dalen *et al.* 2004). All species, with the exception of the lynx, can be identified by species-specific fragment size (black bear 158-164.5 base pairs (bp) and 396-401 bp, coyote 115-120 bp and 362.5-364 bp, wolf/dog 123-128 bp and 367-369 bp, and red fox 342.9-344.5 bp only). PCR conditions are under supplemental information. We tested swabs from carcasses that failed to identify a predator species after initial testing using species-specific mtDNA primers developed for the Iberian lynx (*Lynx pardinus*; Palomares *et al.* 2002) that we documented to work on known Canada lynx samples from Newfoundland. Additional details and PCR conditions are provided in supplemental information.

Any samples that failed the two previous analyses were amplified and sequenced using mtDNA cytochrome B primers that amplify most carnivores (Farrell *et al.* 2000) using conditions described in Onorato *et al.* (2006). These primers were effective in identifying black bears, Canada lynx, and red foxes, but not coyotes. Samples that failed to amplify with the Farrell primers were amplified and sequenced using the canid-specific mtDNA control region ScatID primers using conditions and primers described in Adams *et al.* (2003) to identify coyote samples that failed the initial species ID screening.

A predator species was assigned when detected from a hemorrhaged wound swab or from a feeding wound swab when a carcass did not contain a hemorrhaged wound. We did not use molecular tools to test for the presence of bald eagles, because they frequently scavenge kill sites and are rarely the predator of caribou calves (O'Gara 1994).

Nuclear DNA Individual and Sex Determination

We only detected black bear and coyote DNA at kill sites, and therefore amplified successful samples with black bear or canid specific microsatellite loci to identify individual predators. When both the A and B swab from a single wound positively identified the species, we analyzed the swab for individual identification that amplified best for species identification. We screened 18 loci to evaluate diversity levels in Newfoundland black bears and then developed two bear PCR multiplexes using the most polymorphic loci. Black bear multiplex one includes six microsatellite loci (G10C, G10M, G10P, G10X, CXX20, and Mu23 – Paetkau *et al.* 1998, Taberlet *et al.* 1997, DeBarba *et al.* 2010, and Ostrander *et al.* 1993) and one sex determining locus (Ennis & Gallagher 1994). Black bear multiplex two includes five microsatellite loci (G1A, G10B, Mu15, Mu50, and Mu59 – Paetkau *et al.* 1998, Taberlet *et al.* 1997, and Bellemain & Taberlet 2004). The PCR conditions are provided in supplementary information.

For coyotes, nine microsatellite loci (FH2001, FH2054, FH2088, FH2137, FH2611, FH2670, FH3725, C09.173, Cxx.119 – Breen *et al.* 2001; Guyon *et al.* 2003; Holmes *et al.* 1994) based on the methods of Stenglein *et al.* (2010) and two sex determining loci (DBX6 and DBY7 – Seddon 2005) were combined in one canid PCR multiplex. For PCR conditions, see supplemental information.

Black bear and coyote samples were tested in duplicate for their respective PCR multiplexes. Samples that failed to amplify at $\geq$4 loci were dropped from the analysis. We ran up to 6 PCR replicates for each multiplex and each multiplex replicate included all primers. To obtain a consensus genotype at each locus, we required each allele to be detected twice for heterozygotes and an allele to be detected three times for homozygotes. Samples

that failed to achieve a consensus for ≥9 loci in black bears and ≥6 loci in coyotes were dropped from the analysis. Our consensus genotype thresholds were chosen to meet a probability of identity siblings (PID_{sibs}) (Waits *et al.* 2001) value of less than 0.003. This means that less than 1/333 comparisons of first-degree relatives would have identical genotypes at the loci analyzed and was used to avoid including false recaptures in the dataset. PID_{sibs} values for coyotes ranged from 0.0023 to 0.00025 and for black bears from 0.00012 to 0.0000027 depending on the loci that amplified.

We matched completed genotypes using the software GenAlEx6 (Peakall & Smouse 2006). Replicate PCRs for samples that matched at all but one or two loci were also evaluated to determine if mismatches could be attributed to allelic dropouts or false alleles. Individuals that were only detected once were analyzed using the software RELIOTYPE (Miller *et al.* 2002) to estimate the genotyping error rate and evaluate the reliability of the final consensus genotype. We required consensus genotypes to be ≥95% reliable and retested samples until our threshold was achieved.

Results

We investigated 32 caribou calf mortalities between May 28th and July 22nd. Six of these carcasses were not from our sample of collared individuals, but were found opportunistically on the landscape. There was a large amount of variation in the quantity of caribou calf remains. At 12 kill sites, we found a mostly intact carcass impacted by various degrees of consumption. A dismembered carcass and significant remains were found at an additional five sites, while a severed head was found buried at three other sites. Scant remains of bones, hoof, and hide were found at eight sites and the collar alone was found at four sites. Blood or bite marks were found on three of the four collars.

Molecular Species Identification Success Rates

We collected 12 scat, 3 hair, and 157 swab samples for molecular analysis. None of our extraction negatives elicited a positive result in our species identification test. Sixty-seven percent, 0%, and 54% of scat, hair, and swab samples were successful for species identification (Table 1), and no result was obtained from all negative controls. If the A swab from a wound was successful, we did not always test the B swab, and therefore only tested 139 of 157 swab samples. The success rate for killing wound swabs was 86% and 46% for feeding wound swabs (Table 1). Since multiple swabs were collected from each carcass, we identified a predator species at 100% of carcasses that had a killing wound (10) and 94% of carcasses overall. We only found a collar at one of the sites where a predator species was not identified using molecular tools, and it was unclear if the calf was preyed upon or had slipped its collar since there was no blood or tooth marks on the collar. Only a single predator species was detected at each kill site using molecular tools. Predator scats were only present at a small proportion of kill sites, but predator species identified by scat samples confirmed the predator species identified via swab samples.

Molecular and Field Observation Method Comparison

Molecular methods detected a predator species at 30 of 32 kill sites (94%) while the field observation method assigned a predator species at 20 of 32 kill sites (62.5%). Predator species were assigned for 11 kill sites where the field observation method failed in comparison to 1 kill site where the field observation method alone assigned a predator species. Molecular methods identified a predator species at 3 kill sites despite locating only a collar during field investigations. The molecular and field observation methods both failed for the site where the collar was potentially slipped.

Twelve kills (63%) had a molecular and field observation predator species assignment that agreed, but assignments differed at another 7 kills (37%) where a predator species was assigned by both methods. At 3 kill sites, molecular methods detected coyote DNA when field observation methods assigned Canada lynx or red fox. There was also 1 kill assigned to coyote and 3 kills assigned to black bear via the field observation method that the molecular methods assigned to the opposite species.

Molecular methods assigned 20 caribou calf kills (66.7%) to coyote, including all three severed heads, and 10 to black bear (33.3%), while red fox and Canada lynx were not detected using molecular tools (Fig. 2B). Coyote DNA was detected at 70% of carcasses containing a killing wound with black bears accounting for the remaining proportion. Kill sites assigned using field observation methods (n=20) attributed 8 (40%), 9 (45%), 2 (10%), and 1 (5%) to coyote, black bear, Canada lynx, and red fox, respectively (Fig. 2A). The field observation method did not attribute any kills to bald eagles, but site investigations inferred the occurrence of eagle scavenging at several sites. Field observation and/or molecular methods assigned a mammalian predator species to each of these sites.

Individual and Sex Identification

Swabs that were successful for species identification were also analyzed for individual identification. Overall an individual predator was identified from 62% of swabs and at 70% of carcasses (Table 2). Molecular methods only detected a single individual at each kill, although one swab recovered from a kill site where we visually detected 2 coyotes was mixed as evidenced by the existence of >2 alleles for multiple loci. Individual identification for black bear swabs was slightly lower (52%) than the success rates for coyote swabs (69%) (Table 2). We detected 5 unique, individual black bears, one of which was found at 3 caribou

calf kill sites. Four of these bears were male, including the individual detected 3 times. We detected 11 individual coyotes, and all were male. In addition to 9 single captures, we detected 1 coyote 2 times and another individual 3 times.

Discussion

Our pilot study using DNA analysis to evaluate predation of Newfoundland caribou calves confirmed the utility of molecular methods for improving kill site predator species identification. Species identification was highly successful and could be assigned to 30 of 32 kill sites (94%). Two striking benefits of using molecular methods to study predation are the reduction of mortalities attributed to unknown predators and the increased accuracy of predator species assignments. Molecular methods decreased the proportion of calf mortalities attributed to unknown predators by 31.5% (10 kills), and at 7 kill sites (37%), molecular methods detected a different predator species than was assigned using field observation methods. Molecular methods also provide a means to determine predator-specific kill site observations, which should improve the accuracy of predator species identification using field observations.

In our system, molecular methods changed our understanding of the proportion of calf predation attributed to each predator species. The field observation method determined that black bear (45%) were the primary predator of caribou calves followed by coyote (40%) and attributed a small amount of predation to Canada lynx and red fox (Fig. 2A). However, coyotes were detected nearly twice as frequently (66.7%) as black bears (33.3%) according to the molecular results, while Canada lynx and red fox were not detected (Fig. 2B). The increase in the proportion of coyote kills estimated using molecular methods can be explained by additional DNA-based predator species identifications at kills with non-descript predator

killing and feeding characteristics and the assignment of coyotes to several kill sites that were assigned to Canada lynx and red fox using field observation. Although the proportion of predation attributed to coyote (66.7%) and black bear (33.3%) in our study (Fig. 2B) using molecular methods was similar to a caribou calf study in Quebec (Crete & Desrosiers 1995) and dissimilar to studies in Alaska (Jenkins & Barton 2005) and British Columbia (Gustine *et al.* 2006), the value of these comparisons is limited since our results are based on 1 year of research in comparison to the other studies which spanned 2-7 years. Furthermore, preliminary data from our second year of research indicates there may be a more equal proportion of kills attributed to coyote and black bear.

Field biologist experience, uncertainty between species-specific killing and feeding characteristics, and scavenging may explain why field observation methods assigned a different predator species than molecular methods. Less experienced biologists were less likely to leave a kill site unassigned to a predator species and were more likely to have incongruent molecular and field identifications, which may indicate a failure to recognize similar kill site characteristics between predator species. Overlapping kill site characteristics could explain why coyote DNA was detected from the cervical killing wounds of 3 carcasses where field observations assigned Canada lynx (2 kills) and red fox (1 kill). Coyote DNA was also detected at 2 kill sites with skinned hides that were assigned to black bear via field observations. Detecting coyote DNA at kill sites with skinned remains of intact hide suggests this handling behavior is not specific to black bear, but could result from coyote scavenging a bear kill. Scavenging could also explain the incongruence between molecular and field identifications for 3 carcasses discovered opportunistically that may have been on the

landscape longer and were therefore more prone to scavenging than calves that were regularly monitored.

Inadvertently attributing calf predation to a scavenging species is a potential weakness of both molecular and field methods. Ideally, molecular methods would only use killing wound swabs to determine the predator species. However, we felt that using all of the collected samples was justified, because of the potential for a negative bias in the proportion of black bear kills because black bears tend to consume the majority of the carcass leaving only a few remains and eliminating any evidence of the killing bite wound. In fact, we potentially detected a bias since the proportion of coyote to black bear kills decreased from 2.3 for killing wounds to 1.8 for killing and feeding wounds combined. Furthermore, we think that scavenging was limited overall since 22 of the 26 (85%) collared calf kill sites were recovered early in the study when monitoring was frequent, and molecular methods only detected one individual predator per kill site for both collared calves and calves discovered opportunistically with the exception of the kill site with the mixed swab.

The application of molecular methods to identify individual predators and their sex is an underexplored resource that could help inform predator-prey management. Other studies have shown that one or more specialist predators can have a large impact on prey populations (Ross *et al.* 1997, Ernest *et al.* 2002, Festa-Bianchet *et al.* 2006). Our sample sizes were too small to draw any conclusions regarding individual specialization, but the skewed number of male coyotes detected at kill sites supported our hypothesis that male predators prey on caribou calves more frequently than female predators. This is consistent with Blejwas *et al.* (2006) attributing most domestic sheep kills to territorial, male coyotes. It is possible that the propensity of male predation is related to differences in home range size between males and

females and/or the constraints placed on the female during cub and pup-rearing (Harrison & Gilbert 1985), which coincides with caribou calving in the Newfoundland system. Alternatively, differences in nutritional requirements between sexes have been proposed as an explanation of sex-biased predation for other species (Barboza *et al.* 2000).

Our per sample species identification success rates for hair, scat, and swabs (0-67%) were lower than other studies (85-97%) (Onorato *et al.* 2006, Blejwas *et al.* 2006), but may have been affected by the damp Newfoundland climate, which would lead to increased DNA degradation (Piggot 2005, Murphy *et al.* 2007, Brinkman *et al.* 2010). Furthermore, our swabs were collected from killing and feeding wounds and from carcasses that were likely 1-2 days old in contrast to other studies that sampled carcasses within 24 hours (Blejwas *et al*. 1996, Sunqvist *et al.* 2008). However, our species identification success rates per carcass (94%) were similar to other studies as a result of collecting multiple swabs from every carcass as recommended by Sunqvist *et al.* (2008). Our individual identification success rates for swabs (62%) were slightly higher than other studies (50-58%) (Blejwas *et al.* 2006, Sundquist *et al.* 2008, Caniglia *et al*. 2012). We affirm the suggestion by Sunquist *et al.* (2008) to collect multiple swabs and further recommend sampling multiple locations of each carcass to increase per carcass success rates.

Additional research is necessary to determine the length of time predator DNA stays viable on a carcass. Multiple studies demonstrated amplification success rates for scats decrease with time since deposition (Piggott 2005, Murphy *et al.* 2007, Santini *et al.* 2007, Panasci *et al.* 2011), and we anticipated a similar relationship for swab success rates. However, we did not see a change in molecular identification success rates between carcasses of collared individuals that were monitored every other day (n=22) and once a week (n=4) or

for carcasses discovered opportunistically (n=6). This may suggest that once a week monitoring is sufficient, but the increased likelihood of scavenging must also be considered by researchers hoping to balance amplification success rates with the costs of monitoring.

In summary, we have demonstrated the effectiveness of DNA-based methods for identifying predator species, individual, and sex at caribou calf kill sites in Newfoundland. Molecular methods can increase the reliability and accuracy of predator species identifications and could be particularly informative in sparsely studied, multi-predator systems. We feel that molecular methods are underutilized in the study of predation and recommend their application across a wide range of studies. However, we do not think that molecular methods should replace field observation methods, but must instead be viewed as complementary since both methods inform understanding of predator-prey relationships.

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Tables

Table 1 – Molecular species identification success rates by sample.

Sample Type	# Samples	% Success/Sample	# Carcasses	% Success/Carcass
Scat	12	67	5	80
Hair	3	0	2	0
Killing Wound Swab	28	86	10	100
Feeding Wound Swab	111	46	22	90
Total Swab	139	54	32	94

Table 2 – Swab sample individual success rates by species.

Sample Type	# Samples	% Success/Swab	# Carcasses	% Success/Carcass
Black Bear Swab	21	52	10	70
Coyote Swab	26	69	20	70
Total Swab	47	62	30	70

Figures

Figure 1 – The location of our three study sites (shaded in gray) on the island of Newfoundland, Canada.

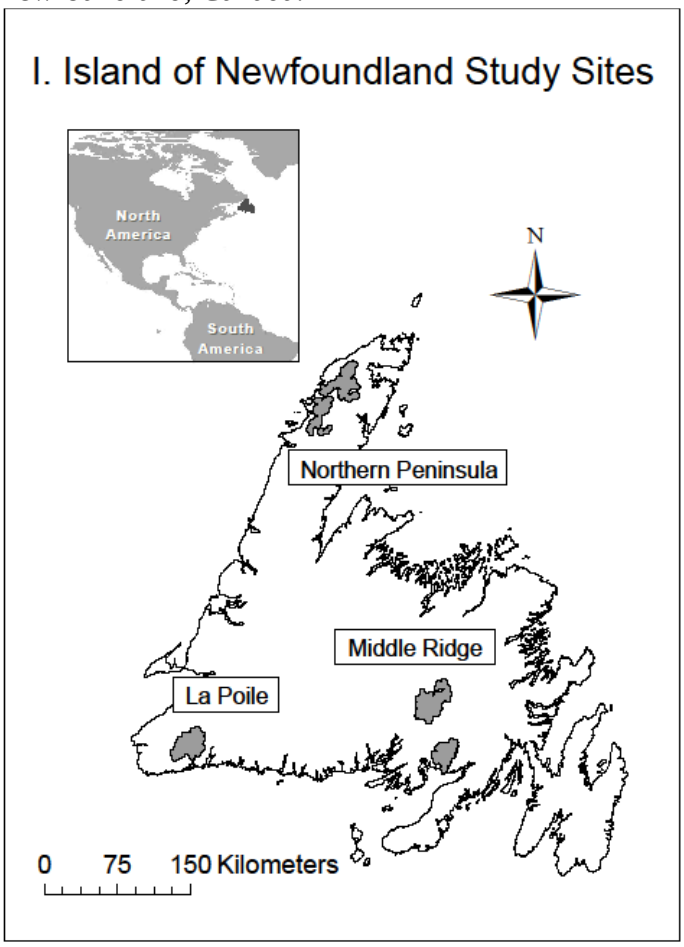
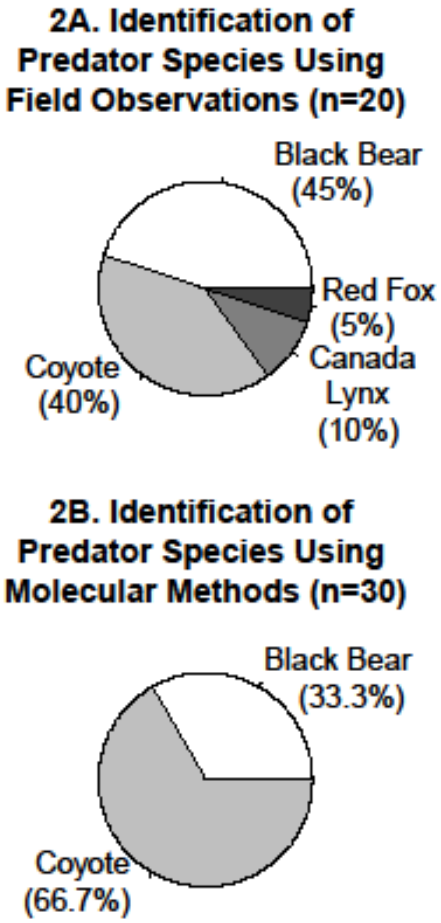


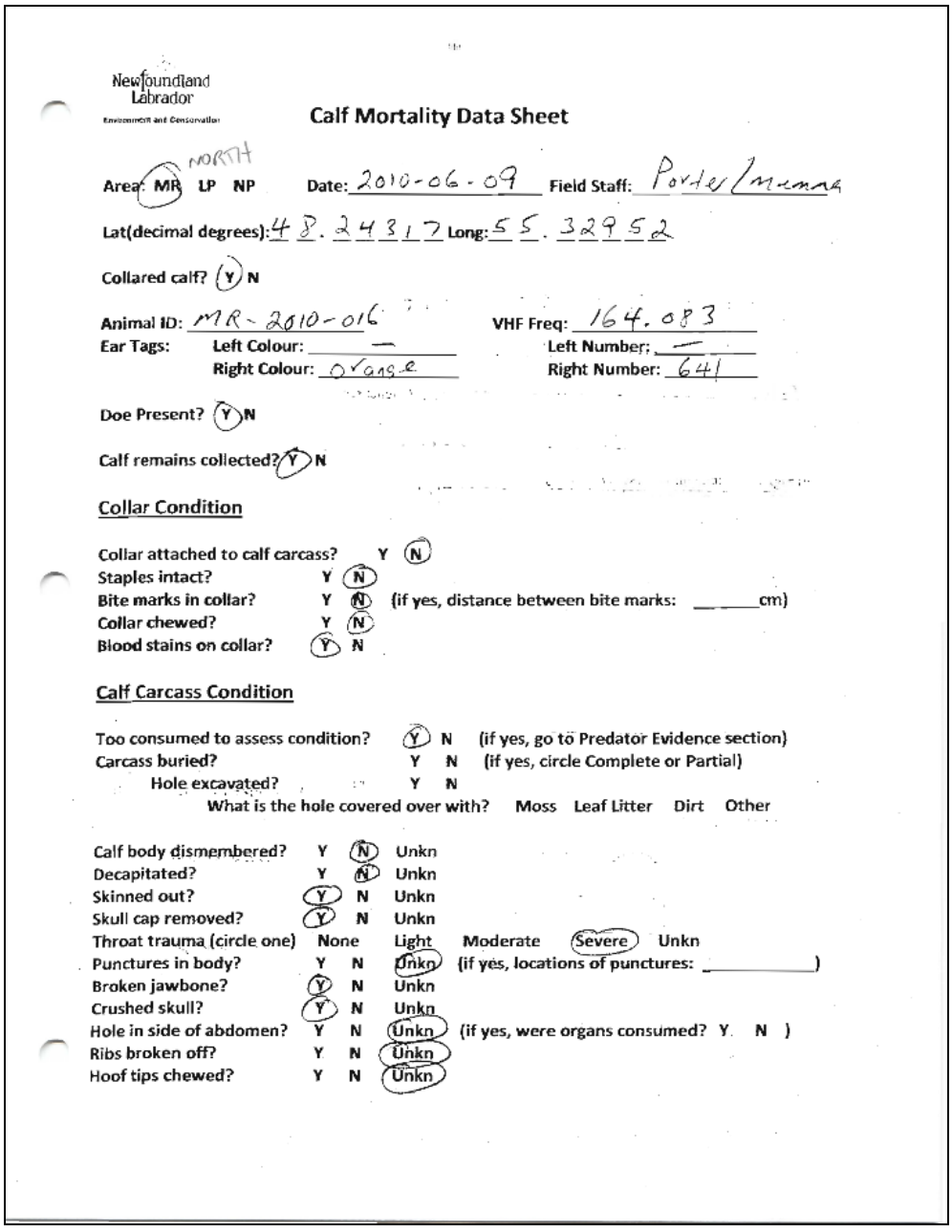
Figure 2A - 2B – The proportion of predation attributed to each predator species via field and molecular methods.



Supplementary Information

Figure S1 – The field observation sheet for caribou calf kill site MR-2010-016. A black bear was assigned via the field observation and molecular methods.

(front)



Newfoundland Labrador
Environment and Conservation

Calf Mortality Data Sheet

Area: MR LP NP (NORTH) Date: 2010-06-09 Field Staff: Porter/Mumma

Lat(decimal degrees): 48.24317 Long: 55.32952

Collared calf? (Y) N

Animal ID: MR-2010-016 VHF Freq: 164.083

Ear Tags: Left Colour: — Left Number: —

Right Colour: Orange Right Number: 641

Doe Present? (Y) N

Calf remains collected? (Y) N

Collar Condition

Collar attached to calf carcass? Y (N)

Staples intact? Y (N)

Bite marks in collar? Y (N) (if yes, distance between bite marks: _____cm)

Collar chewed? Y (N)

Blood stains on collar? (Y) N

Calf Carcass Condition

Too consumed to assess condition? (Y) N (if yes, go to Predator Evidence section)

Carcass buried? Y N (if yes, circle Complete or Partial)

Hole excavated? Y N

What is the hole covered over with? Moss Leaf Litter Dirt Other

Calf body dismembered? Y (N) Unkn

Decapitated? Y (N) Unkn

Skinned out? (Y) N Unkn

Skull cap removed? (Y) N Unkn

Throat trauma (circle one) None Light Moderate (Severe) Unkn

Punctures in body? Y N (Unkn) (if yes, locations of punctures: _____)

Broken jawbone? (Y) N Unkn

Crushed skull? (Y) N Unkn

Hole in side of abdomen? Y N (Unkn) (if yes, were organs consumed? Y N)

Ribs broken off? Y N (Unkn)

Hoof tips chewed? Y N (Unkn)

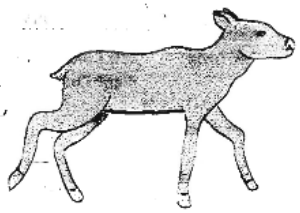
(back)

Predator Evidence:

	Unknown	Bear	Coyote	Lynx	Eagle	Collected?
Sighting						
Scat		✓				(Y) N
Track						
Hair/feathers						Y N

Notes: Typical Bear. Skin out, skull crushed, eaten. Long bones crushed, marrow eaten, Hide left in a "neat" pile. Bear Scat collected nearby

Calf's Right Side



Calf's Left Side

CAUSE OF DEATH: black bear.

Mortality Site Characteristics

80% low scrub / 20% Grass
- Crowberry
- blueberry

Slope: 40 ° (estimated to nearest 5°)

Aspect (circle one) N (NE) NW S SE SW E W

Topography (circle): (hummocky) rocky flat boggy mature softwood blown down & bent over

Estimated distance to nearest tree cover: 100 m

Photo numbers: 1201 → 1212

Camera Owner: Tate

DON'T FORGET TO FILL OUT A GROUND-TRUTHING SHEET FOR EACH MORTALITY SITE

Supplementary Materials and Methods – Swab Technique Optimization

We placed a grid consisting of 2 cm^2 cells across the surface of a pig femur that was chewed for several days by a domestic dog. We tested the amplification success of samples collected with a dry swab (Williams *et al.* 2003, Sundqvist *et al.* 2008, Blejwas *et al.* 2006) and swabs dipped in distilled water (Sweet *et al.* 1997, Glen *et al.* 2010), DETs buffer (Frantzen *et al.* 1998), and ethanol. We also tested a 2 swab method where a wet swab was followed by a dry swab (Sweet *et al.* 1997) and conducted the 2 swab method using 2 wet swabs. We tested the amplification success when processing the 2 swabs in the same extraction and separately. We found wet swabs to have higher amplification success rates and found similar amplification success regardless of whether we processed the 2 swabs together or separately (Table 1 Supplementary Information).

Table S1 – A comparison in amplification strength (fluorescent units) when processing A and B swabs together and individually (Confidence Interval=CI).

Swabs	Average Shorter Band	CI	Average Longer Band	CI
Processed Together	6321	(+/-)880	9057	(+/-)240
Processed Individually	6057	(+/-)705	8321	(+/-)595

Supplementary Materials and Methods – PCR Conditions for Black Bear, Coyote, and Red Fox Species Identification

The PCR conditions for the species ID test were 0.286 uM SIDL, 0.2 uM H16145, 0.1 uM H3R, 1x Qiagen Master Mix, 0.5x Qiagen Q solution, 1.39 uL water, and 1 uL DNA extract in a 7ul reaction. The PCR profile was an initial denaturation step at 95˚C for 15 minutes followed by 40 cycles at 94˚C for 30 seconds, 44˚C for 90 seconds, and 72˚C for 60 seconds before a final elongation at 60˚C for 30 minutes. Allele sizes were determined using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and associated GeneMapper 3.7 software.

Supplementary Materials and Methods – Details and PCR Conditions for Lynx Species Identification

The primers create a lynx fragment of ~126 base pairs. The PCR conditions for the lynx ID test were 0.2 uM DL7F, 0.2 uM CR2bR, 1x Qiagen Master Mix, 0.5x Qiagen Q solution, 0.72 uL water, and 1.8 uL DNA extract in a 7ul reaction. The PCR profile was an initial denaturation step at 95˚C for 15 minutes followed by 40 cycles at 94˚C for 30 seconds, 62.5˚C for 90 seconds, and 72˚C for 60 seconds before a final elongation at 60˚C for 30 minutes. Allele sizes were determined as stated in the previous paragraph.

Supplementary Materials and Methods – Black Bear Multiplex One PCR Conditions

The conditions for the 7 uL PCR were 0.03 uM G10C, 0.2 uM G10M, 0.14 uM G10P, 0.045 uM G10X, 0.09 uM CXX20, 0.21 Mu23, and 0.04 uM SE 47-48 for each primer pair, along with 1x Qiagen Master Mix, 0.5x Qiagen Q solution, and 1 uL DNA extract. The PCR profile was an initial denaturation step at 95˚C for 15 minutes followed by a touchdown of 13 cycles at 94˚C for 30 seconds, 57˚C for 90 seconds, and 72˚C for 60 seconds with a 0.5˚C

decrease in the annealing temperature at each cycle followed by 27 cycles at 94˚C for 30 seconds, 50˚C for 90 seconds, and 72˚C for 60 seconds.

Supplementary Materials and Methods – Black Bear Multiplex Two PCR Conditions

The conditions for the 7 uL PCR were 0.013 uM G10B, 0.04 uM G1A, 0.035 uM Mu15, 0.034 uM Mu50 and 0.032 uM Mu59 for each primer pair, along with 1x Qiagen Master Mix, 0.5x Qiagen Q solution, and 1 uL DNA extract. The PCR profile was an initial denaturation step at 95˚C for 15 minutes followed by a touchdown of 13 cycles at 94˚C for 30 seconds, 63˚C for 90 seconds, and 72˚C for 60 seconds with a 0.5˚C decrease in the annealing temperature at each cycle followed by 27 cycles at 94˚C for 30 seconds, 56˚C for 90 seconds, and 72˚C for 60 seconds.

Supplementary Materials and Methods – Coyote Multiplex PCR Conditions

The conditions for the 7 uL PCR multiplex were 0.2 uM FH2670, 0.1 uM FH2611, 0.1 uM FH2088, 0.05 uM FH2054, 0.1 uM FH3725, 0.05 uM FH2137, 0.1 uM FH2001, 0.3 uM Cxx119, 0.05 uM C09.173, 0.1 uM DBX6, and 0.04 uM DBY7 for each primer pair, along with 1x Qiagen Master Mix, 0.5x Qiagen Q solution, and 1 uL DNA extract. The PCR profile can be found in Stenglein *et al.* (2010). Allele sizes were determined in the manner stated previously.

Chapter III – Predicting Predator Species at Caribou Calf Kill Sites in a Multi-predator System

Authors – Matthew A. Mumma, Steve E. Gullage, Colleen Soulliere, Dennis L. Murray, Shane P. Mahoney, and Lisette P. Waits

Abstract

Predation is a driving force in many communities, but assessing the impact of predator species in multi-predator systems can be difficult. We used molecular identifications of predators (76 kill sites) and kill site observations, along with boosted classification trees to predict the predator species (43 kill sites) at caribou calf kill sites and identify key predator-specific kill site observations in Newfoundland, Canada. We also evaluated kill site observations that have been traditionally used to identify coyote and black bear kills in Newfoundland and tested several hypotheses with respect to predator behavior and kill site observations. We determined that black bears (~48%) and coyotes (~52%) were the 2 primary predators of caribou calves and identified that treatment of the carcass, presence of throat trauma (typically coyote), removal of the skull cap (typically black bear), and which tissues were consumed were all important predictors of the predator species in our model. Consistent with traditional predator-specific kill site observations, we found that black bears more frequently removed hoof tips and coyotes were more likely to bury carcasses. We did not find common trends for skinning carcasses (considered black bear trait) or decapitating carcasses (considered coyote trait), and possible explanations for these findings are discussed. Consistent with our hypotheses, we found that black bears consumed a greater proportion of each carcass and were more likely to crush and eat leg bones. We also hypothesized that black bear predation would decrease as calf age increased and that black bear kills would be

closer to cover in comparison to coyote kills. Although there was a potential trend in calf age, there was not an apparent trend in distance to cover. This study demonstrates that molecular and statistical methods can reduce the subjectivity associated with predator species assignments of kill sites and identify predator-specific kill site observations.

Introduction

Predation is an important force in structuring populations (Terborgh *et al*. 2010), but it can be difficult to distinguish the relative contribution of predators in communities where multiple predator species occupy single or overlapping tropic levels (Whitten *et al*. 1992; Mills & Mills 2014; Smith *et al*. 2014). Researchers have elucidated predator-prey relationships by radio-collaring both predators (Cavalcanti & Gese 2010; Mills & Mills 2014) and prey (Mumma *et al*. 2014; Smith *et al*. 2014). Monitoring of radio-collared prey species facilitates investigation of kill sites and the use of kill site observations to determine predator species. However, predator-specific kill site observations are rarely validated because the true predator species is seldom known, making predator identifications highly subjective and dependent on biologist experience (Mumma *et al*. 2014).

Recent advances and novel applications of molecular techniques can help decrease the uncertainty and subjectivity associated with predator species assignment at kill sites. Several researchers have identified predator species or individual predators from scats at prey kill sites using molecular techniques (Ernest *et al*. 2002; Onorato *et al*. 2006), while others have used cotton swabs to sample and identify predator species from residual saliva left on carcasses (Blejwas *et al*. 2006; Caniglia *et al*. 2012; Glen *et al*. 2009; Mumma *et al*. 2014; Wengert *et al*. 2013; Wengert *et al*. 2014; Williams *et al*. 2003; Sundquist *et al*. 2008).

However, molecular predator species assignment suffers from some of the same limitations as traditional kill site assignment. Both methods risk assigning kill sites to scavenging predators as opposed to killing predators (Mumma *et al*. 2014; Wengert *et al*. 2014). Furthermore, traditional and molecular kill site assignment can fail to assign predator species at some kills, albeit for different reasons. Non-descript and/or overlapping predator-

specific kill site characteristics may not permit identification of a single predator species, while molecular methods can fail as a result of DNA degradation (Blejwas *et al*. 2006; Caniglia *et al*. 2012; Ernest *et al*. 2002; Glen *et al*. 2009; Mumma *et al*. 2014; Onorato *et al*. 2006; Williams *et al*. 2003; Sundquist *et al*. 2008).

We propose a more holistic approach that combines the power of genetic tools with a modeling approach that capitalizes on predator-specific observations at kill sites. Molecular techniques when successful provide a means to positively identify the presence of a predator species. Kill site observations can provide evidence of a predator species if species-specific observations are known. Combining these two data sources can lead to a richer and more complete understanding of predation in predator-prey systems with multiple predator species.

The predator-prey system on the island of Newfoundland, Canada, is an excellent model system for evaluating predator-prey dynamics because of a declining prey population and a changing predator guild. The caribou (*Rangifer tarandus*) population has decreased by >66% since 1998, largely as a result of increased calf predation (Weir *et al*. 2014). Historically, gray wolves (*Canis lupus*), black bears (*Ursus americanus*), and Canada lynx (*Lynx canadensis*) were considered the major predators of caribou, but with the extirpation of wolves in the early 1900s and the arrival of coyotes (*Canis latrans*) in the 1980s (Weir *et al*. 2014), predation on Newfoundland may no longer resemble the predator-prey system under which caribou evolved. Previous research using kill site observations indicated that black bears accounted for the majority of predation followed by Canada lynx and coyotes with a minimal amount of predation attributed to red foxes (*Vulpes vulpes*) and bald eagles (*Haliaeetus leucocephalus*) (Mahoney & Weir 2009). However, a pilot study in 2010 using molecular tools identified coyotes as the most frequent predator followed by black bears with

little to no predation attributed to Canada lynx, red foxes, and bald eagles (Mumma *et al*. 2014).

Our objectives were: 1) to clarify the nature of caribou calf predation on Newfoundland using molecular methods and kill site observations in conjunction with a statistical model to predict predator species at caribou calf kill sites and 2) to enhance our understanding of predator-specific kill site observations by identifying influential observations in our statistical model and evaluating the proportion of predator kills exhibiting specific kill site observations. We explicitly evaluated if observations traditionally used to identify kills by black bears (skinned hide, removed skull cap, and removed hoof tips) and coyotes (throat trauma, decapitation, and buried remains) in Newfoundland were important predictors in our model and/or were more commonly observed at kills of their corresponding species.

In addition, we hypothesized that black bears and coyotes are the major predators of caribou calves and that predation by Canada lynx, red foxes, and bald eagles is rare. We also generated hypotheses for predator-specific kill site observations that align with the biology and behavior of black bears and coyotes. We hypothesized that black bear predation would be more common on younger calves, which is consistent with other studies and has been linked to the inability of bears to consistently catch older, faster calves (Ballard *et al*. 1980). Given the larger size of black bears and increased bite force in comparison to the other predator species (Christiansen & Wroe 2007), we postulated that a greater proportion of the calf would be consumed at black bear kills and that large bones would be more commonly crushed and consumed. Lastly, we hypothesized that black bear kills would be closer to cover than coyote kills as a result of black bear habitat preferences (Bastille *et al*. in review) and the

coursing hunting strategy of canids, which lends itself to long chases in open habitats (Estes & Goddard 1967).

Materials and Methods

Study Site

The island of Newfoundland (111,390 km^2) is located off Canada's eastern coast and has a cool, maritime climate with interspersed coniferous forest, windswept barrens, and peatland (McManus & Wood 1991). Caribou are widely distributed and the only native ungulate on Newfoundland. We selected three (La Poile, Middle Ridge, and the Northern Peninsula) of Newfoundland's caribou calving grounds (Fig. 1) for our study (Rayl *et al*. 2014).

Capture and Monitoring of Caribou Calves

From late May through early June in 2010, 2011, and 2012, we hand-captured 333 one-to-three day old caribou calves across our three study sites (Fig. 1). Our methods followed American Society of Mammalogists' guidelines (Sikes *et al*. 2011) and were approved by the Animal Care and Use Committee at the University of Idaho (Protocol 2011-32). Each calf was fitted with a 200g expandable, breakaway very high frequency (VHF) radio-collar containing a motion sensitive transmitter (Lotek Wireless Inc., New Market, ON, Canada; Telemetry Solutions, Concord, CA). Transmitter pulse rates increase for collars that are stationary for >4 hours signaling calf mortality or a slipped collar. Collar pulse rates were checked regularly via fixed-wing and/or helicopter flights from the date of capture through August. Checks occurred daily early in June, but became less frequent (bi-weekly) in August when mortalities were rare.

When a change in pulse rate was detected, trained field personnel (<1 to >30 years of experience) investigated each location to determine if predation had occurred as supported by calf remains and/or predator sign. Observations and photographs were collected using standard protocols (Fig. S1 Supplementary Information) from each kill site and a predator species was assigned (black bear, coyote, Canada lynx, red fox, bald eagle, or unknown predator). Kill site observations and photographs were reviewed at a later date by multiple biologists, and predator species were adjusted when the evidence did not support the original predator assignment. When present, predator scat and hair samples were collected, and carcass remains were sampled for predator saliva containing predator DNA using sterile, cotton swabs.

Kill Site Field Observations

In Newfoundland, biologists have been assessing collared caribou calf kill sites since 1979 (Mahoney and Weir 2009). Predator species assignment has largely been based on kill site characteristics that are in agreement with what is known about the biology and behavior of each predator species and sometimes confirmed by the presence of identifiable predator scats. Black bears frequently consume the majority of a calf carcass leaving scant bones and tissues remaining, such as the jaw bone, skull cap, hoof tips, and hide. Coyote characteristics include dismembered and/or scattered carcasses that may be severed in half or decapitated and often demonstrate throat trauma. Canada lynx kills are also associated with throat trauma and can be potentially differentiated from coyote kills by canine size and spacing. Punctures on the dorsal surface of carcasses are considered a potential sign of Canada lynx or bald eagle. Diagnostic characteristics of red fox kills are largely unknown.

Although certain characteristics are often associated with specific predators, many characteristics are thought to potentially result from multiple predator species. Additional observations at kill sites that are not consistently indicative of one predator species include a crushed skull, a hole in the abdomen, crushed leg bones, broken ribs, the specific tissues consumed, and even the age of the calf at the time of predation. In our study, we also wanted to explore if site-specific variables, such as slope, topography, distance to cover, and habitat type, could inform predator species assignment.

Sample Collection for Molecular Analysis

We searched an area within a 25-m radius from carcasses for predator scat and hair. Small sticks collected from woody shrubs in the field were used to sample 0.5 ml of total fecal material from multiple locations on the lateral surface of each predator scat (Stenglein *et al.* 2010), and fecal material was then placed in 2 ml collection tubes containing DETS buffer (Frantzen *et al.* 1998). Predator hair samples were placed in individual paper envelopes and stored collectively in sealed plastic bags containing silica desiccant (Roon *et al.* 2005). Clean latex gloves were used during collections to prevent cross-contamination between kill sites and samples.

We sampled carcass remains for residual predator saliva using sterile, cotton swabs soaked in ethanol (Mumma *et al*. 2014). We swabbed up to four locations on each carcass or up to 4 tissues when the majority of the carcass was consumed. We collected 2 swabs from each location to provide a back-up sample in case of laboratory error. Personnel wore clean latex gloves to prevent cross-contamination between carcasses and samples. All swabs were placed in individual paper envelopes that were collectively stored in sealed plastic bags containing silica desiccant.

DNA Extraction and Species Identification

All samples were extracted in a laboratory dedicated to low quantity DNA samples and used the Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA) for scat samples and the Qiagen DNeasy tissue kit for hair and swab samples. We used 10 follicles for each hair extraction when available and each batch of hair, scat, and swab extractions contained a negative control to monitor for contamination. Back-up swabs were only processed when all of the first swabs from a carcass failed to provide a species identification.

We used a mitochondrial DNA (mtDNA) control region fragment analysis method to identify all samples to species (Debarba *et al*. 2014). This analysis uses primers for differentiating black bear, coyote, and two non-target species: brown bears (*Ursus arctos*) and wolves (*Canis lupis*) (Murphy *et al.* 2000; Onorato *et al.* 2006), plus an additional primer (H3R) designed to differentiate red fox (Dalen *et al.* 2004). All failed samples were analyzed with an additional test to identify Canada lynx samples using mtDNA primers developed for Iberian lynx (*Lynx pardinus*) by Palomares *et al.* (2002) (Mumma *et al*. 2014). Additional PCR details can be found in Mumma *et al*. (2014). We determined allele sizes using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and associated GeneMapper 3.7 software.

Samples that failed the initial tests were amplified and sequenced using mtDNA cytochrome B primers that amplify most carnivores (Farrell *et al.* 2000) using conditions described in Onorato *et al.* (2006). These primers can identify black bear, Canada lynx, and red fox, but not coyote. Any remaining failed samples were amplified and sequenced using the canid-specific mtDNA control region ScatID primers using conditions and primers described in Adams *et al.* (2003) to identify coyote samples that failed the initial species ID

screenings. We did not use molecular tools to test for the presence of bald eagles, because they frequently scavenge carcasses and are rarely the predator of caribou calves (O'Gara 1994).

Statistical Model

We built boosted classification trees using the package gbm (Ridgeway *et al*. 2013) in program R (R Core Team, 2014). These methods combine a large number of weakly predictive classification trees in to an ensemble of trees for the purpose of prediction (Natekin & Knoll 2013). We selected boosted classification trees, because they have been shown to provide superior predictive power for some datasets, along with permitting a large number of explanatory variables in comparison to the number of observations. In addition, the predictive power of these methods is not adversely affected by collinear variables or variables that fail to meet assumptions of normality. Conceptually, the goal of boosted classification trees is to estimate the functional relationship between your explanatory variables (x) and the response variable (y) by minimizing a specified loss function $\Psi(y, f)$ (Natekin & Knoll 2013), which in our case was binomial, $y \in \{0,1\}$:

$$\hat{f}(x) = argmin\Psi(y, f(x))$$

In contrast to random forests, boosted methods do not determine the relationship between the explanatory and response variables by averaging across independently built trees, but instead estimate the fit of new trees by the considering the error across all previously built trees (Natekin & Knoll 2013). The related structure of boosted trees leads to increased predictive power, but can limit model generalization; therefore, we used 10-fold cross validation to limit overfitting, which also allowed us to estimate our error rate (misidentification rate). We then adjusted the number of variables and model parameters to minimize our misidentification rate

and ran our final model with 21 explanatory variables, 2000 trees, a shrinkage parameter of 0.0005, and an interaction depth of 2.

In our model, we used the positive molecular predator species identifications (n=76) as our response variable and 21 categorical predictors as our explanatory variables. All of our explanatory variables were categorical or were converted to categorical variables to reduce false precision. These included the proportion consumed (complete, significant, partial, minimal, none, and unknown) slope (flat, moderate, steep, or very steep), and distance to cover (cover, near cover, open, or very open). Additional observations recorded regarding the state of the carcass included tissues consumed (all, bone and meat, meat, viscera, none, and unknown), treatment of the carcass (unburied sparse, unburied scattered, unburied dismembered, unburied halved, unburied intact, buried decapitated, or buried halved), removal of the hide, skull cap, or hoof tips, and whether or not the skull, jaw bone, leg bones, or ribs were crushed or broken. Other variables included if the carcass was buried (yes, partial, or no), decapitated (yes, no, or unknown), or dismembered (yes, no, or unknown), and whether or not the abdomen was opened (yes, no, or unknown) and if there was throat trauma (severe, moderate, light, none, or unknown). Since most caribou calves are born within a single week in late May and early June, we included a variable for the age of the calf based upon the date the carcass was discovered. Calves found in the first half of June, second half of June, first half of July, and second half of July were assumed to be <2 weeks old, 2-4 weeks old, 4-6 weeks old, and 6-8 weeks old, respectively. We did not include calves killed after July 31st in our model to decrease the likelihood of scavenging since calf monitoring was less frequent in August. Originally, we also included an observation regarding the presence and location of punctures, but later removed this variable because it lacked explanatory

power. We also considered a study site variable (La Poile, Middle Ridge, or Northern Peninsula) and site-specific variables of topography (flat, flat-boggy, boggy, hummocky-boggy, hummocky, or rocky) and habitat type (shrub low, shrub tall, coniferous dense, coniferous open, coniferous sparse, wetland treed, wetland shrub, wetland herb, or rocky/rubble). Topography, while not completely independent from slope, was a finer scale assessment. Additional details for explanatory variables can be found in the supplementary information (Table S1).

Once our model was constructed, we then used our model to predict the predator species at 39 kill sites where molecular techniques were not used, failed, or detected red fox DNA, along with 4 additional kill sites assigned to bald eagle via field observations. We ran potential red fox and bald eagle kills through the model because of the propensity of both species to scavenge. We report the odds ratios, which are a ratio of the probability of a black bear kill over the probability of a coyote kill, and relative influence (%) of each variable, which is a measure of the sum of squared improvements at all splits averaged across all trees (Breiman *et al*. 1994). Although collinear variables should not adversely impact model predictions, it is important to recognize that for a specific variable the relative influence may be reduced and odds ratios may be pushed toward 1 by another highly influential, collinear variable. Therefore, we also plotted the proportion of black bear kills and coyote kills (both molecular and predicted identifications) exhibiting specific kill site observations, along with Wald confidence intervals, to further evaluate species-specific kill site observations. Finally, we also conducted a multiple correspondence analysis using the FactoMineR package (Husson *et al*. 2014) in program R (R Core Team, 2014) to evaluate overlap of kill site observations between predator species for kill sites identified via molecular methods (n=76)

and kill sites predicted by our model (n=43). Multiple correspondence analysis is the equivalent of a principal component analysis for categorical data.

Results

From June through August in 2010, 2011, and 2012, we investigated 154 suspected calf mortalities and 7 additional carcasses that were opportunistically found on the landscape. We excluded 42 of these sites from further analyses, because they were either located in August (n=21) when monitoring was infrequent or only a collar was found with no other remains (n=21). Using field observations only, we assigned 44 of these sites to black bear, 44 to coyote, 5 to Canada lynx, 1 to red fox, and 5 to bald eagle (Fig. 2). We suspected predation at an additional 20 sites, but were unable to assign a single predator species using field observations.

Molecular Predator Species Identification

We tested samples from 110 sites suspected of predation using molecular tools. The majority of samples collected were swabs, and the swab predator identifications agreed with hair and scat identifications at the few sites where hair or scat were collected. Unfortunately, samples were not collected from 9 additional sites assigned to black bear (n=3), coyote (n=2), and bald eagle (n=4) via field observations. We detected predator DNA at 88 (80% of carcasses) of the kill sites tested and found a single predator species at 78 kills (black bear-36, coyote-40, Canada lynx-0, and red fox-2, Fig. 2). We found black bear DNA at 4 sites assigned to coyote and 1 site assigned to bald eagle by field observations and found coyote DNA at 3 sites assigned to black bear, 4 sites assigned to Canada lynx, and 1 site assigned to red fox by field observations. We also detected red fox DNA at 1 kill assigned to black bear and 1 kill assigned to coyote via field observations, but suspected scavenging because of the

scarcity of remains. We detected multiple predator species at 10 other kills, and all primary and secondary swabs failed at 22 additional sites.

Predator Species Identification Model

We used the 76 kill sites where black bear or coyote were detected using molecular techniques in our boosted tree model, including several kill sites that were previously assigned to other species via field observations. We wanted to include additional predator species, but Canada lynx, red fox, and bald eagle were precluded as a result of their uncertain and rare kill site assignment. Although kill site observations frequently overlapped between black bear and coyote kills (Fig. 3), we estimated the misidentification rate of our model at 8.3% via cross-validation. We then used our final model to predict the predator species at 43 kill sites (21 black bear and 22 coyote kills – Fig. 2) where swabs were not collected or molecular methods failed to identify a single predator species. The model predicted black bear at 5 sites assigned to coyote and coyote at 2 sites assigned to black bear using field observations. The model also predicted black bear for the 2 kills where red fox DNA was detected and coyote for the 1 remaining Canada lynx and 4 remaining bald eagle kills assigned via field observations.

Relative Influence and Predator-specific Kill Site Observations

In our model, carcass treatment and throat trauma had the highest relative influence followed by tissues consumed and skull cap removal (Fig. 4). Carcass treatment was a broad variable that captured a range of carcass conditions, some of which suggested a coyote kill (buried and decapitated - bd) and others that indicated black bear (unburied carcass with sparse remains - usp) as evidenced by their odds ratios (Fig. 5A). Halving a carcass (buried halved - bh and unburied halved - uh) and an unburied carcass that was scattered also

suggested a coyote kill (Fig. 5A). The presence of throat trauma (light-L, moderate-M, or severe-S) was associated with coyote kills (Figs. 5B and 7A), while a removed skull cap increased the probability of a black bear kill (Fig. 5D and 6A). Not being able to identify throat trauma and/or skull cap removal (unknown-U) was also indicative of a black bear kill as a result of complete or near complete consumption of bones and meat (all-A), which is consistent with our tissues consumed predictor (Fig. 5C). Moderate (M) consumption or unknown (U) consumption, which occurred when part of a carcass was buried and the remaining kill site could not be found, supported a coyote kill prediction (Fig. 5C).

There were many variables that we predicted to be informative that demonstrated moderate to low relative influence. Proportion consumed and calf age demonstrated moderate support, while a skinned carcass, removal of hoof tips, decapitation, carcass burial, distance to cover, and crushed leg bones lent minimal support to the model (Fig. 4). However, complete consumption (com) was more frequently observed at black bear kills (Fig. 9A), and while not significant statistically, there appears to be opposite trends with regards to the proportion of predation attributed to black bears (negative relationship) and coyotes (positive relationship) as calf age increases (Fig. 8A). Despite the low relative influence for hoof tip removal and crushed leg bones, bears kills more commonly exhibited these observations (Figs. 6C and 9C). Likewise, coyote were more likely to bury carcasses (Fig. 7C), but burial was not highly influential in our predictive model (Fig. 4). Clear trends in the proportion of predator-specific observations were not evident for skinned carcasses, decapitated carcasses, and the distance to cover variable (Figs. 6B, 7B, and 8B).

Discussion

Predator species identifications using kill site observations suffer from a degree of subjectivity (Mumma et al. 2014), but to date, we are not aware of any studies that have implemented a modeling framework to decrease the subjectivity of predator species assignment. Furthermore, we are not aware of any research that provides a means to explicitly evaluate predator-specific kill site observations. We believe that our application of molecular tools and boosted classification trees creates a framework for other researchers to increase the accuracy of kill site assignments and identify key characteristics that may aid in the identification of the correct predator species.

Predator Species Identification

In our study, predator species assignments using field observations, molecular techniques, and our statistical model all identified black bear (44-49%) and coyote (44-51%) as the primary predators of caribou calves in Newfoundland, but there were a large number of discrepancies between field observations and our molecular/modeling approach. We found conflicting predator species assignments at 22% of kill sites (n=26) and molecular techniques and/or modeling identified a predator species at another 17% of kills (n=20) where field observations were inconclusive.

Kill sites with conflicting predator species assignments or sites where a predator species could not be assigned by field observations displayed mixed observations some suggesting black bear and others coyote. The propensity of overlapping kill site characteristics was consistent with our multiple correspondence analysis (Fig. 3) and may be partially explained by scavenging by a second predator species. Regardless, we think the data strongly support the need for an objective modeling framework to assess kill site predators in

conjunction with thorough field assessments. For example, molecular methods detected red fox at 2 kill sites that were almost entirely consumed. We thought that complete carcass consumption by a red fox was unlikely, so we used our statistical model to determine that black bears were the most likely predator.

Uncertainty regarding Canada lynx, red fox, and bald eagle predation stems from the lack of confirmed kills and the corresponding limited understanding of species-specific kill site observations. Our findings suggest that some kill sites were incorrectly assigned to these species by field observations and that these species are minor predators of caribou calves (less than the combined 11% assigned via field observations – Fig. 2). However, we recognize that our molecular methods were limited by not including bald eagle, and our model was limited by not being able to incorporate information on Canada lynx, red fox, and bald eagle kills.

Comparison to Other Studies

Our proportions of black bear (48%) and coyote predation (52%) assigned via molecular methods and our predictive model from 2010-2012 were slightly different than those first reported in our 2010 pilot study (black bear - 33.3%, coyote - 66.6%) (Mumma *et al*. 2014). One explanation for this change could be that approximately half of the collars were deployed in 2011 and 2012 to the Middle Ridge study site where black bear predation was more frequent. However, our findings may also have been affected by predator manipulation experiments that occurred for both black bears (diversionary feeding 2010 and 2011) and coyotes (lethal removal 2012) in the southern portion of the Middle Ridge study site, but quantifying the potential impacts of these actions was not straightforward (Lewis *et al*. 2014).

Our findings are similar to a study in southern Quebec, where coyotes colonized following the extirpation of gray wolves, but dissimilar to studies in more northern latitudes were gray wolves have remained. Coyotes were the major predator of caribou in Gaspésie Park, Quebec followed by black bears (Crete & Desrosiers 1995). However, gray wolves (57%) and bears (38%) were identified as the major predators of caribou calves for the Mentasta caribou herd in Alaska (Jenkins and Barten 2005), and gray wolves and wolverines (*Gulo gulo*) were the most common predators, followed by bears and eagles, for a study in northern British Columbia (Gustine *et al*. 2006). How wolf and bear predation pressure on caribou differs from a novel predator, coyotes, along with the subsequent effects on population viability for caribou across their southern range, warrants further study.

Predator-specific Kill Site Observations

We were less surprised by the highly influential variables in our model and more surprised by the variables that proved uninformative. Tissues consumed and carcass treatment were broad categories that captured a significant amount of kill site variability, and skull cap removal and throat trauma were already recognized as characteristic observations for black bear and coyote kills, respectively. However, other characteristic black bear observations, such as hoof tip removal and skinning, and coyote observations, such as decapitation and burying, provided minimal predictive power (Fig. 4), which may indicate an uninformative variable or a variable that is correlated to another highly influential predictor.

We could further evaluate variables with low relative influence by looking for trends in the proportion of black bear and coyote kills displaying specific kill site observations. Hoof tip removal (Fig. 6C) was more commonly observed for black bears and burying was more commonly documented for coyotes (Fig. 7C), which suggests that these two variables

were correlated with other highly influential variables. In fact, information on burying was captured by one of our most informative variables, carcass treatment. In contrast, we did not observe clear trends for skinning (Fig. 6B) or decapitation (Fig. 7B), which suggests that these variables may not be good indicators of the predator species.

The lack of information provided by skinning and decapitation may have been the result of categorizing these variables too broadly. A designation of skinning could occur when a large portion of hide was removed from a carcass, but the designation was also assigned when the hide remained attached to the legs, but most of the meat had been consumed, or when the hide had been skinned off the legs and pulled down over the hooves. Similarly, decapitation was assigned when a buried head was found, but also when the head was missing from a carcass due to removal or consumption. Unfortunately, the resolution of the data did not permit us to tease apart these differences.

Evaluating the proportion of black bear and coyote kills displaying specific kill site observations also allowed us to test our additional kill site observation hypotheses. We did not detect a significant decline in the proportion of black bear kills as calf age increased, but we think this was partially due to the reduced sample sizes and overall reduction in predation on older calves (Fig. 8A). Additionally, we did not observe a clear trend in distance to cover between species, but did observe that most kill sites were located within cover (Fig. 8B). Since caribou in Newfoundland select open habitats for calving (Rayl *et al*. 2014), it is unlikely that the propensity for cover is a result of higher calf availability, but it could be that cover provides predators with greater concealment, thus increasing predator success in these areas. Alternatively, predators may be killing calves in open areas and transporting them to habitats with greater cover to limit thermal exposure or avoid other predators and avian

scavengers, even though we tried to account for this in Fig. 8B by excluding calves that were clearly cached and buried.

Our hypothesis that black bears would consume a greater proportion of the carcass and would be more prone to crush and consume large bones was supported (Figs. 9A, 9B, and 9C). Black bears are significantly larger predators with 4x the bite force of coyotes (Christiansen & Wroe 2007), which may allow them to rapidly crush through and consume larger bones, thereby decreasing handling time even if coyotes were hunting in small family units. These traits would also provide an explanation for the tendency of coyotes to dismember, scatter, and/or cache portions of carcasses.

Conclusion

This study demonstrates the utility of molecular and statistical tools for predator species identification, but also recognizes the insights that are possible through traditional field methods. Combining these three data sources allowed us to improve predator species assignments and provide a fuller understanding of predator biology. In future studies, we recommend that researchers and managers implement frequent monitoring to limit the effects of scavenging and consider multiple lines of evidence when assessing predator species at kill sites to reduce subjectivity and elucidate predation in multi-predator systems.

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Figures

Fig. 1 – A map of Newfoundland, Canada with our 3 study sites (La Poile, Middle Ridge, and Northern Peninsula) and calving grounds.

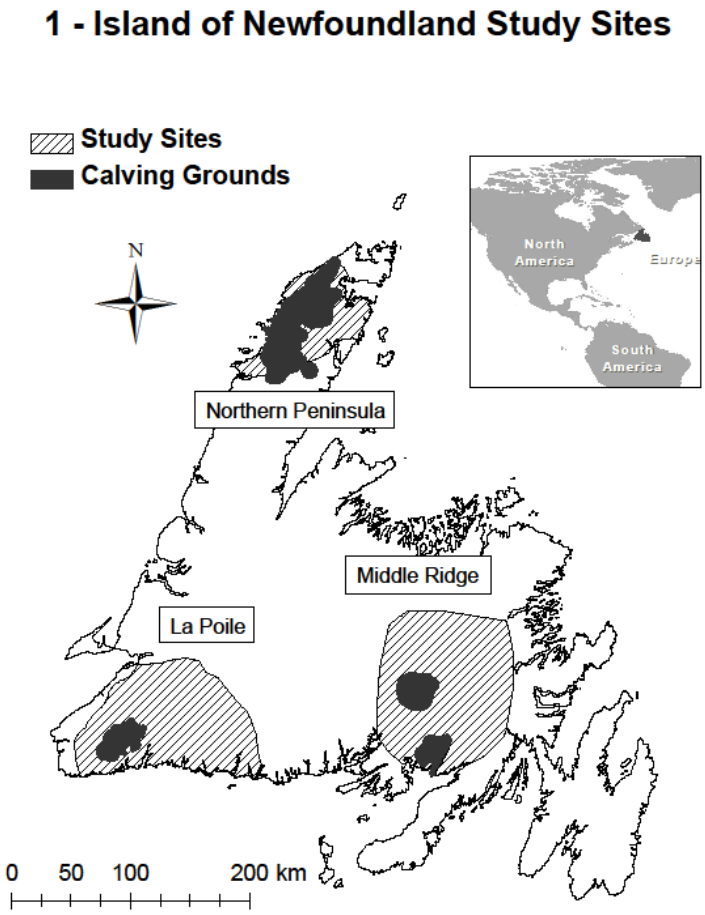


Fig. 2 – The proportion of predation assigned to each predator species using field observations, molecular techniques, and our boosted tree model.

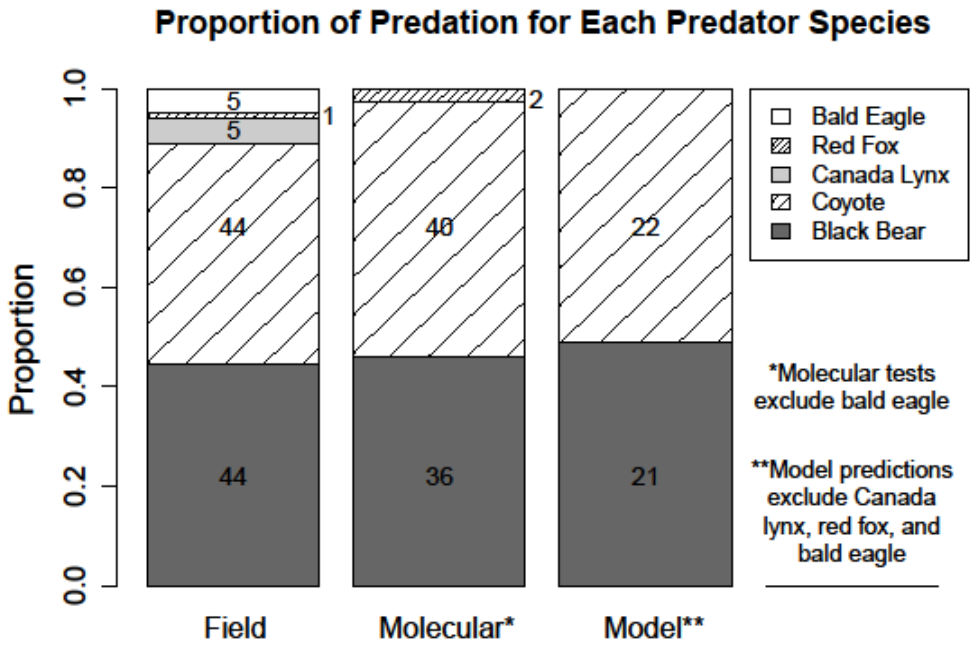


Fig. 3 – A multiple correspondence analysis plotting black bear (O) and coyote (Δ) kills in space (n=119), along with their densities (black bear-solid line, coyote-dotted line), using 21 kill site observations.

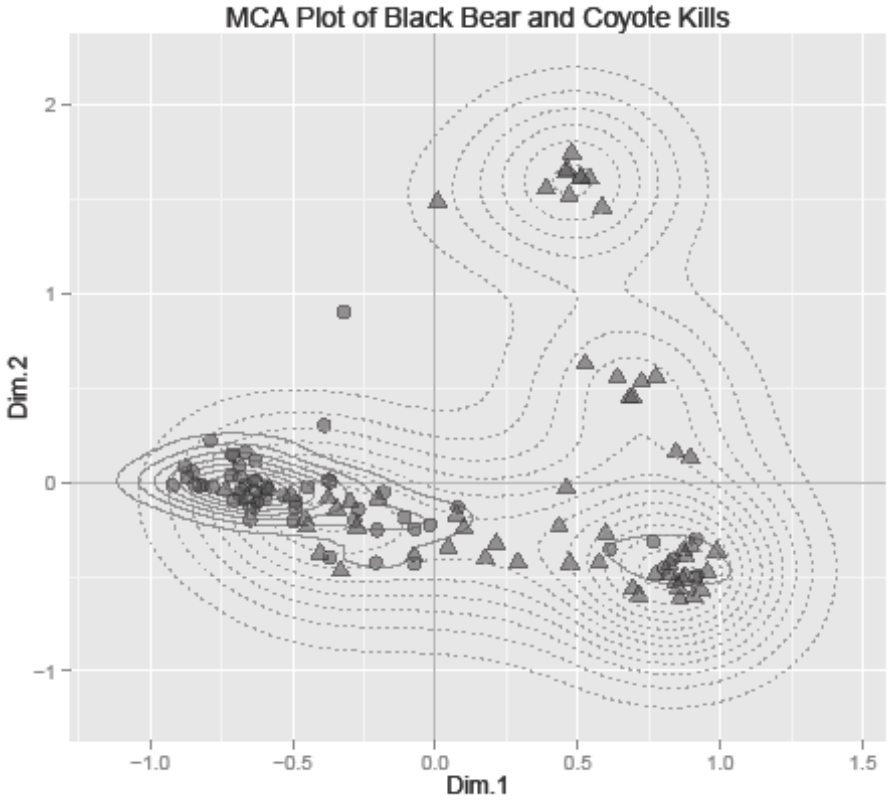


Fig. 4 – The relative influence (%) of each explanatory variable in our boosted classification tree model.

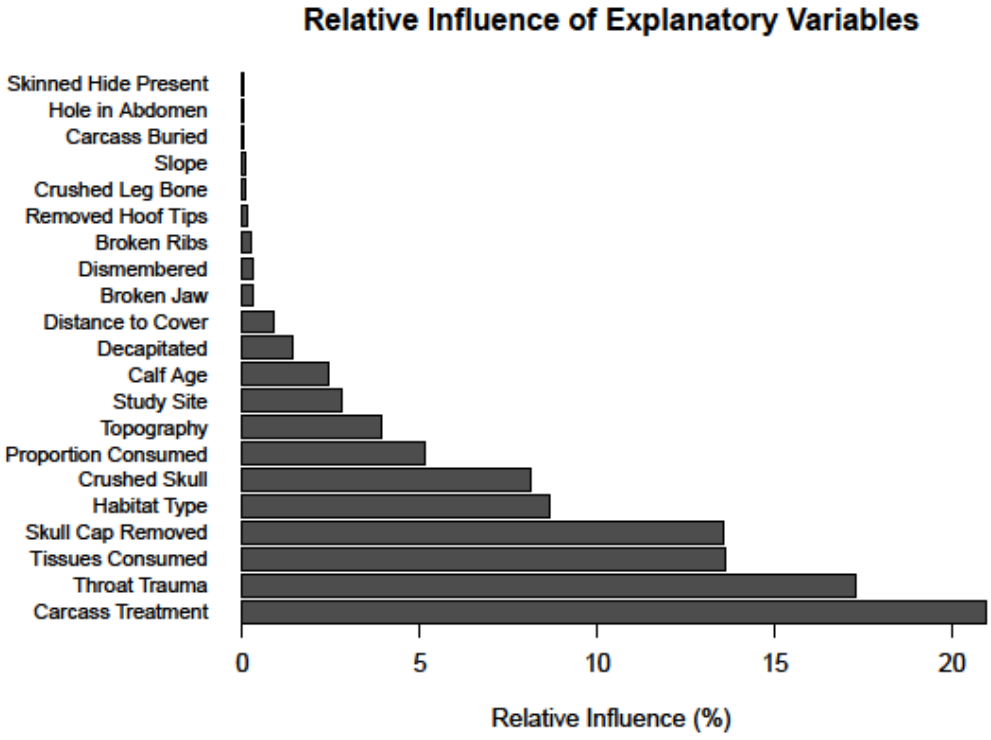


Fig. 5 – Odds ratios for our 4 most influential variables (carcass treatment, throat trauma, tissues consumed, and skull cap removal) in our boosted tree model. Key – Fig. 5A (BD-buried decapitated, BH-buried halved, UD-unburied dismembered, UH-unburied halved, UI-unburied intact, USC-unburied scattered, USP-unburied sparse), Fig. 5B (L-light, M-moderate, N-none, S-severe, U-unknown), Fig. 5C (A-all, B-M-bone-meat, M-meat, N-none, U-unknown, V-viscera), and Fig. 5D (N-no, U-unknown, Y-yes,)

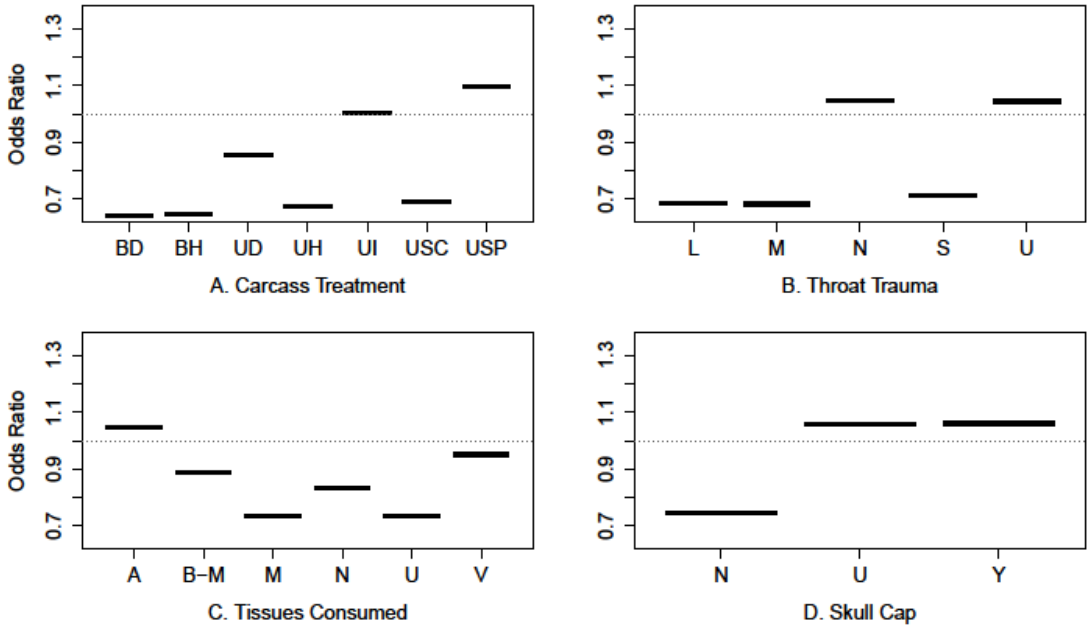


Fig. 6 – The proportion of black bear and coyote kills demonstrating common black bear kill site observations. Key – Figs. 8A. Skull Cap Removal, 8B, and 8C (Y-yes, N-no).

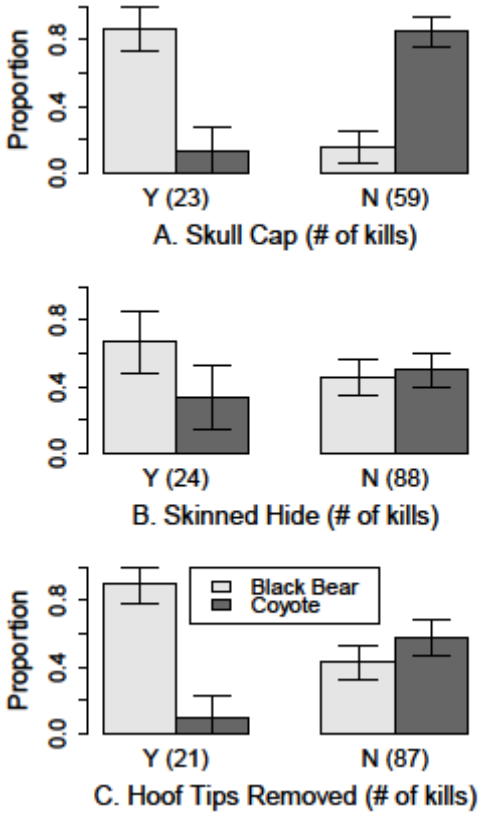


Fig. 7 – The proportion of black bear and coyote kills demonstrating common coyote kill site observations. Key – Figs. 9A, 9B, and 9C (Y-yes, N-no).

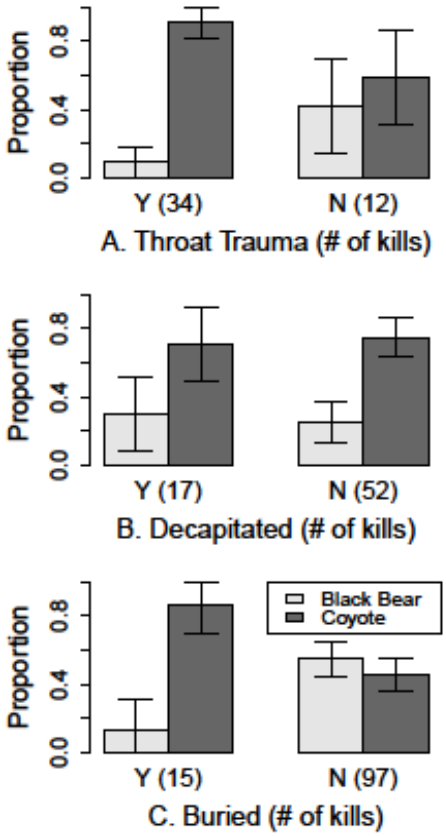


Fig. 8 – The proportion of black bear and coyote kills across 10A) calf ages and 10B) varying distances to cover. Please note that cached and buried carcasses were excluded from Figure 10B.

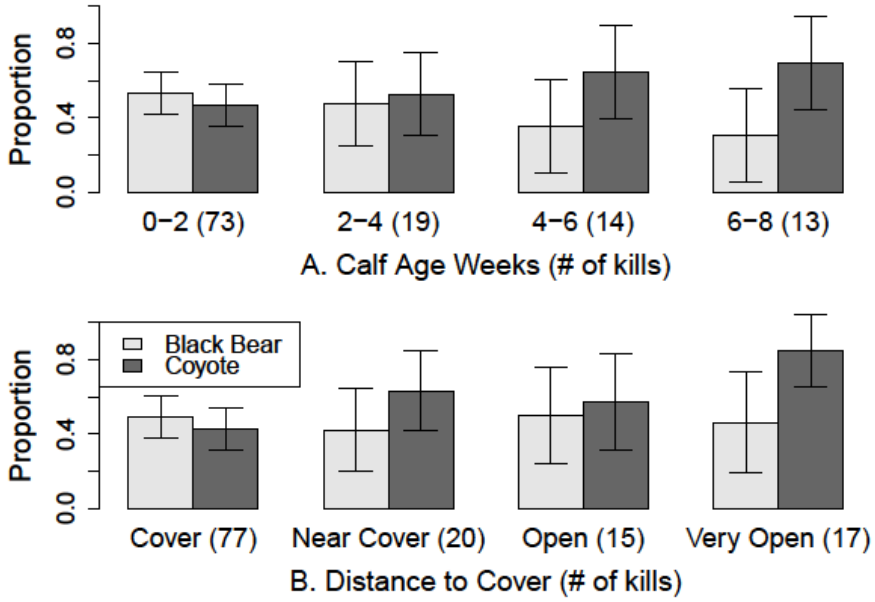
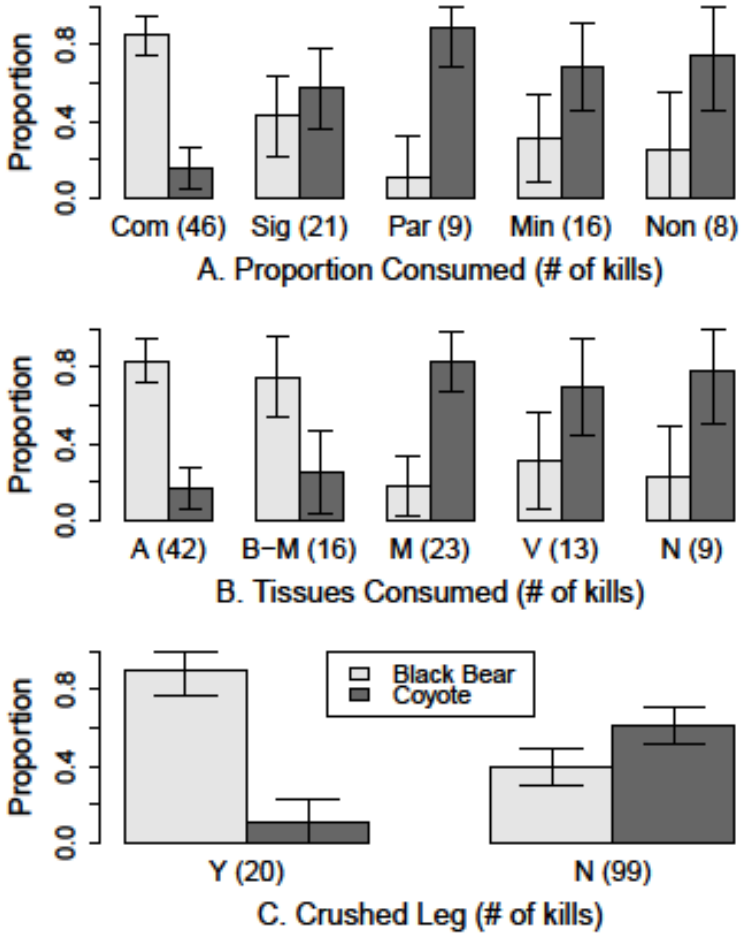


Fig. 9 – The proportion of black bear and coyote kills for 11A) varying degrees of (proportion) consumption, 11B) different tissues consumed, and 11C) the presence or absence of crushed leg bones. Key – Fig. 11A (Com-complete, Sig-significant, Par-partial, Min-minimal, Non-none), Fig. 11B (A-all tissues, B–M-bones and meat, M-meat only, V-viscera, N-none), and Fig. 11C (Y-yes, N-no).



Supplementary Information

Table S1 – Descriptions for explanatory and response variables.

Model Variables					
Carcass-specific Explanatory Variables	**Categories (description)**				
Calf Age	June 1-15 (~0-2 weeks)	June 16-30 (~2-4 weeks)	July 1-15 (~4-6 weeks)	July 16-31 (~6-8 weeks)	
Amount Consumed (increments of 5)	Complete (≥90% consumed)	Significant (70-85% consumed)	Partial (35-65% consumed)	Minimal (5-30% consumed)	None (0% consumed)
Tissues Consumed	All (consumed portions of viscera, muscle, and bone)	Bone-Meat (consumed some bones and portions of muscle)	Meat (consumed portions of muscle)	Viscera (consumed portions of viscera)	None (no consumption)
Tissues Consumed (continued)	Unknown (buried head or front half of carcass)				
Carcass Treatment	Unburied Sparse (sparse bones, hair, hide, and muscle)	Unburied Scattered (messy carcass with pieces of carcass torn apart and strewn around kill site)	Unburied Dismembered (carcass divided into multiple sections often with entire legs separated from carcass)	Unburied Halved (carcass severed at mid-section with both or one half remaining)	Unburied Intact (carcass in different states of consumption but skeletal structure mainly intact)
Carcass Treatment (continued)	Buried Decapitated (head removed and buried)	Buried Halved (front half of carcass severed and buried)			
Carcass Buried	Yes (not clearly distinguishable)	Partial (debris covering remains but clearly distinguishable)	No		

Dismembered (carcass divided into multiple sections often with entire legs separated from carcass)	Yes	No	Unknown		
Decapitated (head removed)	Yes	No	Unknown		
Throat Trauma	Severe (Punctures and/or tearing of throat with visible blood and extensive hemorrhaging)	Moderate (Punctures present and hemorrhaging)	Light (punctures visible by little to no hemorrhaging)	None	Unknown
Hole in Abdomen (hole opened in abdomen)	Yes	No	Unknown		
Punctures (removed from model uninformative)	Head	Neck	Body	None	Unknown
Skinned Hide Present (30 cm section of hide removed from carcass)	Yes	No	Unknown		
Skull Cap Removed (skull cap removed and remaining in entirety of in several large pieces)	Yes	No	Unknown		
Removed Hoof Tips	Yes	No	Unknown		
Crushed Skull (broken, cracked or chewed skull	Yes	No	Unknown		

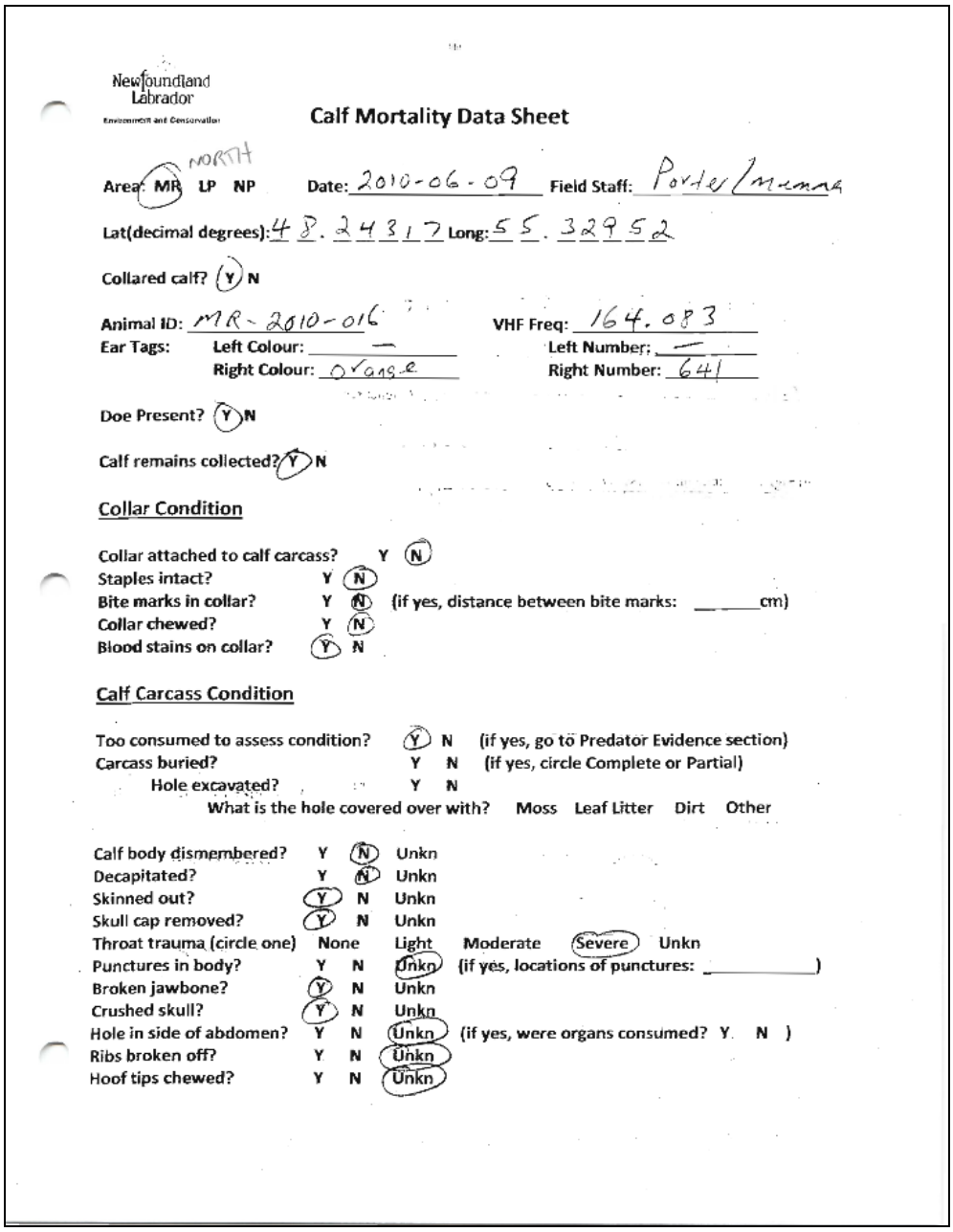
Broken Jaw (jaw crushed in skull or removed from skull, but crushed)	Yes	No	Unknown		
Crushed Leg Bone (leg bone fragments evident)	Yes	No	Unknown		
Broken Ribs (Ribs chewed or snapped off)	Yes	No	Unknown		
Site-specific Explanatory Variables	**Categories (Description)**				
Study Site	La Poile	Middle Ridge	Northern Peninsula		
Habitat Type	Shrub Low - at least 20% ground cover which is at least one-third shrub, average shrub height <2 meters	Shrub Tall - at least 20% ground cover which is at least one-third shrub, average shrub height ≥2 meters	Coniferous Dense - >60% crown closure, coniferous trees are 75% or more of total basal area	Coniferous Open - 26-60% crown closure, coniferous trees are 75% or more of total basal area	Coniferous Sparse - 10-25% crown closure, coniferous trees are 75% or more of total basal area
Habitat Type (continued)	Wetland Treed - land with a water table near, at, or above the soil surface, the majority of vegetation is stunted trees	Wetland Herb - land with water table near, at, or above the soil surface, the majority of vegetation is herb	Wetland Shrub - land with a water table near, at, or above the soil surface, the majority of the vegetation is shrub	Rock/Rubble - bedrock, rubble, rocky beaches	No Observations for Water, Exposed Land, Herb, Bryoids, Broadleaf Dense, Broadleaf Open, Broadleaf Sparse, Mixed Wood Dense, Mixed Wood Open, or Mixed Wood Sparse

Distance to Cover (distance to shrub or coniferous habitat types, increments of 5 meters)	cover (0-5 meters)	near cover (10-45 meters)	open (50-195 meters)	very open (≥200 meters)	
Topography	flat (flat, dry surface)	flat-boggy (flat, boggy surface)	boggy (uneven, boggy surface)	boggy-hummock (lumpy hummock interspersed with bog)	hummock (dry, lumpy hummock)
Topography (continued)	rocky (rocky, uneven surface)				
Slope (increments of 5)	flat (0-5°)	moderate (10-30°)	steep (35-45°)	very steep (≥50°)	

Response Variable		
Predator Species	Black Bear	Coyote

Fig. S1 – The field observation sheet for caribou calf kill site MR-2010-016. A black bear was assigned via the field observation and molecular methods.

(front)



Newfoundland Labrador
Environment and Conservation

Calf Mortality Data Sheet

Area: MR (NORTH) LP NP Date: 2010-06-09 Field Staff: Porter/Mumma

Lat(decimal degrees): 48.24317 Long: 55.32952

Collared calf? (Y) N

Animal ID: MR-2010-016 VHF Freq: 164.083

Ear Tags: Left Colour: — Left Number: —

Right Colour: Orange Right Number: 641

Doe Present? (Y) N

Calf remains collected? (Y) N

Collar Condition

Collar attached to calf carcass? Y (N)
Staples intact? Y (N)
Bite marks in collar? Y (N) (if yes, distance between bite marks: _____cm)
Collar chewed? Y (N)
Blood stains on collar? (Y) N

Calf Carcass Condition

Too consumed to assess condition? (Y) N (if yes, go to Predator Evidence section)
Carcass buried? Y N (if yes, circle Complete or Partial)
Hole excavated? Y N
What is the hole covered over with? Moss Leaf Litter Dirt Other

Calf body dismembered? Y (N) Unkn
Decapitated? Y (N) Unkn
Skinned out? (Y) N Unkn
Skull cap removed? (Y) N Unkn
Throat trauma (circle one) None Light Moderate (Severe) Unkn
Punctures in body? Y N (Unkn) (if yes, locations of punctures: _____)
Broken jawbone? (Y) N Unkn
Crushed skull? (Y) N Unkn
Hole in side of abdomen? Y N (Unkn) (if yes, were organs consumed? Y N)
Ribs broken off? Y N (Unkn)
Hoof tips chewed? Y N (Unkn)

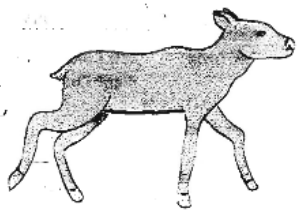
(back)

Predator Evidence:

	Unknown	Bear	Coyote	Lynx	Eagle	Collected?
Sighting						
Scat		✓				(Y) N
Track						
Hair/feathers						Y N

Notes: Typical Bear. Skin out, skull crushed, eaten. Long bones crushed, marrow eaten, Hide left in a "neat" pile. Bear Scat collected nearby

Calf's Right Side



Calf's Left Side

CAUSE OF DEATH: black bear.

Mortality Site Characteristics

Slope: 40 ° (estimated to nearest 5°)

Aspect (circle one) N (NE) NW S SE SW E W

Topography (circle): (hummocky) rocky flat boggy

Estimated distance to nearest tree cover: 100 m

80% low scrub. / 20% Grass
- Crowberry
- blueberry

mature softwood blown down & overgrown

Photo numbers: 1201 → 1212

Camera Owner: Me

DON'T FORGET TO FILL OUT A GROUND-TRUTHING SHEET FOR EACH MORTALITY SITE

Chapter IV – Evaluating Noninvasive Genetic Sampling Techniques to Estimate the Abundance of Three Large Carnivores

Under consideration in Molecular Ecology Resources

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Abstract

Monitoring large carnivores is difficult because of intrinsically low densities and can be dangerous if physical capture is required. Noninvasive genetic sampling (NGS) is a safe and cost-effective alternative to physical capture. We evaluated the utility of two NGS methods (scat detection dogs and hair sampling) to obtain genetic samples for abundance estimation of coyotes, black bears, and Canada lynx in three areas of Newfoundland, Canada. We calculated abundance estimates using program CAPWIRE, compared total costs, and the cost/sample for each method relative to species and study area, and performed simulations to determine the optimal sampling to achieve abundance estimates with coefficients of variation (CV) of <10%. Scat sampling was effective for both coyotes and bears and hair snags effectively sampled bears in two study sites. Rub pads were ineffective in sampling coyotes and lynx. The precision of abundance estimates was dependent upon the number of captures/individual, which was higher for scat. Our study suggested that ~3.4 captures/individual will result in a CV of <10% for abundance estimates when populations are small (23-39), but fewer captures per individual may be sufficient for larger populations. We found scat sampling was more cost-effective for sampling multiple species and for coyotes, but that hair sampling was less expensive per sample for bears at a study site with higher abundance and less accessibility by roads. We recommend that researchers implement pilot

studies to determine the most effective means of sampling for population monitoring and preserve the scarce resources available for species conservation.

Introduction

Large carnivore populations occur intrinsically at low densities (MacKay *et al*. 2008; Mondol *et al*. 2009) and have been further reduced by direct and indirect anthropogenic influences (Weaver *et al*. 1996). Managers are frequently tasked with monitoring population size and distribution to guide management actions for large carnivores. Traditional methods of large carnivore monitoring depend on capture and handling, which can be difficult, expensive, and dangerous for both animals and handlers (Gompper *et al*. 2006). Noninvasive sampling techniques are often a viable alternative that can increase sampling success, reduce sampling cost, and increase animal and handler safety (Waits 2004; MacKay *et al*. 2008; Kelly *et al*. 2012).

A myriad of noninvasive techniques are available to provide researchers with the ability to determine the distribution, abundance, and population trends of carnivores (Long *et al*. 2008). Track and scat surveys have a long history of use in determining carnivore occupancy (MacKay *et al*. 2008), and species detection via scat surveys has been improved through the use of trained scat detection dogs (Smith *et al*. 2003). Camera trapping has been used for occupancy and abundance estimation of species with distinct, individual markings (e.g., Mondol *et al*. 2009). With the advent of the polymerase chain reaction (PCR), species, sex, and individual were able to be identified from scat, hair, urine, and saliva (Waits & Paetkau 2005) for the purpose of improving occupancy surveys (Gompper *et al*. 2006), evaluating genetic diversity and structure (Kohn *et al*. 1995), estimating species abundance (Kohn *et al.* 1996), and identifying diet items (Deagle *et al.* 2005).

Numerous studies have used noninvasive genetic sampling (NGS) methods, and some have combined multiple sampling methods to estimate population parameters (De Barba *et al*.

2010a; Long *et al*. 2011; Reed 2011), but few have explicitly compared the effectiveness of multiple NGS techniques. De Barba *et al*. (2010b) found that baited hair snags resulted in more samples and higher amplification success than sampling scat and hair opportunistically or along transects for brown bears (*Ursus arctos*); however, opportunistic sampling was less expensive than hair snags, while identifying a similar number of unique individuals (De Barba *et al*. 2010b). Other studies have shown that the collection of scats along trails or at bait sites is superior to hair collection and/or saliva collection from prey kill sites (Vine *et al*. 2009; Sugimoto *et al*. 2012), while Lathan *et al*. (2012) demonstrated that optimal hair sampling methods may differ between bear species (*Ursus* spp.). Several additional studies determined that sampling with scat detection dogs provided the highest capture rates (Wasser *et al*. 2004 – bears), but was also the most costly (Harrison 2006 – bobcats (*Lynx rufus*); Long *et al*. 2007 – black bears (*Ursus americanus*), fishers (*Martes pennanti*), and bobcats). Our objective was to evaluate the utility of scat detection dogs and hair sampling to provide viable, genetic samples for abundance estimation of three large carnivore species in three study sites across Newfoundland, Canada.

Determining large carnivore abundance in Newfoundland has become a priority because of a declining caribou (*Rangifer tarandus*) population and a changing predator guild. Numbers of caribou in Newfoundland have declined >66% since 1998 and increased calf predation is considered a potential driver (Lewis & Mahoney 2014). Historically, gray wolves (*Canis lupus*), black bears, and Canada lynx (*Lynx canadensis*) were Newfoundland's apex predators, but gray wolves were extirpated from the island prior to the 1930s (Lewis & Mahoney 2014). Beginning in the 1980s, coyotes (*Canis latrans*) colonized Newfoundland

(Lewis & Mahoney 2014) and have become a major caribou calf predator (Mumma *et al*. 2014).

In 2009, we used scat detection dogs and hair sampling to collect samples in 3 caribou calving grounds in Newfoundland for the purpose of estimating abundances of coyote, black bear, and Canada lynx. We wanted to identify the most efficient means and appropriate sampling intensity to monitor these populations by answering the following questions: 1) which method provides the greatest number of samples overall, per species, and per study site; 2) how many individually identified samples and captures/individual are necessary to precisely estimate population abundances; and 3) what is the total cost and the cost/sample for each method?

Materials and Methods

Study Site

The island of Newfoundland (111,390 km^2) is characterized by a cool, maritime climate and interspersed coniferous forest, windswept barrens and peatland (McManus & Wood 1991). Caribou are widely distributed and are the only native ungulate on Newfoundland. Three caribou calving grounds (La Poile – LP, Middle Ridge – MR, and Northern Peninsula – NP, Fig. 1) ~500-1,500 km^2 in size were selected after delineation via telemetry data (Rayl *et al*. 2014). Scat detection dogs were used to sample coyotes, black bears, and Canada lynx in the LP and NP study sites. Hair snags and rub pads were used to sample coyotes, black bears, and Canada lynx at all three study sites.

Scat Detection Dog Sampling

A scat detection dog was trained to locate scats from coyotes, black bears, and Canada lynx. In the NP, 15 12 x 12 km grid cells were overlaid across the study site. The size of our

grid cells was a compromise between the average home range size of black bears (391 km^2) and coyotes (110 km^2) in Newfoundland and followed a consistent methodology to a previous study which used scat detecting dogs to sample fisher (*Martes pennanti*) scats in California (Thompson *et al*. 2012). Each cell was sampled 1-2 times in 2009 during June, July and/or August. The scat detection dog team was flown via helicopter when grid cells were not accessible by roads. Scat sampling of the LP study site occurred in adjacent roaded areas, because of limited road access to the LP calving ground (Fig. 1). Locations were strategically chosen to provide similar sampling intensity and coverage as was achieved in the NP.

The scat detection dog was permitted to search freely, while the handler ensured coverage of a range of habitat types. Search length and time varied depending on the number of scats found and the weather, but generally consisted of a 2-6 hour search spanning 5-10 km. Samples were placed in plastic bags using clean, latex gloves and frozen at the end of each day when possible. Prior to lab processing, scats were thawed and faecal material from multiple locations on the lateral surface of each scat (Stenglein *et al*. 2010a) was collected and placed in a 2-milliliter (ml) tube containing DETS buffer to prevent DNA degradation (Frantzen *et al.* 1998). Scats were given a sample ID and electronic records were generated that included study site, grid cell, date, and GPS coordinates.

Hair Sampling

We overlaid 5 x 5 km grid cells across the LP (20 cells), MR (44 cells), and NP (22 cells) study sites. A single transect was placed in each cell using a random starting location and orientation that was subsequently adjusted for some cells to permit reasonable access by field personnel and increase the probability of capture by selecting nearby locations containing game trails. Our transects were 600 m long and contained bear hair snags at each

end and a coyote rub pad and a Canada lynx rub pad at 150 m, 300 m, and 450 m (Fig. 2). Complete descriptions of hair snags and rub pads can be found in the supplementary material.

Study sites were accessed using helicopters, and snags and pads were set-up in mid-May and checked and re-baited in late June, early August, and late September with a final check in mid-October of 2009. Recovered samples were placed in paper envelopes using clean, latex gloves and labeled with the study site, grid cell, transect number, station type, and sample number and date. Paper envelopes were placed collectively in plastic bags filled with silica desiccant to prevent DNA degradation until lab processing (Roon *et al.* 2005).

DNA Extraction and Species Identification

We extracted samples in a low quantity and quality DNA laboratory to limit occurrence of contamination using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA) for scat samples and the Qiagen DNeasy tissue kit for hair samples. We used up to 10 follicles for hair extractions when available and used a negative control in all scat and hair batches to monitor for contamination.

We used a mitochondrial DNA (mtDNA) control region fragment analysis test to identify each sample to species (Mumma *et al*. 2014; De Barba *et al*. 2014). This test could not detect Canada lynx, so we tested all failed samples using mtDNA primers developed for Iberian lynx (*Lynx pardinus*) by Palomares *et al.* (2002).

Nuclear DNA Individual and Sex Determination

For coyotes, we combined nine microsatellite loci (FH2001, FH2054, FH2088, FH2137, FH2611, FH2670, FH3725, C09.173, Cxx.119 – Breen *et al.* 2001; Guyon *et al.* 2003; Holmes *et al.* 1994) based on the methods of Stenglein *et al.* (2010a) with two sex

determining loci (DBX6 and DBY7 – Seddon 2005) to form a single canid PCR multiplex (Mumma *et al*. 2014).

We developed 2 black bear PCR multiplexes (Mumma *et al*. 2014). Black bear multiplex 1 included six microsatellite loci (G10C, G10M, G10P, G10X, CXX20, and Mu23 – Paetkau *et al.* 1998; Taberlet *et al.* 1997; De Barba *et al.* 2010; Ostrander *et al.* 1993) and a sex-determining locus (Ennis & Gallagher 1994). Black bear multiplex 2 included six microsatellite loci (G1A, G10B, Mu15, Mu50, and Mu59 – Paetkau *et al.* 1998; Taberlet *et al.* 1997; Bellemain & Taberlet 2004). Additional PCR and allele scoring details for species identification and individual identification of black bears and coyotes can be found in Mumma *et al*. (2014).

We combined nine felid microsatellite loci (FCA096, FCA275, F85, FCA043, F124, FCA132, FCA082, FCA0008, F53 – Menotti-Raymond *et al*. 1999) and a sex-determining locus (Amel – Pilgrim *et al*. 2005) into a single multiplex to individually identify Canada lynx samples. Additional details of the reaction and PCR conditions can be found in the supplementary material. We determined allele sizes for species and individual identification using an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA) and associated GeneMapper 3.7 software.

We tested all samples in duplicate for their first respective PCR multiplex. We dropped samples that failed to amplify at ≥4 loci. Black bear samples that amplified at ≥4 loci were tested in duplicate for the second bear multiplex. We required each allele to be detected twice for heterozygotes and three times for homozygotes to obtain a consensus genotype. We ran up to six PCR replicates for each multiplex and dropped samples that failed to achieve a consensus genotype at ≥6 loci for coyotes and ≥9 loci for black bears. We

selected our minimum number of consensus loci for coyotes and black bears to assure that all samples had a probability of identity siblings (PID_{sibs}) (Waits *et al.* 2001) value of less than 0.01 as estimated by the software GenAlEx6 (Peakall & Smouse 2006) regardless of which loci were complete. This means that less than 1/100 comparisons of first degree relatives would result in identical genotypes for our minimum number of consensus loci and was used to avoid false recaptures in the dataset. When all loci were completed for an individual, our estimated PID_{sibs} values for black bear and coyote were <0.001. We did not establish a minimum number completed loci for lynx samples or calculate PID_{sibs} for Canada lynx, because we only identified 1 individual.

We used the software GenAlEx6 (Peakall & Smouse 2006) to match completed genotypes. We also evaluated replicate PCRs for samples that matched at all but one or two loci to determine if mismatches could be attributed to allelic dropouts or false alleles. We analyzed individuals that were only detected once using the software RELIOTYPE (Miller *et al.* 2002) to estimate the genotyping error rate and evaluate the reliability of the final consensus genotype. We required consensus genotypes to be ≥95% reliable and retested samples until we achieved our threshold to reduce the likelihood that single captures were the result of genotyping error. We also calculated error rates (frequency of allelic dropouts and false alleles) for hair and scat samples across species and study site by comparing consensus genotypes from randomly selected individually identified samples to their first 2 PCR replicates.

Abundance Estimation and Simulations

We used hair and scat samples to generate black bear and coyote abundance estimates via the package CAPWIRE (Pennell *et al*. 2013) in program R. Program CAPWIRE (Miller

et al. 2005) and the corresponding R package CAPWIRE were developed specifically for the analysis of non-invasively collected samples by permitting abundance estimation using recaptures of individuals from a single sampling session (Miller *et al*. 2005). CAPWIRE is based on a simple urn model where individuals are drawn with replacement and requires that all individuals are correctly identified, all samples are independent, and the population is closed during the period of sample deposition and collection. We designed our previously discussed individual identification protocols to satisfy the assumption that all individuals were identified correctly. For scat sampling, we assumed that all samples were independent and assumed that the population was closed during deposition and collection. Scat degradation studies suggest a low probability of obtaining a complete genotype after 30 days for wolf and brown bear scats (Santini *et al*. 2007; Murphy *et al*. 2007). We expected similar to more extreme degradation patterns for coyote and black bear scats in our study due to Newfoundland's damp climate, which is likely to exacerbate degradation (Murphy *et al*. 2007). Therefore, we assumed that successfully identified scats were deposited no earlier than 30 days prior to our initial sampling periods in the LP and NP, which was 11-12 weeks from the date of our last sampling periods. An insufficient number of scat samples was collected in the LP for black bears and in the LP and NP for Canada lynx to permit abundance estimation.

To maintain our assumption of independence for hair samples, we only included one sample per individual per snag per session. However, we did consider black bear hair samples collected in the same session from opposite hair snags along the same transect to be independent, because we detected very few individual black bears at both snags (13/189 opportunities) of a single transect. Similar to the approach taken by Robinson *et al*. (2009),

we combined hair collection sessions to increase our average number of captures/individual to the frequency (≥1.7) recommended by Stenglein *et al*. (2010b) when using CAPWIRE. In the MR, we combined the first three sessions, but eliminated the fourth session to limit our sampling period to ~16 weeks, which is comparable to the duration of other studies that assumed population closure (Boersen *et al*. 2003; Boulanger *et al*. 2008). In the NP, we used all four sessions to maximize our number of recaptures, which extended the duration of our study to ~20 weeks and may increase the likelihood of violating our assumption of population closure. Hair snags failed to provide a sufficient number of samples to estimate abundance of black bear in the LP and coyote and Canada lynx in all of the study sites.

We ran models under the assumption of an equal capture (ECM) rate for all individuals and two innate rates (TIRM) of capture within the population and used likelihood ratio tests to determine the best supported model. Following preliminary analyses, we parameterized the MR black bear model using a maximum population size of 200 individuals and set the maximum population size to 100 individuals for coyote and black bear models using hair or scat in the LP and NP study sites. We estimated 95% confidence intervals for all population size estimates using 1,000 bootstraps. We also plotted the number of captures/individual against the estimated coefficient of variation (CV) for our four smaller populations, but excluded the much larger MR black bear population for reasons explained later.

Following abundance estimation, we performed simulations to estimate the number of samples necessary to achieve a <10% CV (Sokal & Rohlf 1995). We used the parameters (abundance, # of individuals with the lower capture rate, # of individuals with the higher capture rate, and the ratio of capturability between the two classes) estimated under the better

supported two innate rates model to generate 100 theoretical capture histories at five different sampling intensities of each population for which abundance was estimated. We then averaged the estimates of abundance and 95% confidence intervals and determined the coefficient of variation for each set of capture histories.

Sampling costs

We estimated and compared the total cost (Tables S1 & S2 Supplementary Information) and cost/individually identified and independent (hereafter referred to as III) sample across study sites and collection methods. We evaluated the cost/III sample, because our goal of abundance estimation was reliant on sample independence. Since sampling for all three species occurred concurrently, we determined the cost/III sample by dividing the total sampling cost over the number of III samples for each species in each study site and by dividing the total sampling cost over III samples for all species in each study site. Our scat detection dog sampling was performed by a graduate student, so we used rates provided by an independent conservation dog company (Find It Detection Dogs) to estimate the cost of conducting a comparable level of scat detection dog sampling (Table S1 Supplementary Information). Since hair sampling was performed by a combination of Newfoundland provincial biologists and graduate students, we estimated costs for hair sampling using only provincial biologists (Table S2 Supplementary Information). We also report total lab costs and lab costs/III sample for each method.

Results

Sampling and Molecular Identification Success

Scat detection dogs located 185 and 193 samples in the LP and NP study sites (Table 1). Ninety-five percent of these samples were successful for species identification (Table 1).

Fifty-eight coyote samples, 94 black bear samples, and one Canada lynx sample were identified in the LP, and 62 coyote, 87 black bear, and 6 Canada lynx samples were identified in the NP (Table 1). Coyote samples were more successful for individual identification (76% – LP, 86% – NP) than black bear samples (33% – LP, 29% – NP) (Table 1). False allele and allelic dropout PCR error rates were similar across study sites, and were 0.007 and 0.060 for black bears and 0.006 and 0.03 for coyotes, respectively. We did not estimate error rates for lynx because of the limited number of samples collected. No individual lynx were identified in the LP, and only 1 of the 6 NP lynx samples were individually identified (Table 1). The average number of captures/individual was 3.7, 1.3, 2.4, and 1.9 for LP coyotes, LP black bears, NP coyotes, and NP black bears, respectively (Table 1).

Eighteen, 678, and 139 hair samples were collected via hair snags in the LP, MR, and NP. Only three samples total (one black bear and two failed samples) were collected from rub pads across all three study sites. Thirty-three percent of hair samples were successful for species identification in the LP vs. 68% and 63% in the MR and NP, respectively (Table 1). Five hundred and forty-nine of the hair samples identified to species were from black bears with eight remaining samples from the NP attributed to coyotes (Table 1). Individual identification success rates from hair samples were 71%, 38%, 60%, and 56% for LP black bear, MR coyotes, MR black bear, and NP black bear (Table 1). False allele and allelic dropout PCR error rates were similar across study sites and were estimated at 0.04 and 0.002, respectively, from a selection across all 3 study sites of individually identified bear hair samples. We did not estimates error rates for coyotes, because of the limited number of samples collected. Once we accounted for sample independence, the number of III samples was 3, 2, 170, and 25, and the average number of captures per individual was 1.5, 1.0, 1.7,

and 1.6 for LP black bears, MR coyotes, MR black bears, and NP black bears, respectively (Table 1).

Abundance Estimates and Simulations

We estimated a population size of 24 (7.5% CV) coyotes for the LP area sampled via scat detection dogs (71 samples), which was consistent with simulations that indicated an III sample size of ~75 would result in an estimate with a <10% CV (Fig. 3A). An abundance estimate of 32 (19.2% CV) was determined for NP coyotes using scat sampling, and ~100 III samples would be necessary to achieve our desired level of precision (<10% CV) (Fig. 3B). Twenty-three (28% CV) and 39 (39.6% CV) black bears were estimated using scat and hair samples, respectively, for the Northern Peninsula, and we estimated that approximately 75 and 100 III samples would be necessary reduce estimates below a 10% CV (Fig. 3C & 3D). One hundred and thirty-five (14% CV) black bears were estimated for the MR using hair samples, and ~150 III samples would be necessary to achieve a <10% CV (Fig. 3E). In our plot of captures/individual vs. CV, our data suggests a CV of <10% could be achieved with ~3.4 captures/individual for populations between 23 and 39 individuals (Fig. 4).

Sampling Costs

The total estimated cost of scat sampling was $16,363 (USD) and $28,182 for the LP and NP study sites (Table 2). We estimated a per III sample cost for scat samples of $230, $531, $861, and $1,126 for LP coyotes, NP coyotes, LP bears, and NP bears, respectively (Table 2). Our cost/III samples for scat from all species was $182 for the LP and $352 for the NP (Table 2). Our estimated costs for hair sampling was $86,382, $166,235, and $86,340 for the LP, MR, and NP study sites (Table 3). We estimated a per III sample cost for hair samples of $972 and $3,454 for MR black bears and NP black bears, respectively (Table 3).

The cost per III samples for hair from all species was $961 for the MR and $3,454 for the NP (Table 3). The total lab costs (and the cost/III sample) for all samples across study sites was $4,809 (and $28.29) for scat and $6,195 (and $30.52) for hair.

Discussion

In our study, we found that optimal sampling methods differed between species and across study sites in conjunction with variable predator densities and accessibility. Scat sampling yielded sufficient sample sizes to estimate the abundance of multiple species and estimate the abundance of coyotes in multiple study sites (Table 1 & Fig. 3), which is similar to the findings of other studies (Harrison 2006; Long *et al*. 2007). Hair sampling was only successful for black bears in the MR and marginally successful for black bears in the NP (Table 1). Neither scat nor hair sampling was successful for Canada lynx (Table 1).

We were able to estimate coyote abundance in the LP and NP and black bear abundance in the NP using scat samples (Fig. 3). We did not detect differences in coyote abundance between the NP and LP study sites, even after accounting for the number of locations sampled within each study site. Although there were 58 black bear samples collected in the LP, the lower individual identification success rates for black bear scats, in comparison to coyote scats, yielded a low number of III samples and captures/individual (1.3) (Table 1). Although not evaluated in a single study within the same system, other studies do not indicate higher amplification success rates for canids than bears (Broquet *et al*. 2007). Potential reasons for this trend could be differences in diet (Murphy *et al*. 2003) or the chemical composition of coyote and black bear scats, either of which could lead to inhibition of DNA amplification or increased degradation (Huber *et al*. 2002; Murphy *et al*. 2003). Alternatively, the morphology of scats could explain these differences (Murphy *et al*. 2003),

since a firm coyote scat may be more prone to slough off the epithelial cells necessary for molecular identification than a softer, less formed black bear scat. We were not able to generate abundance estimates from the few Canada lynx samples collected via scat detecting dogs and propose that there could be a biological or methodological explanation. Canada lynx densities may be lower than coyote or black bear densities in the Newfoundland system or there may have been lower rates of detection for lynx scats resulting from the limited number of positive lynx scats available during scat detecting dog training.

Black bear hair sampling was more successful in the MR than in the LP and NP study sites (Table 1). We speculate that this was the result of higher black bear densities in the MR than in the LP and NP, as demonstrated by the higher MR abundance estimate (Fig. 3) even after considering that the MR contained over twice the number of hair snags as either the LP or NP study sites. Improved abundance estimates for the LP and NP study sites would be possible by increasing the number of hair sampling sessions and by shortening the time between sessions, which should increase amplification success. Individual identification success rates ranged from 56-71% for hair samples identified as black bear. However, this does not account for the 281 (34%) hair samples that failed species identification. Since nearly all the hair samples identified to species were from black bears (Table 1), individual identification success rates may have been as poor as 40% (328 individually identified black bear/830 potential black bear samples). Other studies have reported a wide range (14%-99%) of hair genotyping success for bears (De Barba *et al*. 2010b; Roon *et al*. 2003). The long periods between collections coupled with Newfoundland's wet environment likely increased DNA degradation (Stetz *et al.* In Press) and decreased amplification success in our study.

Rub pads were extremely unsuccessful in our study for coyotes and Canada lynx (Table 1). Low Canada lynx densities may partially explain our inability to capture lynx, but methodological differences for coyotes and Canada lynx sampling in comparison to other studies could also explain our low success rates. Other studies reported successful sampling of coyotes (Ausband *et al*. 2011) and Canada lynx (McDaniel *et al*. 2000) in the fall and/or winter when food resources are scarce and scents and attractants may be more effective. Sampling could also have been improved through more frequent application of lures, different lures, and a natural substrate for coyotes (McDaniel *et al*. 2000; Ausband *et al*. 2011).

The large confidence intervals of our abundance estimates and the lack of overlap between sampling methods reduced our ability to distinguish study site differences and differences between abundance estimates generated via scat vs. hair sampling (Fig. 3). Point estimates of abundance in the NP were lower for scat sampling than hair sampling, but confidence intervals overlapped (Fig. 3). Considering the much larger extent of scat sampling in the NP (~4x), we anticipated a much larger abundance estimate from scat, but this outcome could be the result of violating CAPWIRE's closure assumption for hair sampling, which we risked to increase our number of captures/individual. However, it is also plausible that this result could be explained by differences between methods in the accumulation of new individuals as sample size increases in conjunction with the lower amplification success rates for black bear scat samples.

Inferences can be drawn with regards to the effect of sampling intensity on the precision of abundance estimates. Scat sampling on average yielded a higher number of captures per individual than hair sampling (Table 1), which had a direct impact on the precision of abundance estimates for comparable population sizes (Fig. 3 & 4). The high

number of captures/individual for coyotes in the LP study site resulted in the only abundance estimate that was below our 10% CV threshold (Figure 3A). If we disregard the much larger MR black bear population, a clear trend is apparent indicating a decrease in the CV with increasing captures/individual and a 10% CV is likely to be achieved around 3.4 captures/individual (Figure 4). However, this recommendation appears highly dependent upon the size of the population. When comparing black bear abundance estimates using hair samples from the MR and NP, we have a similar number of captures/individual (Table 1) and a 1.2-fold increase for the size of the MR confidence interval (Fig. 3), but we find a nearly three-fold decrease in the CV as a result of the much larger MR population size. A similar trend is suggested by our simulations, which indicated that a 3:1 ratio of III samples to population size provides sufficient power to estimate small populations (23-39) with a CV of <10% (Fig. 3). This ratio may be a reflection of increasing the number of captures/individual or increasing the proportion of unique individuals detected, both of which should increase with increased sample size. However, we also find that a ~1:1 ratio may be sufficient to achieve a 10% CV for the MR black bear population (Fig. 3E). This makes intuitive sense, because CV is a normalized measure of dispersion. Therefore, we would expect the CV to decrease for a set number of captures/individual as population size increases. In practice, researchers also need to consider the percentage of samples that fail to amplify and collect a sufficient number of total samples to provide the required III samples for accurate and precise abundance estimates as suggested by Solberg *et al*. (2006). Although outside the scope of our study, the distribution of capture/rates across individuals is also likely to be important and further research is necessary to explore how variation in the distribution of captures affects abundance estimates.

Additional considerations need to be recognized when comparing the costs between scat and hair sampling in our study. The total cost of scat sampling and the cost/III sample from all species and cost/III sample from coyotes were considerably less expensive than the hair sampling cost/III sample (Table 2 & 3). However, cost/III sample from black bears in the MR was less expensive than scat sampling for black bears in the LP and NP study sites (Table 2 & 3). Other researchers found that scat detection dogs were the most costly (Harrison 2006; Long *et al*. 2007), but we found scat detection dogs to be the most cost-effective for sampling multiple species at one time and for sampling coyotes (Table 2). However, scat sampling only occurred in areas where most sampling locations could be accessed via roads. Road access markedly reduced helicopter time, which was the primary expenditure for hair sampling. Furthermore, only 2 hours/day were charged to scat sampling in the NP when helicopter support was necessary (Table S1 Supplementary Information), because helicopters were already being used for additional research in the area. If helicopters were hired for scat detection dog sampling alone, additional costs would have accrued during the 4- to 8-hour window between transporting and retrieving the scat detection dog team to and from sampling locations. In the NP, where only one sampling location required helicopter support, scat detection dog sampling would remain the best option for sampling multiple species and coyotes regardless of whether or not helicopter costs could be buffered by other research activities. In the more remote MR study site, helicopter costs could become prohibitively expensive if not buffered by other research activities; however, our results indicated that these costs may be necessary to sample for coyotes. Regardless, hair sampling for black bears in the MR was more cost-effective than black bear scat sampling in the LP and NP study sites, which can be attributed to the higher capture rates associated with a more dense population.

Lab costs were slightly higher for hair samples as a result of reduced amplification success, but overall lab costs were relatively minor in comparison with field sampling costs.

Evaluating multiple sampling methods is important to ensure efficient use of resources when monitoring populations. Another study, found that multiple methods may be necessary to achieve sampling goals and combined methods across their study area (De Barba *et al*. 2010a). Our results suggest that scat sampling is more efficient for sampling multiple species in study areas accessible via roads, but that hair snags may be more efficient for sampling black bears in remote locations. We think that our findings can be used to guide sampling approaches in other study areas, but would recommend that researchers implement pilot studies to identify the most effective sampling approaches for their study systems.

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Tables

Table 1 – Number of samples and species and individual success rates by study site (La Poile (LP), Middle Ridge (MR), and Northern Peninsula (NP)), sample type (scat or hair), and species (coyote, black bear (B. Bear), Canada lynx (C. Lynx)). (Individually identified and independent samples = III Samples, Individual=IND)

Study Site	Sample Type	Total Samples	% Identified to Species*	Species	# of Samples	# IND Identification (%)	III Samples **	# of Unique INDs	Captures /IND
LP	Scat	185	95%	Coyote	94	71 (76%)	71	19	3.7
				B. Bear	58	19 (33%)	19	15	1.3
				C. Lynx	1	0	NA	NA	NA
LP	Hair	18	39%	Coyote	0	NA	NA	NA	NA
				B. Bear	7	5 (71%)	3	2	1.5
				C. Lynx	0	NA	NA	NA	NA
MR	Hair	679	68%	Coyote	8	3 (38%)	2	2	1.0
				B. Bear	453	273 (60%)	171	99	1.7
				C. Lynx	0	NA	NA	NA	NA
NP	Scat	193	95%	Coyote	62	53 (86%)	53	22	2.4
				B. Bear	87	25 (38%)	25	13	2.5
				C. Lynx	6	1 (17%)	1	1	1.0
NP	Hair	141	63%	Coyote	0	NA	NA	NA	NA
				B. Bear	89	50 (56%)	25	16	1.6
				C. Lynx	0	NA	NA	NA	NA

*Unidentified scats failed to amplify or were non-target species

**All scat samples were considered III samples, but hair samples from the same individual and session were only considered III samples if collected from different hair snags

Table 2 – Total cost ($USD) and cost/sample estimates for scat detecting dog sampling in the La Poile (LP) and Northern Peninsula (NP) study sites for coyote, black bear, and Canada lynx. (NA = cost/sample not recorded due to minimum sampling success, Individually identified and independent samples = III Samples)

Category	Item	LP Scat Costs*	NP Scat Costs*
Transportation	Roundtrip Travel to NF	2993	3233
	Roundtrip Ferry Costs	597	597
	Travel Costs within NF	507	507
	Helicopter	NA	11559
Staff	Roundtrip Travel Per Diem	2800	2800
	Camper Per Diem	900	900
	Hotel + Per Diem	615	615
	Scat Dog Team Cost	7800	7800
	Dog Insurance	150	150
	Total	16363	28162

Sample Info	Species	LP Samples	NP Samples
III Samples	Coyote	71	53
	Black Bear	19	25
	Canada Lynx	0	1
	All	90	80
Cost/III Sample	Coyote	230	531
	Black Bear	861	1126
	Canada Lynx	NA	NA
	All	182	352

*Based on estimates from "Find It Detection Dogs" for one dog and handler

(See Supplemental Information for itemized descriptions)

Table 3 – Total cost ($USD) and cost/sample estimates for hair sampling in the La Poile (LP), Middle Ridge (MR), and Northern Peninsula (NP) study sites for coyote, black bear, and Canada lynx. (NA = cost/sample not recorded due to minimum sampling success, III Samples = individually identified and independent samples)

Category	Item	LP Hair Costs*	MR Hair Costs*	NP Hair Costs*
Transportation	Helicopter	74765	149529	74765
	Gas	251	109	210
Staff	Salary	5612	7808	5612
	Lodging	3137	4531	3137
	Per Diem	1603	2231	1603
Supplies	Lures	115	229	115
	Posts	276	552	276
	Barbed Wire	233	466	233
	Bait	368	736	368
	Misc. Supplies	22	44	22
	Total	86382	166235	86340
Sample Info	Species	LP Samples	MR Samples	NP Samples
III Samples	Coyote	0	2	0
	Black Bear	5	171	25
	Canada Lynx	0	0	0
	All	5	173	25
Cost/III Sample	Coyote	NA	NA	NA
	Black Bear	NA	972	3454
	Canada Lynx	NA	NA	NA
	All	NA	961	3454

*Based on four sessions and two biologists

(See Supplemental Information for itemized details)

Figures

Figure 1 – The location of our three study sites and calving grounds on the island of Newfoundland, Canada. The location of scat sampling in the La Poile (LP) study site is shown because it was outside of the LP calving ground.

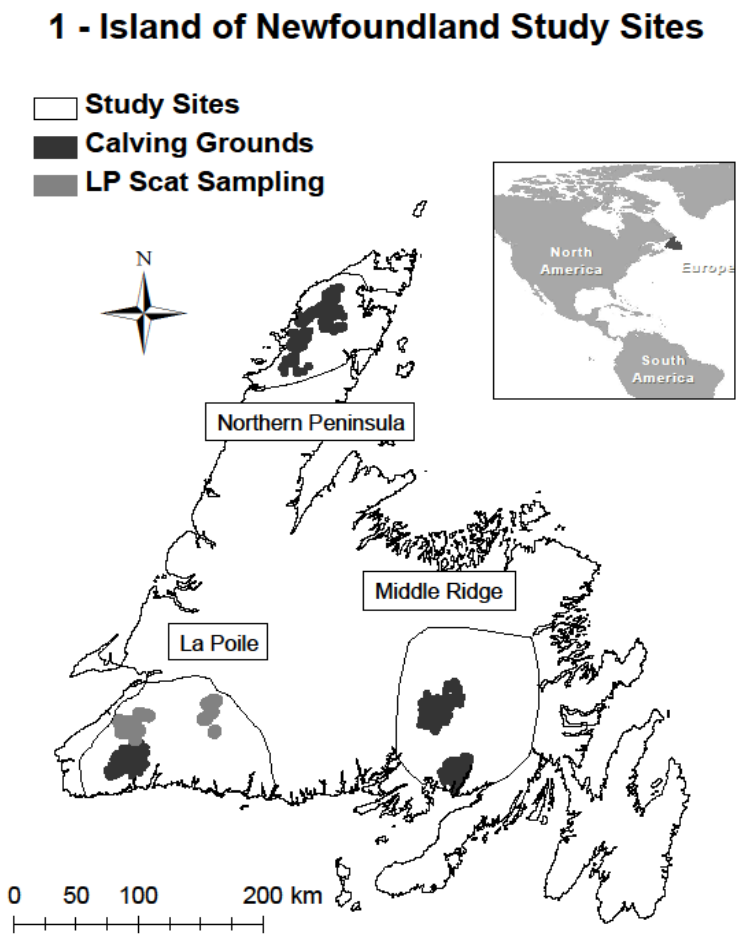


Figure 2 – Transect configuration for black bear hair snags and coyote and Canada lynx rub pads.

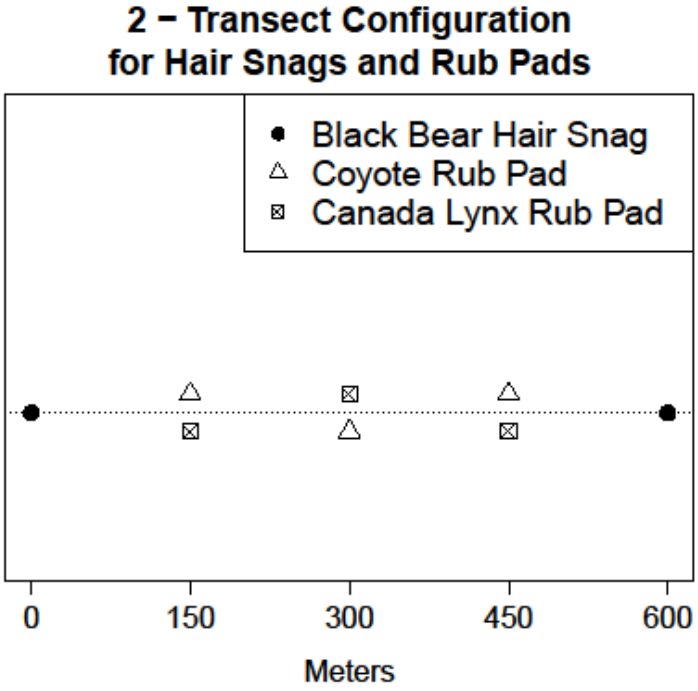


Figure 3 – Abundance estimates and simulations via the R package CAPWIRE using scat detecting dog and hair snag sampling for coyotes and black bears in the LP (La Poile), MR (Middle Ridge), and NP (Northern Peninsula) study sites.
*Only 3 sessions in MR study site were used to satisfy the closure assumption of CAPWIRE, thereby creating the mismatch between the total number of hair samples collected and individually identified and the number of samples used in the MR black bear population size estimate.

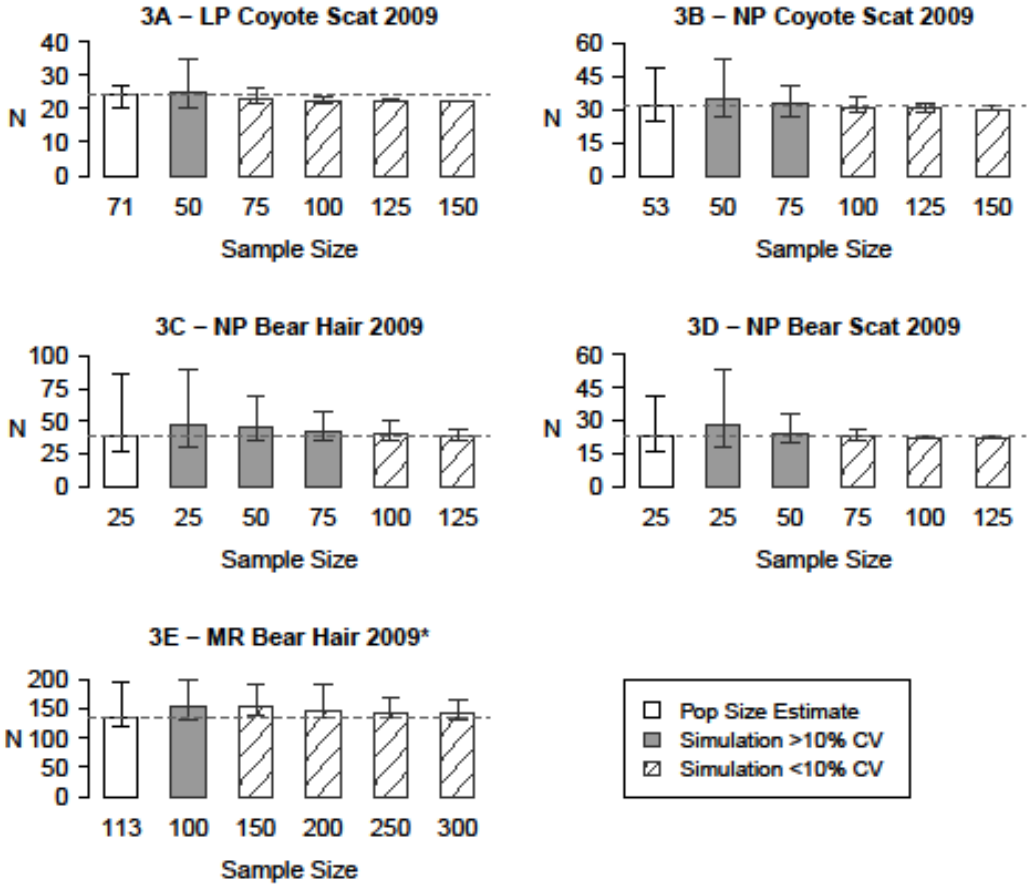
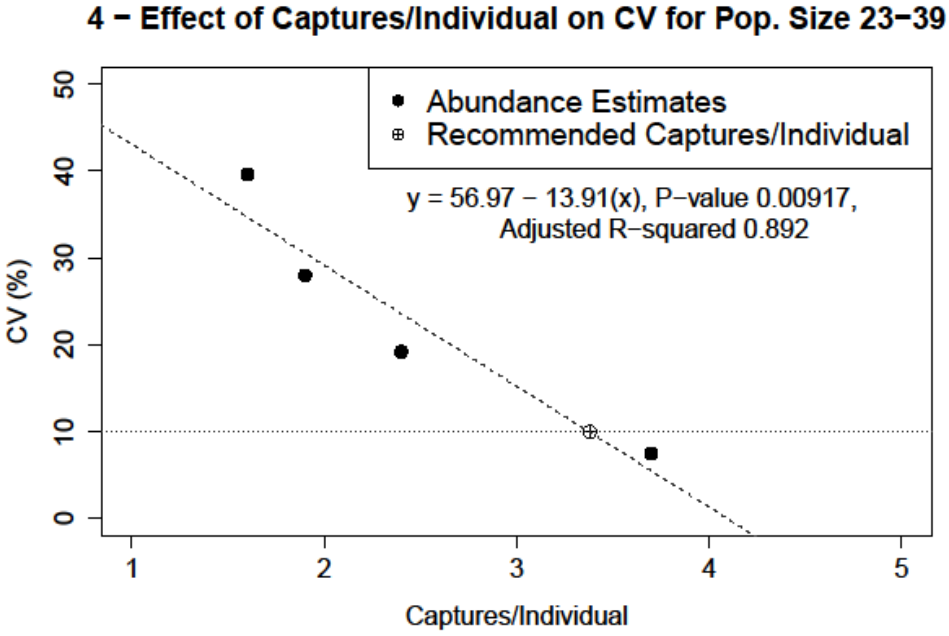


Figure 4 – The effect of the average number of captures/individual on the coefficient of variation (%) of abundance estimates. The horizontal dashed line represents our desired coefficient of variation (%) for abundance estimates, and the decreasing dashed line is a fitted linear model predicting the coefficient of variation (%) by the average number of captures/individual.



Supplementary Information

Hair Snag and Rub Pad Descriptions

Bear hair snags were constructed using wooden stakes to hang barbed-wire fencing at 0.5 m and 1 m to create a circular structure with an approximate 5-m diameter. Three cans of sardines were placed at the center of each bear hair snag as an attractant. Coyote and Canada lynx rub pads were approximately 50 m apart on opposite sides of the transect (Fig. 2). Coyote rub pads were constructed by affixing a 9 cm x 12 cm coconut fiber mat with exposed metal barbs to the ground on top of a cotton ball saturated with skunk oil. Two ml of the trapping lure Hawbaker's Wiley red 500 was applied to the mat and white feathers or a CD were hung on a nearby tree if present or on a wooden stake. Canada lynx rub pads were constructed by attaching 9 cm x 12 cm coconut fiber mats with exposed metal barbs to trees, if present, or stakes at a height of 50 cm approximately (McDaniel *et al*. 2000). Two ml of Halford's Pikauba scent lure was applied to the mat and white feathers or a CD were hung above the mat (McDaniel *et al*. 2000). At some coyote and lynx sites, coyote urine was sprayed in the surrounding area.

Lynx individual identification reaction and PCR conditions

Our 7 uL reaction included the following concentrations: 0.14 uM FCA096, 0.10 uM FCA275, 0.16 uM F85, 0.16 uM FCA043, 0.06 uM F124, 0.09 uM FCA132, 0.13 uM FCA082, 0.10 uM FCA0008, 0.20 uM F53, and 0.16 uM Amel for each primer pair, along with 1x Qiagen Master Mix, 0.5x Qiagen Q solution, and 1 uL DNA extract. The PCR profile began with an initial denaturation step at 95˚C for 15 minutes followed by a touchdown of 13 cycles at 94˚C for 30 seconds, 60˚C for 90 seconds, and 72˚C for 60 seconds with a 0.8˚C decrease in the annealing temperature at each cycle followed by 31 cycles at 94˚C for 30

seconds, 50˚C for 90 seconds, and 72˚C for 60 seconds and a final elongation step at 60˚C for 30 minutes.

Table S1 – A list of itemized descriptions for cost estimates displayed in Table 2 of the main text by study site.

Study Site	Item	Description ($USD unless indicated $CAN)
LP	Roundtrip Travel to NF	$0.317/km (9440 km)
LP	Roundtrip Ferry Costs	($33/person+$313.09/>12 m)*2 ($CAN)
LP	Travel Costs within NF	$0.317/km (50 km/day/field days+100 km/day/off day)
LP	Helicopter	NA
LP	Roundtrip Travel Per Diem	$200*14 days
LP	Camper Per Diem	$45/day (20 days)
LP	Hotel + Per Diem	$100/day hotel ($CAN) +$15/day per diem (6 days)
LP	Scat Dog Team Cost	$390/day (20 days)
LP	Dog Insurance	$150/month
NP	Roundtrip Travel to NF	$0.317/km (10200 km)
NP	Roundtrip Ferry Costs	($33/person+$313.09/>12 m)*2 ($CAN)
NP	Travel Costs within NF	$0.317/km (50 km/day/field days+100 km/day/off day)
NP	Helicopter	$1100/hr (6 days 2 hours each) ($CAN)
NP	Roundtrip Travel Per Diem	$200*14 days
NP	Camper Per Diem	$45/day (20 days)
NP	Hotel + Per Diem	$100/day hotel ($CAN) +$15/day per diem (6 days)
NP	Scat Dog Team Cost	$390/day (20 days)
NP	Dog Insurance	$150/month

Table S2 - A list of itemized descriptions for cost estimates displayed in Table 3 of the main text by study site.

Study Site	Item	Description ($CAN)
LP	Helicopter	$1100/hour (6 hours*13 days) - 2 days/session*4 sessions+3 days/set-up+2 days/breakdown)
LP	Gas	$1.129/liter (255 liters) - 1915 km/(30 km/liter)*(4 sessions+breakdown)
LP	Salary	$17.50/hour (8 hours*46 days) - 2 people*((4 days(2 collection + 2 travel)*4 sessions)+5 days(3 set-up+2 travel)/set-up+2 days/breakdown)
LP	Lodging	$100/night (36 nights) - 2 people*((3 nights*4 sessions)+4 nights/set-up+2 nights/collection)
LP	Per Diem	$40/day (46 days) - 2 people*((4 days*4 sessions)+5 days/set-up+2 days/breakdown)
MR	Helicopter	$1100/hour (6 hours*26 days) - 4 days/session*4 sessions+6 days/set-up+4 days/breakdown
MR	Gas	$1.129/liter (111 liters) - 668 km/(30 km/liter)*(4 sessions+breakdown)
MR	Salary	$17.50/hour (8 hours*64 days) - 2 people*((5 days(4 collection + 1 travel)*4 sessions)+8 days(6 set-up+2 travel)/set-up+4 days/breakdown)
MR	Lodging	$100/night (32 nights) - 2 people*((4 nights*4 sessions)+6 nights/set-up+4 nights/collection)
MR	Per Diem	$40/day (64 days) - 2 people*((5 days*4 sessions)+8 days/set-up+4 days/breakdown)
NP	Helicopter	$1100/hour (6 hours*13 days) - 2 days/session*4 sessions+3 days/set-up+2 days/breakdown)
NP	Gas	$1.129/liter (213 liters) - 1276 km/(30 km/liter)*(4 sessions+breakdown)
NP	Salary	$17.50/hour (8 hours*46 days) - 2 people*((4 days(2 collection + 2 travel)*4 sessions)+5 days(3 set-up+2 travel)/set-up+2 days/breakdown)
NP	Lodging	$100/night (36 nights) - 2 people*((3 nights*4 sessions)+4 nights/set-

		up+2 nights/collection)
NP	Per Diem	$40/day (46 days) - 2 people*((4 days*4 sessions)+5 days/set-up+2 days/breakdown)
All	Lures	Hawbaker's Wiley Red Lure 500, Halford's Pikauba, etc.
All	Posts	4 foot wooden stakes
All	Barbed Wire	
All	Bait	Cans of sardines and mollasses
All	Misc. Supplies	Flagging tape, rub pads, nails, mallets, gloves, visual attractants (white feathers, CDs), etc.

Appendix

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